The reproductive ecology of *Icmadophila splachnirima*, including aspects of the reproduction in additional members of Icmadophilaceae

Dissertation submitted by
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Abstract

This thesis describes a journey of discovery of the reproductive mechanisms used by certain members of the Icmadophilaceae – a group of lichens about whose reproductive mechanisms there has been much confusion in the academic literature. This thesis focuses on *Icmadophila splachnirima*, a rare lichen that occurs mostly in endangered subalpine boggy habitats of New Zealand and South-East Australia. It grows on continuously moist peaty soil, over mosses and plant debris, within a landscape dominated by tussock and low shrubs. It is found in Victoria, Tasmania, the South Island of New Zealand, Stewart Island, Chatham Islands and the subantarctic Auckland and Campbell Islands. A comprehensive geo-referenced compilation of all currently known localities of this species provided. This thesis describes a molecular genetic reassessment of the genus *Icmadophila* and provides evidence supporting the reinstatement of the genus *Knightiella*, comprising the single species *Knightiella splachnirima*. Also the recently described *Icmadophila eucalypti* belongs in a genus of its own, leaving only three species in *Icmadophila* s. str. Contrary to previous reports, it is shown that the type species of the genus, *I. ericetorum*, does most likely not occur in New Zealand.

A population genetic approach was used to examine whether gene flow exists between the very disjunct known populations of the focal species and to assess the relative importance of sexual versus vegetative propagules for long distance dispersal. Evidence is provided that *I. splachnirima* is most likely not self-fertile. Sexual reproduction via ascospores is the primary mechanism for long-distance dispersal, and the spores can combine with multiple algal partners of *Coccomyxa* s. lat. Furthermore, field experiments show that under unfavourable environmental conditions, particularly insufficient hydration, the species can reversibly resort to vegetative reproduction.

Based on this reproductive strategy, the author assumes that populations of *I. splachnirima* will be able to maintain themselves in their natural environment, in spite of potentially deteriorating habitat conditions in the course of climatic change. However, especially small and isolated populations may not be able to recover from genetic bottlenecks and habitat loss due to anthropogenic activity. Therefore, monitoring of small and isolated populations is advised, and if necessary, transplants of compatible mating partners should be considered to ensure their long-term survival.

In the course of the research undertaken for this thesis, the author observed and proved that sexual reproduction occurs in two other members of the family Icmadophilaceae, *Siphula decumbens* and *S. fastigiata*, whose ability for sexual reproduction had previously been unknown. The resurrection of the genus *Nylanderiella* is proposed for the *Siphula decumbens* group.
1. Lichen reproduction

Lichens have been described as ‘fungi that have discovered agriculture’ (Goward 2003), with the fungal partner (mycobiont) dominating the symbiotic relationship. That means the term lichen does not refer to a taxonomic unit, but to a fungal life style or nutritional mode. The same fungus can be associated with several different algal species, and consequently the scientific name of a lichen refers to the fungus only. The algal partner (photobiont) is just the ‘crop’ of the fungal ‘farmer’. When incorporated into the lichen symbiosis, sexual reproduction of green-algal photobionts is usually suppressed, even though it can be observed in isolated cultures (Friedel & Büdel 2008) and is likely to occur in the free-living photobiont. Therefore, discussion of reproduction in lichens must largely concern the activities of the fungal partner, with reproduction of the photobiont being something of a sideshow.

This thesis describes a journey of discovery of the reproductive mechanisms used by certain members of the Icmadophilaceae, in particular Icmadophila splachnirima – a subject that has given rise to considerable confusion and controversy in the academic literature.

1.1 Sexual reproduction of lichens

Unreferenced statements in this general introduction are from lichenological standard text books like Henssens & Jahns (1973, in German) and Nash et al. (2008, in English) or a general lichen review like Büdel (2010, in German).

1.1.1 The sexual reproductive cycle

In the vast majority of lichens (over 99%), the mycobiont (fungal partner) is an ascomycete. Ascomycetes are haploid during most of the life cycle, and only the zygote, during sexual reproduction, is diploid. Ascomycetes are characterized (and distinguished from other fungi) by the production of ascospores during sexual reproduction. Ascospores are formed by cells called asci (sing. ascus) inside sexual fruiting bodies, collectively called ascomata. The two main types of ascomata are disc-shaped apothecia (sing. apothecium) and urn-shaped perithecia (sing. perithecium). Apothecia are the most common sexual fruiting bodies among Ascomycota, and important for this study.

In the following description of the sexual reproductive cycle the morphological structures of flowering plants, which are analogous to the respective fungal structures, are included in quotation marks and parentheses. This is only for ease of understanding and does not imply any evolutionary
relatedness of these structures. The sexual reproductive cycle is explained as follows (Henssens & Jahns 1973, Dyer et al. 1992, Honegger & Scherrer 2008):

The crucial step during lichen mating is the coincidental contact of a passively dispersed spermatium or conidium (“pollen-equivalent”, “male” dispersal unit) and the receptive hypha called trichogyne (“stigma”) of an ascogonium (“carpel”, “female” part). Spermatia are released in masses from fungal structures called pycnidia (“anthers”). Pycnidia are flask shaped microscopic structures immersed into the lichen thallus, usually visible only as tiny pores on the upper thallus surface. An ascogonium (“carpel”) is a microscopic fruiting body primordium, which will give rise to an apothecium after spermatization (“pollination”). It is entirely immersed in the thallus, and only its receptive hypha, the trichogyne (“stigma”), protrudes above the upper cortex. Each lichen thallus can produce both types of gametes or gametangia, ‘male’ pycnidia shedding pycnospor (also called spermatia or conidia) and ‘female’ trichogynes, i.e. they would be considered ‘monoeccious’, if they were flowering plants. However, as in flowering plants, this does not mean that all lichens can self-fertilise.

After a spermatium establishes contact with a trichogyne, dikaryon formation takes place, i.e. the pairing of the haploid nuclei via plasmogamy, but without their fusion. This process has no equivalent in flowering plants, and its result is a dikaryotic ascogonium consisting of ascogenous (i.e. ascus-forming) hyphae. The monokaryotic (haploid), vegetative hyphae surrounding the dikaryotic, ascogenous hyphae, will now build up the apothecial fruiting body. When the fruiting body is preformed by vegetative tissue, the ascogenous hyphae will grow into the top layer of the apothecium called the hymenium, where their tips will differentiate into meiospore producing cells called asci (sing. ascus). It is in the young ascus where karyogamy (fusion of the nuclei) occurs, i.e. the formation of a diploid zygote. However, this is only a transitional state, immediately followed by meiosis. After meiosis, the four recombined haploid nuclei undergo another mitotic nuclear division. The ascus now contains eight haploid nuclei. Within the ascus, portions of the cytoplasm will be separated, each containing one haploid nucleus. Each of these compartments will develop into an ascospore. After maturation, the ascospores get released and passively dispersed by wind or water. The ascospore must germinate in the vicinity of compatible free-living algal cells, to form a new lichen thallus via re-lichenization.

The photobiont (algal partner of the lichen symbiosis) is not involved in the sexual reproduction of the mycobiont, apart from providing nutrients and being essential for survival of the germinating spore. However, as mentioned before, sexuality of green-algal photobionts is suppressed inside a thallus, even though it occurs in isolated cultures and in free living algae.
1.1.2 Mating Systems

Among sexually reproducing lichens (and non-lichenized ascomycetes), two groups with different mating systems are distinguished based on their ability to self-fertilise (Dyer et al. 1992, Seymour et al. 2005b, Honegger & Scherrer 2008, Werth 2010). Lichens capable of self-fertilization are referred to as homothallic, while those which rely on cross-fertilization with different individuals are heterothallic. The genetic background of this difference lies in the constitution of the mating type locus (MAT), which exists in two varieties called idiomorphs – not alleles, because the genes are entirely different, although located in the same place of the genome. After fertilization, the receptive cell (trichogyne) must contain two haploid chromosome sets with both MAT idiomorphs, in order to initiate dikaryon formation and apothecial development. Heterothallic (cross-fertilizing) lichens have individuals with two ‘genetic sexes’, each possessing only one MAT idiomorph per haploid chromosome set (i.e. individual), and therefore must be fertilized by an ‘opposite sex’ spermatium contributing the complementary MAT idiomorph. Homothallic lichens already contain two different MAT idiomorphs in each haploid chromosome set; i.e. the MAT genotype of the mating partner is irrelevant for mating success, and fertilisation serves merely to add a second haploid chromosome set (which can be identical) for sexual recombination.

Presently, it is not possible to predict the mating system of a lichen species, as it can differ among closely related species, and no correlation was found with the frequency of thalli bearing apothecia or their abundance per thallus (Honegger & Scherrer 2008). Because lichens grow very slowly, are difficult to culture, and have microscopically small pycnidia and trichogynes, the mating system cannot be examined using an approach equivalent to pollination-experiments in flowering plants. Instead elaborate molecular-genetic procedures are necessary to examine whether a lichen can self-fertilise or not.

1.2 Vegetative reproduction

Lichens have evolved a variety of structures for vegetative reproduction, i.e. the co-dispersal of mycobiont and photobiont in clonal fragments of the thallus. The two most common types of vegetative propagules are called isidia (sing. isidium) and soredia (sing. soredium); further types are known but irrelevant for this overview (see Büdel & Scheidegger 2008).

Isidia are fairly large (up to ca. 1 mm), often cylindrical, fully differentiated (cortex, algal layer, often with medulla) thalline projections; these are attached to the parent thallus by a very small, and often constricted attachment point, so they can easily be detached by physical contact. Isidia are not important for this thesis, since they are absent from the studied species, in contrast to soredia.
Soredia are much smaller, undifferentiated, dust-like particles consisting of loosely entangled fungal hyphae wrapped around some algal cells. They are shed from special ecorcicate zones of the thallus called soralia (sing. soralium), which can be located on different parts of the thallus depending on the species. They are easily detached and dispersed by wind and water.

The obvious advantage of these propagules over ascospores is the simultaneous dispersal of both symbionts. However, soredia are usually larger than ascospores, and can therefore be considered to serve mainly short distance dispersal and local population establishment, while ascospores are more suitable for long distance dispersal (Bailey 1966, Armstrong 1987, Armstrong 1991, 1994, Walser et al. 2001, Seymour et al. 2005b).

Although soralia are very common among lichens generally, it is noteworthy, that there is a tendency towards sterility in sorediate lichens, while vegetative propagules are commonly absent in sexually reproducing species (Bowler & Rundel 1975, p.336). This led to Poelt’s (1970) taxonomic concept known as Species Pair Theory; this accommodates pairs of species, which are morphologically indistinguishable, except that the first species reproduces only sexually while the second one reproduces only by soredia or isidia. However, this concept is controversial (Tehler 1982), and may be applicable to some cases and not applicable to others. Several recent molecular studies found no support for it, and therefore Printzen (2014) considers the Species Pair concept obsolete now.

1.3 Reproductive and Life Strategies in Lichens

A major difference between lichenised ascomycetes and vascular plants as well as many non-lichenised fungi is that lichen fruiting bodies are (in the vast majority) perennial structures, i.e. there is no annual growing season and they shed spores for several consecutive years. In spite of this fundamental difference, there seems to be a remarkable diversity of life strategies and reproductive strategies among lichens, analogous to that known from vascular plants (Rogers 1990, Jahns & Ott 1997). The similarities go far beyond the simple ‘choice’ of sexual versus vegetative reproduction emphasised by Bowler & Rundel (1975). Jahns & Ott (1997) have attempted to apply the C-S-R model (Grime 1974) and the concept of r- and K-strategists (MacArthur & Wilson 1967, Gadil & Solbrig 1972) to a number of lichens. The C-S-R model distinguishes between three classes: competitors, stress tolerators and ruderals (i.e. disturbance tolerators); each species can be assigned a position in a triangle cornered by these extremes. The concept of r- and K-strategists compares species with regard to their relative resource allocation towards either abundant reproduction and dispersal (short-lived r-strategists) or persistence of mature specimens in their original habitat with comparatively low numbers of offspring (long-lived K-strategists).
Also the vascular plant classification by Frey & Hensen (1995) into the following 8 groups of varying life history and reproductive strategy seems to be applicable to lichens: annual shuttle species, fugitives, cryptophytes, short-lived shuttle species, colonists, perennial colonists, perennial shuttle species, perennial stayers.

Jahns & Ott (1997) have reviewed the lichenological literature and give various examples showing that although lichens are mostly using the K-strategy, the whole group does cover the entire r-K continuum as well as the C-S-R model (Rogers 1990). Examples are known for some ephemeral lichens (completing their life cycle within one or very few years), many pioneer species, stress-tolerant and competitive perennials, short-lived and long-lived shuttle species (Jahns & Ott 1997). Even the equivalent of hapaxanth plants is found among lichens, i.e. species that live for a couple of years before reaching sexual maturity, and they die after sporulation.

2. Introduction to the lichen family Icmadophilaceae and the main study species *Icmadophila splachnirima*

The present PhD project focuses on members of the lichen family Icmadophilaceae. Members of this family are characterised amongst others by pink apothecia without a margin containing photobiont cells, which produce rather small, colourless ascospores (Rambold *et al.* 1993). Thallus morphology is very variable, comprising species with crustose, foliose and fruticose thalli. Four of the currently eight recognised genera are believed to have lost the ability for sexual reproduction; these are *Thamnolia, Siphula, Endocena* and the recently described *Chirleja*. In the absence of fruiting body characters, their placement in the family Icmadophilaceae is based on molecular-genetic evidence. The circumscription of the genus *Icmadophila* has changed a lot since about 1990, and it still has not settled (for details see introduction of Chapter 2).

The main focus of this thesis is on *Icmadophila splachnirima* (Hook.f. & Taylor) D.J. Galloway emend. L. Ludw., a rare lichen that occurs mostly in endangered subalpine boggy habitats; it has recently received the conservation status ‘Nationally Vulnerable’ in New Zealand (De Lange *et al.* 2012). It grows on continuously moist peaty soil, over bryophytes and plant debris, within a landscape dominated by tussock and low shrubs. It has a disjunct Australasian distribution in Victoria, Tasmania, the South Island of New Zealand, Stewart Island, Chatham Island and the subantarctic Auckland and Campbell Islands. The disjunctions in New Zealand could be partly explained by plate tectonics (Galloway 2008: 334), but also by incomplete distribution data. The species can be readily recognised by the combination of eye-catching, bright pink, sessile apothecia and a small-foliose, lettuce-green thallus (Figure 1; Galloway & Elix 1980, Galloway 2007). All other lichens with pink, sessile apothecia have a crustose thallus. Another prominent morphological character, and important to recognise
sterile specimens, is the lack of a lower cortex or any specialized attachment structures on the lower surface, i.e. the arachnoid (spider web-like), white medullary hyphae are fully exposed and also serve as attachment to the substratum.

The photobiont is believed to belong to the unicellular green algal genus *Coccomyxa* (Galloway & Elix 1980, Galloway 1985, illustrated in Tschermak-Woess 1988, p. 71). This genus is characterised by an elongated ellipsoid cell shape and parietal chloroplast; a good scaled colour illustration was published by Büdel (2010: 328). The algal partner of *I. splachnirima* stands out among other *Coccomyxa* algae in having exceedingly small cell size of only 4-5 × 2-3 μm, which is smaller than many prokaryotes.

A formerly unrecognised vegetative reproductive mode via marginal soralia was recently reported for the first time for *I. splachnirima* (Ludwig 2011), in addition to the previously known sexual reproduction by apothecia. In the field these two reproductive modes appeared more or less mutually exclusive, with vegetative propagation prevailing in more exposed and dryer micro-habitats than sexual thalli. It was therefore hypothesized that the observed transition from sexual to vegetative reproduction might be a response to the apparently contrasting micro-environmental conditions. According to this hypothesis, the species’ bimodal reproductive strategy involves an environmentally triggered switch to vegetative reproduction when apothecial development is arrested in an early stage, due to adverse growth conditions. This hypothesis found initial support from data collected by the author during a British Lichen Society-funded long-term reciprocal transplant experiment prior to this PhD project. The results were outlined by Ludwig (2012) and presented in a talk at the 7th International Association for Lichenology Symposium in January 2012. Briefly, the pilot study showed that it was possible to induce the alternate reproductive mode by swapping sexual (with apothecia, not sorediate) and vegetative (sorediate, only apothecium initials) thalli in the field, to let them grow under micro-climatic conditions usually associated with specimens exhibiting the alternate reproductive mode. This gave the impetus for the present PhD study, to explore the reproductive ecology of *I. splachnirima* in detail.

The frequent co-occurrence of both reproductive modes (either in nearby thalli or rarely on the same thallus) makes *I. splachnirima* an exceptional species, because most lichen species can only propagate by one reproductive mode, either sexual or vegetative (see above).

With regard to its life history and reproductive strategy, *I. splachnirima* may be considered a perennial pioneer, as it often colonises freshly exposed soil and forms lasting colonies. It seems not to be a competitive species, since it gets often overgrown by other lichens (e.g. *Cladia* and *Cladonia*), bryophytes and vascular plants. In turn, disturbance appears to be important for *I. splachnirima*, as it keeps the soil open and eliminates competitors (at least temporarily); for example the species is
frequently found along track cuttings or directly on regularly used tramping tracks. The potential role of environmental stress on the species’ reproduction is hoped to be clarified in course of this study. The usually abundant fruiting body production (often in very small thalli) may indicate an r-strategy; however, the numerous, large, sterile and sorediate thalli discovered recently are rather suggestive of a K-strategist. Perhaps this species combines traits of both life strategies, and the switch between sexual and vegetative reproduction implies a simultaneous switch between r- and K-strategies.

Nothing is known about the mating system of *I. splachnirima*. However, mating is most likely mediated by pycnospores shed from pycnidia, which are considered to act as ‘male’ gametes (‘spermatia’) during fertilisation (Honegger & Scherrer 2008) and were first reported to exist in this species by Ludwig (2011). Previously unpublished illustrations of these pycnidia are shown in Figures 1-3.
Figure 1. Thallus portion of *I. splachnirima* with pink apothecia, apothecial initials (ai) and pycnidia (p), which are illustrated here for the first time. Conidiospores shed from pycnidia are believed to act as ‘male’ gametes during fertilisation. Some details are shown in Figures 2 and 3.

Figure 2. Detection of inconspicuous pycnidia of *I. splachnirima*. Young apothecial initials (ai) and pycnidia (p) appear only as ‘colour irregularities’ of the upper cortex. The absence of a lower cortex in this species allows light to shine through the cavities and ostioles of the pycnidia (see Figure 3). Scale bar is 1 mm for both pictures.
3. Relevance of Research

This dissertation is mainly of conservational relevance for the studied species, and also for the family Icmadophilaceae and for species growing in similar habitats. Understanding the significance of both modes of reproduction and their impact on the genetic population structure and dispersal ability is critical to conserving this species in the face of climate change and the declining extent and degradation of wetlands and wet tussock grasslands generally. Furthermore, the study reveals the mating system and clarifies the phylogenetic relationships of *I. splachnirima*.

This thesis contributes to lichen conservation in general, as ground dwelling lichen communities in bogs and wetter subalpine tussock grasslands are very much understudied. The relationships between micro-climate and reproductive mode in *I. splachnirima* may serve as an example for related species, or species with similar habitat requirements.

Additionally, the genetic work contributes to a growing body of knowledge on the phylogenetic relationships, dispersal patterns and molecular-genetic basis of sexuality in lichens, aspects of lichen biology that have received a lot of attention from lichenologists in the past two decades. It also clarifies the phylogenetic relationships of *I. splachnirima* within the Icmadophilaceae and scientifically supports a replacement of the species in the genus *Knightiella*. Further, the mating system of *I. splachnirima* is clarified.
Apothecia are described for the first time for members of the genus *Siphula*, a discovery that puts an end to more than one century of speculation about the reproduction of the species, which was considered to be strictly sterile before.

The genetic work of this thesis also contributes one methodological ‘novelty’, or at least an improvement compared to similar commercial as well as conventional non-commercial methods the author is aware of. That is a genomic DNA extraction method, which mimics quick but expensive commercial DNA extraction kits with silica spin-columns, but at a fraction of the cost (see Chapter 2). The method combines separately purchasable silica spin-columns sold for use with self-made buffers in PCR-clean-up, and initial conventional DNA extraction using a CTAB buffer. The general idea of purifying CTAB-extracted DNA raw extracts using a PCR clean-up kit has been published before. The improvement added here is to use very affordable separately purchased spin-columns and self-made buffers based on very cheap chemicals, instead of a commercial kit. The genomic DNA raw extract is purified as if it were a PCR product, and as if the components were from a commercial kit. It saves a lot of money compared to commercial kits, and a lot of time compared to conventional DNA extraction methods using isopropanol precipitation. Another advantage compared to classic isopropanol precipitation is that very small or old samples can be extracted without the danger of losing the pellet after the final washing step. This quick and very affordable method was successfully used dozens of times, and will be attractive especially to grad students and young researchers with a limited budget.

### 4. Thesis outline

This PhD thesis consists of 7 chapters plus a General Introduction and General Conclusions section. Each of the 7 chapters is written in paper-style, i.e. with separate introduction, material & methods, results, discussion and summary sections. To facilitate comprehension by academic readers, which are not necessarily experts in the particular fields examined, a little more detail is provided than would usually be the case in an article of a peer-reviewed international journal.

The first 5 chapters examine the reproductive ecology of *Icmadophila splachnirima*, the lichen species on which this dissertation is focused. The last two chapters deal with aspects of the reproduction of a few other lichens placed in the same family Icmadophilaceae, which have relevance to the previous chapters. These additional two chapters were not part of the original project, as they are based on original observations made in the course of the main project. It seemed appropriate, however, to include them in this thesis, as they were directly derived from the core thesis work and address closely related topics in Icmadophilaceae.
Chapter 1 provides a comprehensive compilation of the entire known global distribution of *Icmadophila splachnirima*, based on public herbarium records, literature records, and personal observations. This compilation is an asset in itself, but it mainly served to locate potential study sites for field work (chapter 5) and sampling sites or herbarium specimens for a population genetic approach (chapter 3). Herbarium studies also contributed to a re-assessment of the distribution of a related species of *Icmadophila* in New Zealand (Chapter 6).

Chapter 2 is a molecular genetic reassessment of the genus *Icmadophila*. The current generic placement of *I. splachnirima* is very recent and still controversial, since a previous genetic study on the nuclear ribosomal small subunit (nuSSU) found indications that the species should stay in the monotypic genus *Knightiella*, as prior to its transfer to *Icmadophila*. However, this previous study included only a single sample of *I. splachnirima* and examined only one locus. Its findings remained without the according nomenclatorial consequences, since *Icmadophila splachnirima* is still the officially valid name of the species. The purpose of this chapter is to settle this conflict, by providing further genetic data of several ribosomal DNA loci and from several specimens. Since the genus *Icmadophila* as currently circumscribed consist of only five species, of which the generitype *I. ericetorum* had to be examined anyway, specimens of all these species were included in the study, together with several other members of Icmadophilaceae, which increases the value of this thesis substantially. Confidence in the phylogenetic relationships of *I. splachnirima* to the other species in *Icmadophila* and the remaining Icmadophilaceae is essential when trying to compare their ecological and reproductive behaviour (Chapters 4 and 5).

Chapter 3 describes a population genetic approach used to examine whether gene flow exists between the very disjunct known populations of *I. splachnirima* (cf. Chapter 1), and if so to assess the relative importance of sexual versus vegetative propagules for long distance dispersal. Fungal and algal sequences of the ribosomal Internal Transcribed Spacer region (ITS1, 5.8S, ITS2) were used as molecular markers for genetic similarity. Samples originating from almost the entire geographical range of the species were examined. Samples of several sterile and sorediate specimens were included as well, to address the issue of a potential Species Pair being involved, with one sexual and a second sorediate species. The phylogenetic work in Chapter 2 was derived from this chapter, after specimens of the remaining *Icmadophila* species became available.

Chapter 4 examines the mating system of *I. splachnirima*, i.e. whether the species is capable of self-fertilisation or not. The importance of this question is self-evident in context of the reproductive
ecology and conservation of the species, and its answer is important for the interpretation of results from the population genetic approach (chapter 3) and the long-term reciprocal transplant experiment described in Chapter 5. Molecular-genetic techniques were used to characterise the mating type locus (MAT), and to screen for the presence of both MAT genes in haploid thalline tissue.

Chapter 5 is the central chapter of this thesis. It tests the hypothesis that a switch from sexual to vegetative reproduction in *Icmadophila splachnirima* occurs as a response to micro-environmental conditions. Four field-based approaches were used: (1) a long-term reciprocal transplant experiment similar to the pilot study mentioned above, (2) *in situ* quantification of the light environment and (3) moisture regime related to specimens of both reproductive modes, and (4) quantification of a cellular stress marker following short-term reciprocal transplants. This chapter is much more extensive than the other chapters, and it explores how the micro-environmental conditions relate to the species’ reproductive ecology. This thesis was originally based on this chapter and was supposed to involve much more field ecological work (e.g. more replicates) and much less of the molecular-genetic work described in the previous chapters. For various reasons, however, the project shifted more and more into a genetic direction, but importantly without losing the connection to the species’ reproductive ecology.

Chapter 6 investigates the presence and distribution in New Zealand of *Icmadophila ericetorum*, which has previously been reported as being present there. This work was derived from the phylogenetic and population genetic approaches (Chapters 2 and 3), because it was intended to include NZ *I. ericetorum* specimens in the genetic analyses. This was expected to show how much Northern Hemisphere and NZ *I. ericetorum* differ from *I. splachnirima*, and whether both species associate with the same algae. Furthermore, *I. ericetorum* and *I. splachnirima* have very similar substrate and habitat preferences, which raised hopes for an opportunity to compare the relationship between micro-habitat and reproduction in *I. ericetorum*, parallel to the work on *I. splachnirima* in chapter 5. However, the author failed to find *I. ericetorum* in suitable habitats of reported localities, and initial re-examination some herbarium specimens showed that these were misidentified *Dibaeis absoluta* (Icmadophilaceae), a superficially similar species. Therefore, serious doubts about the presence of *I. ericetorum* in NZ arose, which had to be followed up.

Chapter 7 reports for the first time on the discovery of genuine apothecia in members of the genus *Siphula* (Icmadophilaceae), which is renowned among lichenologists for being strictly sterile. Fertile specimens of *Siphula decumbens* were found during searches for additional localities of *I. splachnirima
(Chapter 1 and 3) since both are terricolous and often grow together or not far apart from each other in the same general habitat. More importantly, it appeared to the author that the occurrence of apothecia in the *S. decumbens* may also be correlated to certain micro-habitat conditions, a noteworthy parallel to the suspected environmental dependence of the reproductive mode in *I. splachnirima* (Chapter 5). Molecular-genetic analyses were used to test whether the apothecia were really part of the lichen, or just the fruits of a parasitic fungus, which was the case in previous reports on fertile *Siphula* specimens. The genetic work went hand in hand with the phylogenetic work on the genus *Icmadophila* (Chapter 2), since sequences of additional related taxa were needed to optimise cladistic resolution; the generic placement of the *S. decumbens* group was shown by previous studies to be in need of a revision as well. The multiple overlaps with aspects of the main project justify the inclusion of this chapter in a dissertation focused primarily on the reproductive biology of *I. splachnirima*.

**Chapter 8** presents the overall conclusions of this thesis by linking the findings of the previous 7 chapters into a coherent bigger picture.

The references of all chapters are combined in a single bibliography following Chapter 8. An Electronic Appendix (DVD) is supplied with this thesis containing images, raw data and R-scripts, DNA sequence alignments and copies the author’s publications related to this thesis.
5. Aims

Original aims:

- Produce a comprehensive compilation of the known global distribution of *I. splachnirima*.
- Clarification of the phylogenetic position of the *I. splachnirima* mycobiont and photobiont.
- Population genetic examination of gene flow among disjunct localities of *I. splachnirima*.
- Examination of the mating type locus (MAT) and mating system of *I. splachnirima*.
- Testing the hypothesis that the bimodal reproduction of *I. splachnirima* is influenced by the micro-environment.

Additional aims:

- Clarify the doubtful occurrence and distribution of *Icmadophila ericetorum* in New Zealand and the Southern Hemisphere.
- Examine the apparently first fertile collections of *Siphula decumbens* and *S. fastigiata*, in order to verify their ability of ascoma formation.
CHAPTER 1
The Biogeography of Icmadophila splachnirima

1. Introduction

The lichen Icmadophila splachnirima is endemic to south-eastern Australasia, i.e. the southern part of New Zealand including its subantarctic off-shore islands, as well as the Australian states of Tasmania and Victoria. However, the detailed biogeographic information on this species is currently dispersed among various types of literature, in a range of herbaria and ecological databases.

It is the purpose of this chapter, to provide a compilation of the scattered biogeographic information available on this species. This compilation is an essential part of this dissertation, in order to identify suitable study sites, and to identify localities to gather specimens for a population-genetic approach, but also to better understand the ecology and natural abundance of this rare lichen.

2. Material & Methods

The New Zealand and Australian Virtual Herbaria (http://www.virtualherbarium.org.nz/home, http://avh.chah.org.au/) were initially searched, to determine which herbaria hold specimens of I. splachnirima. The online output of the virtual herbaria provides only incomplete specimen details, therefore all full details were requested from the respective herbaria directly. Full specimen data was supplied by the following herbaria: AK (04.04.2011, Dhahara Ranatunga, Auckland), BM (01.05.2014, Holger Thüs, London), CHR (16.10.2010, Jane Cruickshank, Christchurch), FH (27.07.2014, Michaela Schmull, Cambridge, USA), HO (13.04.2011, Gintaras Kantvilas, Hobart), MEL (05.05.2011, Alison Vaughan, Melbourne), WELT (05.04.2011, Antony Kusabs, Wellington). Data from CANB, CBG (both Canberra) and NSW (Sydney) was obtained via the Australian National Herbarium Specimen Information Register (15.03.2011, Maggie Nightingale).

Otago Regional Herbarium (OTA) specimen data had not yet been data-based, therefore it was directly obtained from the herbarium packets. The identity of all specimens held at New Zealand (NZ) herbaria was checked prior to including the specimen information in this analysis.

Specimen data from the following herbaria was downloaded from the Global Biodiversity Information Facility database (http://data.gbif.org/occurrences/), using the species’ current accepted name and synonyms as search terms: AZU, BG, CABI, S, TSB. Specimen data from the Michigan State University Herbarium (MSC) was obtained via the herbarium database (http://herbarium.lib.msu.edu/, accessed 09.05.2011), and the same information was available through the Consortium of North American Lichen Herbaria (http://symbiota.org/nalichens/, accessed 06.04.2011).

In order to augment the data obtained from the sources described above, searches for new localities were conducted in New Zealand between 2010 and 2014; representative specimens were collected and GPS coordinates recorded where the species was found.

Distribution maps were generated using the free software R 2.10.1 with the package ‘maps’ and the Pacific-centric map template ‘world2Hires’. The geographic coordinates plotted in the maps, were either provided as geo-referenced public herbarium records, or manually inferred using Google Earth and topographical maps of New Zealand, which are freely accessible via [http://www.topomap.co.nz/](http://www.topomap.co.nz/) or [http://www.linz.govt.nz/topography/topo-maps/index.aspx](http://www.linz.govt.nz/topography/topo-maps/index.aspx). When coordinates of NZ herbarium specimens were provided in a different format (e.g. NZTM), these were converted to World Geodetic System 1984 (WGS84) format using the website [http://apps.linz.govt.nz/coordinate-conversion/](http://apps.linz.govt.nz/coordinate-conversion/).

### 3. Results

All results are compiled in Figure 4 and Table 1 to Table 7. Tasmania stands out as the centre of distribution and abundance, with over 70 known localities spread over almost the entire island. In contrast, *I. splachnirima* is known from only 4 sites in Victoria, which are however widely spread, and include the globally Northernmost and Westernmost records in the Grampian Mountains National Park. The species’ known distribution in New Zealand is very disjunct as well. It is centred around Stewart Island and the far South of the South Island, but more recently collections were gathered from the Northern West-coast of the South Island. It is also known from subantarctic Auckland and Campbell Island, the latter being its Southernmost locality. The Easternmost collections were recently gathered from the Chatham Islands. Currently, no records exist for the New Zealand North Island, Antipodes Island and Snares Island. No confirmed record exists from Macquarie Island Kantvilas & Seppelt (1992), but Dodge (1948: 78) identified a poorly developed thallus as *Thelidea* sp. (at that time *Thelidea corrugata* was the commonly used name of *I. splachnirima*).

Several new localities were found by the author in New Zealand: the Blue Mountains summit plateau wetlands, Ajax Hill in the Catlins region, Bald Hill in the Longwood Range, the Heaphy Track between Karamea and Golden Bay, Oban on Stewart Island. Furthermore, the author has gathered collections from Denniston Plateau near Westport, Takitimu Mts. near Te Anau, and Rakeahua Flat on
Stewart Island; these localities were previously reported in the literature Galloway (1968a, 2007), but no voucher specimens were cited or seemed to exist. A collection from Croesus Track in the Paparoa Range near Greymouth from 1999 by Barbara Polly was not included in the list of Galloway (2007: 651-652), and the author has gathered further specimens from this site as well.

The author visited the localities Black Swamp and Mt. Maungatua in Otago, but was not able to find the species there, although Black Swamp is only a very small bog of about 300 m diameter, and the exact area where he collected the species in 1966 was pointed out by Dr D.J. Galloway on a field trip. Also the Awarua wetlands near Invercargill were unsuccessfully searched by the author. However, only a tiny fraction of this vast area had been briefly visited. It is noteworthy, however, that draining of the Awarua plain by local farmers seems to have facilitated the growth of manuka shrubs in particular, which now form a low forest cover over huge areas, which used to be open bog in the 1960s (pers. comm. D.J. Galloway).
Figure 4. Dot map showing the known global distribution of *Icmadophila splachnirima* in south-east Australia (Victoria and Tasmania) and New Zealand. Red dots are localities of the species, blue squares are cities or towns, + symbols represent intersections of every 5th meridian and parallel.
Table 1. Records from the New Zealand South Island. Annotations in [...] were missing and have been inferred/emended by the author when possible or appropriate. Collection dates are given in the standard format dd.mm.yyyy, except when day or day and month were missing. [ref] indicates refined coordinates. Records are arranged according to collection date and locality.

<table>
<thead>
<tr>
<th>locality in North-West of South Island</th>
<th>Coordinates</th>
<th>altitude</th>
<th>Collector</th>
<th>coll. date</th>
<th>Herb. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heaphy Track, 32 km SW of Collingwood</td>
<td>40°53’59.2&quot;S 172°24'20.4&quot;E</td>
<td>885 m</td>
<td>Lars Ludwig</td>
<td>01.01.2014</td>
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<td>Heaphy Track, ca. 40 km NEN of Karamea</td>
<td>40°53’11.8&quot;S 172°13'01.8&quot;E</td>
<td>695 m</td>
<td>Lars Ludwig</td>
<td>04.01.2014</td>
<td>OTA 064214</td>
</tr>
<tr>
<td>Stockton Plateau</td>
<td>41°40.930’S 171°54.020’E</td>
<td>850 m</td>
<td>David Glenny</td>
<td>09.03.1992</td>
<td>WELT L003689</td>
</tr>
<tr>
<td>Croesus Track from end of Blackball Road [Paparoa Range]</td>
<td>42°20’18&quot;S 171°23'23.55&quot;E [ref]</td>
<td>300 m</td>
<td>Barbara Polly</td>
<td>27.10.2000</td>
<td>WELT L006393</td>
</tr>
<tr>
<td>Croesus Track, Blackball end, Paparoa Range</td>
<td>42°20’05&quot;S 171°23'48&quot;E</td>
<td>~330 m</td>
<td>Lars Ludwig</td>
<td>07.01.2014</td>
<td>OTA 064240</td>
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<tr>
<td>Denniston Plateau near Westport</td>
<td>41°46’25&quot;S 171°23'55.5&quot;E</td>
<td>300 m</td>
<td>Barbara Polly</td>
<td>27.10.2000</td>
<td>WELT L003689</td>
</tr>
<tr>
<td>Denniston Plateau near Westport, Whareatea Mine area</td>
<td>41°45’13.5&quot;S 171°47'13.5&quot;E</td>
<td>800 m</td>
<td>Lars Ludwig</td>
<td>05.03.2012</td>
<td>OTA 062505</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>locality in Otago</th>
<th>Coordinates</th>
<th>altitude</th>
<th>Collector</th>
<th>coll. date</th>
<th>Herb. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maungatua, S. of Dunedin [actually West of Dunedin]</td>
<td>[45°52’40&quot;S 170°07'00&quot;E]</td>
<td>[&lt;895 m]</td>
<td>H.H. Allan</td>
<td>12.1936</td>
<td>CHR 373970</td>
</tr>
<tr>
<td>Mt. Maungatua, near Dunedin</td>
<td>[45°52’40&quot;S 170°07'00&quot;E]</td>
<td>2800 ft [850 m]</td>
<td>D.J. Galloway</td>
<td>16.04.1966</td>
<td>BM001085476</td>
</tr>
<tr>
<td>on track near Pulpit Rock E side [Silver Peaks, ca. 13 km N of Dunedin]</td>
<td>[45°44’48&quot;S 170°27'28&quot;E]</td>
<td>2200’ [670 m]</td>
<td>J. Murray</td>
<td>03.1959</td>
<td>OTA 053083</td>
</tr>
<tr>
<td>Silver Peaks North of Dunedin</td>
<td>45°44’32.1&quot;S 170°27'04.6&quot;E</td>
<td>740 m</td>
<td>Lars Ludwig</td>
<td>15.09.2013</td>
<td>OTA 064245</td>
</tr>
<tr>
<td>Silver Peaks near Dunedin</td>
<td>45°44’48.0&quot;S 170°27'27.7&quot;E</td>
<td>675 m</td>
<td>Lars Ludwig</td>
<td>15.09.2013</td>
<td>OTA 064246</td>
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<tr>
<td>Swampy Summit</td>
<td>[45°48’00&quot;S 170°28'45&quot;E]</td>
<td>2260’ [688 m]</td>
<td>J. Murray</td>
<td>03.1959</td>
<td>OTA 053084</td>
</tr>
<tr>
<td>Swampy Hill, Dunedin [= Swampy Summit]</td>
<td>[45°47’45&quot;S 170°28'52&quot;E]</td>
<td>2200 ft. [670m]</td>
<td>D.J. Galloway</td>
<td>19.03.1966</td>
<td>BM001085480</td>
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<tr>
<td>Swampy Hill (Swampy Summit Dunedin)</td>
<td>[45°47’45&quot;S 170°28'52&quot;E]</td>
<td>2200’ [670 m]</td>
<td>D.J. Galloway</td>
<td>19.03.1966</td>
<td>OTA 046895</td>
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<tr>
<td>Swampy Hill – Dunedin [= Swampy Summit]</td>
<td>[45°48’00&quot;S 170°28'45&quot;E]</td>
<td>2240’ [682 m]</td>
<td>D.J. Galloway</td>
<td>01.1969</td>
<td>CHR 373973</td>
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<tr>
<td>Swampy Summit, Dunedin</td>
<td>45°48’03.3&quot;S 170°28'57.5&quot;E</td>
<td>~720 m</td>
<td>Lars Ludwig</td>
<td>07.09.2012</td>
<td>OTA 063988</td>
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<td>Swampy Summit, Dunedin</td>
<td>45°47’46.3”S 170°29'00.2&quot;E</td>
<td>~720 m</td>
<td>Lars Ludwig</td>
<td>29.03.2012</td>
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<tr>
<td>Swampy Summit, Dunedin</td>
<td>45°47’45.7”S 170°28'56.0&quot;E</td>
<td>~720 m</td>
<td>Lars Ludwig</td>
<td>29.03.2013</td>
<td>OTA 062508</td>
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<tr>
<td>Black Swamp, Otago. Map Ref. H45 696628</td>
<td>[46°00’03&quot;S 169°53'54&quot;E]</td>
<td>[390 m]</td>
<td>A. Pelletier, C. Haas</td>
<td>01.04.2003</td>
<td>OTA 058013</td>
</tr>
<tr>
<td>South of Tapanui Hill Summit [Blue Mountains], &lt; 0.5 km from bog car park A</td>
<td>[45°53’31.2”S 169°23'19.8&quot;E] A</td>
<td>994 m</td>
<td>Janet Ledingham</td>
<td>23.02.2008</td>
<td>private herbarium of D.J. Galloway</td>
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<tr>
<td>Blue Mts. near Tapanui</td>
<td>45°55’56.3”S 169°20'18.9&quot;E</td>
<td>~900 m</td>
<td>Lars Ludwig</td>
<td>26.01.2012</td>
<td>OTA 062506 OTA 062507</td>
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</table>
Blue Mts. near Tapanui 45°56'43.8"S 169°21'07.2"E ~900 m Lars Ludwig 03.02.2012 OTA 062503
between “organ pipes” and Butter’s Peak, Mt. Cargill area, Dunedin 45°48'40.2"S 170°33'57.8"E 590 m Lars Ludwig 12.05.2013 OTA 062510

<table>
<thead>
<tr>
<th>locality in Southland</th>
<th>Coordinates</th>
<th>Altitude</th>
<th>collector</th>
<th>coll. date</th>
<th>Herb. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Awarua Plain 1 mile from Bay</td>
<td>[46°32'30&quot;S 168°27'00&quot;E]</td>
<td>[&lt;20m]</td>
<td>W. Martin</td>
<td>24.12.1958</td>
<td>OTA 053080</td>
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<tr>
<td>at Awarua Bay, near road end</td>
<td>[46°34'30&quot;S 168°31'00&quot;E]</td>
<td>c. 1-5 m</td>
<td>D.J. Galloway</td>
<td>02.1961</td>
<td>CHR 373971</td>
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<tr>
<td>at Awarua Bay, near road end</td>
<td>[46°34'30&quot;S 168°31'00&quot;E]</td>
<td>c. 1-5 m</td>
<td>R. Johnson</td>
<td>02.1961</td>
<td>CHR 373972</td>
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<tr>
<td>Borland Bog</td>
<td>[45°47'00&quot;S 167°33'24&quot;E]</td>
<td>180m</td>
<td>J. Steel</td>
<td>29.03.1996</td>
<td>OTA 046889</td>
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<td>Aparima Forks bog, Takitimu Mts.</td>
<td>45°41'25.2&quot;S 167°57'34.1&quot;E</td>
<td>550 m</td>
<td>Lars Ludwig</td>
<td>26.10.2011</td>
<td>OTA 063975</td>
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<td>Aparima Hut bog, Takitimu Mts.</td>
<td>45°42'17.5&quot;S 167°58'58.5&quot;E</td>
<td>490 m</td>
<td>Lars Ludwig</td>
<td>26.10.2011</td>
<td>OTA 063974</td>
</tr>
<tr>
<td>between East Ajax Spur and Ajax Hill, Catlins</td>
<td>46°26'00&quot;S 169°19'04&quot;E</td>
<td>610 m</td>
<td>Lars Ludwig</td>
<td>14.12.2012</td>
<td>OTA 062511</td>
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<tr>
<td>Ajax Hill bog, Catlins</td>
<td>46°25'49.6&quot;S 169°17'43.9&quot;E</td>
<td>665 m</td>
<td>Lars Ludwig</td>
<td>14.12.2012</td>
<td>OTA 062512</td>
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<tr>
<td>Longwood Range, &quot;Little Baldy Hill&quot; (map ref.: D46 121 318 [NZMS 260])</td>
<td>[46°12'36&quot;S 167°50'35&quot;E]</td>
<td>730 m</td>
<td>Peter N. Johnson, via Sue Maturin?</td>
<td>10.1996</td>
<td>PNJ 3103</td>
</tr>
<tr>
<td>near summit of Bald Hill, Longwood Range</td>
<td>46°10'10.8&quot;S 167°49'44.1&quot;E</td>
<td>783 m</td>
<td>Lars Ludwig</td>
<td>08.12.2010</td>
<td>photo</td>
</tr>
<tr>
<td>near summit of Bald Hill, Longwood Range; banks of tarn</td>
<td>46°10'07.5&quot;S 167°49'37.5&quot;E</td>
<td>781 m</td>
<td>Lars Ludwig</td>
<td>17.05.2011</td>
<td>photo</td>
</tr>
<tr>
<td>Coalburn, ca. 48 km West of Hump Ridge</td>
<td>46°10'42.6&quot;S 166°42'10.8&quot;E</td>
<td>250 m</td>
<td>Morgan McLean</td>
<td>26.03.2013</td>
<td>DoC collection</td>
</tr>
</tbody>
</table>

A – Unnumbered collection in D.J. Galloway’s private herbarium; a grid reference from her field book was provided by J. Ledingham, and converted to coordinates.

B – Allison Knight was the first to find it there, and the Takitimu Mts. record in Galloway (2007: 651-652) refers to this observation (pers. comm. A. Knight, D.J. Galloway).

C – Private herbarium of Peter N. Johnson

D – Collected in course of the Department of Conservation TIER1 biodiversity monitoring program, for which the author had been employed to identify lichens. Two very small, fertile specimens were collected, and these vouchers (plot G171, specimen identifiers NV201300029 and NV201300189) are still with the DoC non-vascular plant collection, currently housed in the Otago University Botany Department.

Table 2. Records from Stewart Island. See caption of Table 1 for specifications.

<table>
<thead>
<tr>
<th>locality in Stewart Island</th>
<th>Coordinates</th>
<th>Altitude</th>
<th>collector</th>
<th>coll. date</th>
<th>Herb. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>W ridge Mt. Anglem</td>
<td>[46°44'20&quot;S 167°54'40&quot;E]</td>
<td>[&lt;980 m]</td>
<td>D.J. Galloway</td>
<td>01.02.1966</td>
<td>BM001085483</td>
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<tr>
<td>Bald Cone, Port Pegasus</td>
<td>[47°13'29&quot;S 167°35'50&quot;E]</td>
<td>700' [213m]</td>
<td>D.J. Galloway</td>
<td>10.02.1967</td>
<td>CHR 608294</td>
</tr>
<tr>
<td>Bald Cone, Port Pegasus</td>
<td>[47°13'29&quot;S 167°35'50&quot;E]</td>
<td>[&lt;230 m]</td>
<td>D.J. Galloway</td>
<td>10.02.1967</td>
<td>CHR 608291</td>
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<tr>
<td>Bald Cone, Port Pegasus</td>
<td>[47°13'29&quot;S 167°35'50&quot;E]</td>
<td>[&lt;230 m]</td>
<td>D.J. Galloway</td>
<td>10.02.1967</td>
<td>CHR 608296</td>
</tr>
<tr>
<td>Bald Cone, Port Pegasus</td>
<td>[47°13'29&quot;S 167°35'50&quot;E]</td>
<td>&lt;230 m</td>
<td>D.J. Galloway</td>
<td>02.1967</td>
<td>BM001085479</td>
</tr>
<tr>
<td>Sylvan Cove - Port Pegasus</td>
<td>[47°13'50&quot;S 167°33'40&quot;E]</td>
<td>[cf. CHR 608295]</td>
<td>D.J. Galloway</td>
<td>10.02.1967</td>
<td>CHR 608293</td>
</tr>
<tr>
<td>Sylvan Cove, Port Pegasus</td>
<td>[47°13'50&quot;S 167°33'40&quot;E]</td>
<td>[cf. CHR 608295]</td>
<td>D.J. Galloway</td>
<td>10.02.1967</td>
<td>BM001085473</td>
</tr>
</tbody>
</table>
Table 3. Records from Chatham Islands. See caption of Table 1 for specifications.

<table>
<thead>
<tr>
<th>locality in Chatham Islands</th>
<th>Coordinates</th>
<th>altitude</th>
<th>collector</th>
<th>coll. date</th>
<th>Herb. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tuku, near Awatotara Creek; [old] Chatham Sheet 2 / 405455</td>
<td>[44°02'20&quot;S 176°37'00&quot;W]</td>
<td>180 m</td>
<td>P.N. Johnson</td>
<td>03.02.2000</td>
<td>CHR 528225</td>
</tr>
<tr>
<td>Rekohu (Chatham Island), Tuku-a-Tamatea Nature Reserve</td>
<td>44°03’S 176°36’W</td>
<td>c. 256 m</td>
<td>P.J. de Lange, P.B. Heenan</td>
<td>02.06.2008</td>
<td>CHR 548855</td>
</tr>
<tr>
<td>Rekohu (Chatham Island), Tuku-a-Tamatea Nature Reserve</td>
<td>44°03’S 176°36’W</td>
<td>256 m</td>
<td>P.J. de Lange, P.B. Heenan</td>
<td>02.06.2008</td>
<td>CHR 303501</td>
</tr>
<tr>
<td>Rangiauria (Pitt Island), Hakepa Hill (Walkemup)</td>
<td>44°16’00’S 176°10’30”W</td>
<td>c. 220 m</td>
<td>P.J. de Lange, P.B. Heenan</td>
<td>30.11.2008</td>
<td>CHR 548847</td>
</tr>
<tr>
<td>Rangiauria (Pitt Island), Hakepa Hill (Walkemup)</td>
<td>44°16’00’S 176°10’30”W</td>
<td>220 m</td>
<td>P.J. de Lange, P.B. Heenan</td>
<td>30.11.2008</td>
<td>CHR 304001</td>
</tr>
</tbody>
</table>

G – Inference of coordinates from a 1981 map was assisted by Peter N. Johnson.
### Table 4. Records from Campbell Island. See caption of Table 1 for specifications.

<table>
<thead>
<tr>
<th>Locality in Campbell Island</th>
<th>Coordinates</th>
<th>Altitude</th>
<th>Collector</th>
<th>coll. date</th>
<th>Herb. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beeman Hill</td>
<td>[52°32'50&quot;S 169°09'00&quot;E]</td>
<td>250' [76m]</td>
<td>G.P. Poppleton</td>
<td>23.10.1959</td>
<td>OTA 061865</td>
</tr>
<tr>
<td>Beeman Hill</td>
<td>[52°32'50&quot;S 169°09'00&quot;E]</td>
<td>&lt;187 m</td>
<td>G.P. Poppleton (per Jas Murray)</td>
<td>-</td>
<td>CHR 608292</td>
</tr>
<tr>
<td>N.W. slopes Mt. Beeman</td>
<td>[52°32'43&quot;S 169°08'53&quot;E]</td>
<td>&lt;187 m</td>
<td>1959 party</td>
<td>027.1959</td>
<td>OTA 053078</td>
</tr>
<tr>
<td>North of Beeman Station</td>
<td>[52°32'52&quot;S 169°09'06&quot;E]</td>
<td>&lt;187 m</td>
<td>Richard C. Harris</td>
<td>22.12.1969</td>
<td>MSC 0113061</td>
</tr>
<tr>
<td>Beeman Station</td>
<td>[52°32'52&quot;S 169°09'06&quot;E]</td>
<td>ca. 20 m</td>
<td>R.C. Harris</td>
<td>-</td>
<td>TSB 28088</td>
</tr>
<tr>
<td>Along road to old Tucker Cove Station</td>
<td>[52°33'00&quot;S 169°08'45&quot;E]</td>
<td>&lt;60 m</td>
<td>Richard C. Harris</td>
<td>23.12.1969</td>
<td>MSC0001954</td>
</tr>
<tr>
<td>road to old Tucker Cove Station</td>
<td>[52°33'00&quot;S 169°08'45&quot;E]</td>
<td>&lt;60 m</td>
<td>R.C. Harris</td>
<td>23.12.1969</td>
<td>HO 310092</td>
</tr>
<tr>
<td>On point between Beeman Station and Tucker Cove.</td>
<td>[52°33'06&quot;S 169°09'00&quot;E]</td>
<td>&lt;20 m</td>
<td>Richard C. Harris</td>
<td>17.01.1970</td>
<td>MSC 0001957</td>
</tr>
<tr>
<td>Old Homestead on point between Tucker and Camp Coves.</td>
<td>[52°33'17&quot;S 169°08'45&quot;E]</td>
<td>&lt;40 m</td>
<td>Richard C. Harris</td>
<td>27.12.1969</td>
<td>MSC 0001955</td>
</tr>
<tr>
<td>Below first large outcrop on Lyall ridge.</td>
<td>[52°32'10&quot;S 169°10'00&quot;E]</td>
<td>&lt;400 m</td>
<td>Richard C. Harris</td>
<td>23.12.1969</td>
<td>MSC 0001953</td>
</tr>
<tr>
<td>Beeman boardwalk</td>
<td>52.54371°S 169.15179°E</td>
<td>~80 m</td>
<td>K. Vincent, S. King</td>
<td>23.11.2012</td>
<td>OTA 063971</td>
</tr>
<tr>
<td>Between Garden Cove and Filhol-Honey saddle</td>
<td>[52°34'10&quot;S 169°08'45&quot;E]</td>
<td>&lt;300 m</td>
<td>Henry A. Imshaug</td>
<td>23.12.1969</td>
<td>MSC 0113057</td>
</tr>
<tr>
<td>Between Garden Cove and Mt. Fihol</td>
<td>[52°34'09&quot;S 169°08'04&quot;E]</td>
<td>&lt;558 m</td>
<td>Henry A. Imshaug</td>
<td>11.01.1970</td>
<td>MSC 0113056</td>
</tr>
<tr>
<td>Between Mt. Azimuth and Courrejolles Peninsula</td>
<td>[52°29'30&quot;S 169°09'08&quot;E]</td>
<td>&lt;479 m</td>
<td>Henry A. Imshaug</td>
<td>30.12.1969</td>
<td>MSC 0113060</td>
</tr>
<tr>
<td>on slope above Venus Cove</td>
<td>[52°33'50&quot;S 169°08'40&quot;E]</td>
<td>&lt;558 m</td>
<td>H.A. Imshaug</td>
<td>15.01.1970</td>
<td>AK 241610</td>
</tr>
<tr>
<td>On slope above Venus Cove</td>
<td>[52°33'50&quot;S 169°08'40&quot;E]</td>
<td>&lt;558 m</td>
<td>H.A. Imshaug</td>
<td>15.01.1970</td>
<td>ASU 565447</td>
</tr>
<tr>
<td>On slope above Venus Cove.</td>
<td>[52°33'50&quot;S 169°08'40&quot;E]</td>
<td>&lt;558 m</td>
<td>Henry A. Imshaug</td>
<td>15.01.1970</td>
<td>MSC 0001950</td>
</tr>
<tr>
<td>On Filhol-Honey saddle.</td>
<td>[52°34'40&quot;S 169°09'00&quot;E]</td>
<td>&lt;300 m</td>
<td>Henry A. Imshaug</td>
<td>23.12.1969</td>
<td>MSC 0001959</td>
</tr>
<tr>
<td>At head of Garden Cove toward Filhol Peak.</td>
<td>[52°33'40&quot;S 169°08'16&quot;E]</td>
<td>-</td>
<td>Richard C. Harris</td>
<td>09.01.1970</td>
<td>MSC 0001949</td>
</tr>
</tbody>
</table>
Chapter 1 – Biogeography of *Icmadophila splachnirima*

On summit of Moubray Hill  
[52°33'10"S 169°14'50"E]  
<245 m  
H.A. Imshaug  
12.01.1970  
ASU 565417

NW of Sorenson Hut.  
[52°28'20"S 169°11'55"E]  
<200m  
Henry A. Imshaug  
18.01.1970  
MSC 0001951

South East Harbour catchment  
52.59124°S 169.16098°E  
~100 m  
S. King, K. Vincent, A. Whittaker  
OTA 062513

Table 5. Records from Auckland Islands. See caption of Table 1 for specifications.

<table>
<thead>
<tr>
<th>locality in Auckland Islands</th>
<th>coordinates</th>
<th>altitude</th>
<th>Collector</th>
<th>coll. date</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>[no details, but see $^H$]</td>
<td>[50°33'10&quot;S 166°14'50&quot;E]</td>
<td>[&lt;114 m]</td>
<td>P.W. James NZ/741f</td>
<td>[1963]$^1$</td>
<td>Galloway &amp; Elix (1980)$^1$</td>
</tr>
<tr>
<td>between Mt Eden and Meggs Hill</td>
<td>[50°29'50&quot;S 166°17'40&quot;E]</td>
<td>[&lt;43 m]</td>
<td>P.W. James NZ/368c</td>
<td>[1963]$^1$</td>
<td>Galloway &amp; Elix (1980)$^1$</td>
</tr>
</tbody>
</table>

$^H$ – the collector and 'Auckland Islands' was later emended in hand-writing by D.J. Galloway; Galloway & Elix (1980: 485) have erroneously cited this collection under its former accession OTA 2572:7 as originating from Campbell Island, but Du Rietz never went there (pers. comm. D.J. Galloway, also see Galloway 1985: xxi), also there is no 'North-East Harbour' on Auckland Islands; regarding the collection date see UPS L-089390 and Galloway (1985: xxi).


$^J$ – Galloway & Elix (1980: 485) cite "(BM)", but these collection were not found when I requested all BM specimen details, perhaps they are in James’ private Herbarium.
### Table 6. Records from Victoria, Australia. See caption of Table 1 for specifications.

<table>
<thead>
<tr>
<th>locality in Victoria</th>
<th>coordinates</th>
<th>altitude</th>
<th>collector</th>
<th>coll. date</th>
<th>Herb. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mt. William, Australia [Grampian Mts., holotype of <em>Knightiella leucocarpa</em>]</td>
<td>[37°17'40&quot;S 142°36'00&quot;E]</td>
<td>[&lt;1167 m]</td>
<td>Sullivan n.85</td>
<td>[before 1886]</td>
<td>G00292348 (was G 002214)</td>
</tr>
<tr>
<td>Little Morwell River, Strzelecki State Forest, 4 km NNE of Mirboo North</td>
<td>38°22'S 146°11'E</td>
<td>160 m</td>
<td>H. Streimann</td>
<td>14.12.1992</td>
<td>HO 513624</td>
</tr>
<tr>
<td>Little Morwell River, Strzelecki State Forest, 4 km NNE of Mirboo North</td>
<td>38°22'S 146°11'E</td>
<td>160 m</td>
<td>H. Streimann</td>
<td>14.12.1992</td>
<td>CBG 9219427</td>
</tr>
<tr>
<td>Little Morwell River, Strzelecki State Forest, 4 km NNE of Mirboo North</td>
<td>[cf. HO 513624]</td>
<td>160 m</td>
<td>[cf. HO 513624]</td>
<td>[cf. HO 513624]</td>
<td>BGBM 64914</td>
</tr>
<tr>
<td>Lilly Pilly Gully Track, Wilsons Promontory National Park</td>
<td>39°01'S 146°19'E</td>
<td>[&lt;755 m]</td>
<td>K. Ralston</td>
<td>31.10.1997</td>
<td>MEL 2064911A</td>
</tr>
</tbody>
</table>

K – Specimen/description states ‘5000 ped.’ [ped. = feet], which is an obvious overestimate, as Mt. William is just 1167m high (3829 ft.). Another Australian Mt. William in North-East Tasmania is only 197 m high, and can be ruled out as origin of this specimen.

### Table 7. Records from Tasmania, Australia. See caption of Table 1 for specifications.

<table>
<thead>
<tr>
<th>locality in Tasmania</th>
<th>Coordinates</th>
<th>altitude</th>
<th>collector</th>
<th>coll. date</th>
<th>Herb. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Van Diemen's Land [an obsolete name for Tasmania; lectotype of <em>Parmelia splachnirima</em> fide Galloway &amp; Elix 1980]</td>
<td>[42°S 146°E]</td>
<td>-</td>
<td>R.C. Gunn M</td>
<td>[&lt;1856?]</td>
<td>M M FH 00377334 FH 00377335 FH 00377336 BM 00109763</td>
</tr>
<tr>
<td>Embankment, Old Huon Road, near Hobart.</td>
<td>42°52'S 147°19'E</td>
<td>-</td>
<td>R.A. Bastow</td>
<td>23.07.1887</td>
<td>MEL 1066763A</td>
</tr>
<tr>
<td>Margate</td>
<td>43°02'S 147°15'E</td>
<td>-</td>
<td>L. Rodway</td>
<td>08.1911</td>
<td>HO 69387</td>
</tr>
<tr>
<td>Kingston</td>
<td>42°59'S 147°18'E</td>
<td>-</td>
<td>L. Rodway</td>
<td>07.1915</td>
<td>HO 69187</td>
</tr>
<tr>
<td>Cascades, near Hobart</td>
<td>42°54'S 147°17'E</td>
<td>-</td>
<td>L. Rodway</td>
<td>05.1921</td>
<td>HO 69386</td>
</tr>
<tr>
<td>Picton Hut to Picton Bridge</td>
<td>43°05'S 146°42'E</td>
<td>-</td>
<td>G.C. Bratt, J.A. Cashin</td>
<td>21.09.1963</td>
<td>HO 61356</td>
</tr>
<tr>
<td>Tullah - behind town</td>
<td>41°44’S 145°37’E</td>
<td>-</td>
<td>G.C. Bratt, F.N. Lakin</td>
<td>29.03.1964</td>
<td>HO 33741</td>
</tr>
<tr>
<td>MacGregor Peak</td>
<td>42°59’S 147°56’E</td>
<td>120-420 m</td>
<td>G.C. Bratt, J.A. Cashin</td>
<td>29.08.1964</td>
<td>HO 33742</td>
</tr>
<tr>
<td>Vicinity of Forest Shute and Skree Shute round the top of the Northern Lakes, Federation Peak area, Eastern Arthurs.</td>
<td>43°16’S 146°27’E</td>
<td>-</td>
<td>Filson, R.B.</td>
<td>29.12.1964</td>
<td>MEL 1013756A</td>
</tr>
<tr>
<td>Arve Road - Huon River</td>
<td>43°06’S 146°44’E</td>
<td>70 m</td>
<td>G.C. Bratt, J.A. Cashin</td>
<td>31.07.1965</td>
<td>HO 33743</td>
</tr>
<tr>
<td>Cradle Mountain, first ridge south of Waterfall Valley Hut</td>
<td>41°43’S 145°57’E</td>
<td>900 m</td>
<td>G.C. Bratt</td>
<td>19.12.1966</td>
<td>HO 33744</td>
</tr>
<tr>
<td>Dove Lake, Cradle Mountain National Park.</td>
<td>41°39’S 145°57’E</td>
<td>-</td>
<td>D. McVean</td>
<td>02.1967</td>
<td>CANB 770783</td>
</tr>
<tr>
<td>Cradle Mountain area, Ballroom Forest Track</td>
<td>41°40’S 145°57’E</td>
<td>960 m</td>
<td>G.C. Bratt, J.A. Cashin</td>
<td>09.12.1967</td>
<td>HO 33746</td>
</tr>
<tr>
<td>Cradle Mountain National Park: above Waldheim, near Dove Lake.</td>
<td>41°39’S 145°57’E</td>
<td>-</td>
<td>W.A. Weber</td>
<td>22.02.1968</td>
<td>CANB 253537</td>
</tr>
<tr>
<td>Adamsfield Track between Churchill Spur and Florentine River</td>
<td>42°42’S 146°27’E</td>
<td>426 m</td>
<td>G.C. Bratt</td>
<td>13.04.1968</td>
<td>HO 61962</td>
</tr>
<tr>
<td>Location Description</td>
<td>Coordinates</td>
<td>Elevation</td>
<td>Collector</td>
<td>Date</td>
<td>Catalogue Number</td>
</tr>
<tr>
<td>----------------------</td>
<td>--------------</td>
<td>-----------</td>
<td>-----------</td>
<td>------</td>
<td>------------------</td>
</tr>
<tr>
<td>Adamsfield Track between Churchill Spur and Florentine River, south-western Tasmania.</td>
<td>42°36'S 146°25'E</td>
<td>430 m</td>
<td>G.C. Bratt</td>
<td>13.04.1968</td>
<td>MEL 1013757A</td>
</tr>
<tr>
<td>Adamsfield Track between Churchill Spur and Florentine River, SW of Tasmania.</td>
<td>42°41'S 146°26'E</td>
<td>-</td>
<td>G.C. Bratt</td>
<td>13.04.1968</td>
<td>CANB 211248</td>
</tr>
<tr>
<td>Adamsfield Track between Churchill Spur and Florentine River</td>
<td>[cf. HO 61962]</td>
<td>430 m</td>
<td>G.C. Bratt</td>
<td>13.04.1968</td>
<td>ASU 236515</td>
</tr>
<tr>
<td>Adamsfield Track, near Gap</td>
<td>42°42'S 146°28'E</td>
<td>-</td>
<td>G.C. Bratt, J.A. Cashin</td>
<td>02.1974</td>
<td>HO 33779</td>
</tr>
<tr>
<td>Adam Falls</td>
<td>42°43'S 146°18'E</td>
<td>320 m</td>
<td>G.C. Bratt, J.A. Cashin</td>
<td>24.09.1972</td>
<td>HO 33755</td>
</tr>
<tr>
<td>Conglomerate outcrops, south of Adam Falls, on Ragged Range</td>
<td>42°45'S 146°18'E</td>
<td>420 m</td>
<td>G.C. Bratt, J.A. Cashin</td>
<td>24.09.1972</td>
<td>HO 33756</td>
</tr>
<tr>
<td>Pipers Brook Road, about 6 km north of Lebrina</td>
<td>41°09'S 147°14'E</td>
<td>60 m</td>
<td>G.C. Bratt, M.H. Bratt</td>
<td>11.06.1967</td>
<td>HO 33745</td>
</tr>
<tr>
<td>Gordon River Road, Sentinel Ridge, northern slopes, eastern side</td>
<td>42°52'S 146°13'E</td>
<td>800 m</td>
<td>G.C. Bratt, J.A. Cashin</td>
<td>16.03.1968</td>
<td>HO 33747</td>
</tr>
<tr>
<td>Mathinna - Ringarooma Road, just SE of Mt Saddleback</td>
<td>41°24'S 147°47'E</td>
<td>780 m</td>
<td>G.C. Bratt, M.H. Bratt, K. Mackay</td>
<td>21.11.1970</td>
<td>HO 33773</td>
</tr>
<tr>
<td>Cape Sorell</td>
<td>42°12'S 145°10'E</td>
<td>[&lt;100 m]</td>
<td>J.E.S. Townrow</td>
<td>05.1971</td>
<td>HO 33749</td>
</tr>
<tr>
<td>Jane River Track</td>
<td>42°13'S 146°01'E</td>
<td>360 m</td>
<td>G.C. Bratt, J.A. Cashin</td>
<td>12.06.1971</td>
<td>HO 33748</td>
</tr>
<tr>
<td>Track at end of Feilton Road [typo &quot;Fielston Road&quot; in Galloway &amp; Elix 1980]</td>
<td>42°49'S 146°55'E</td>
<td>400 m</td>
<td>G.C. Bratt, J.A. Cashin</td>
<td>27.11.1971</td>
<td>HO 33750</td>
</tr>
<tr>
<td>Forest on Mt Wedge, 29 km WSW of Maydena</td>
<td>42°51'S 146°17'E</td>
<td>600 m</td>
<td>G.C. Bratt</td>
<td>04.12.1971</td>
<td>HO 33751</td>
</tr>
<tr>
<td>Hastings Caves Road</td>
<td>43°24'S 146°52'E</td>
<td>80 m</td>
<td>G.C. Bratt, J.A. Cashin</td>
<td>30.01.1972</td>
<td>HO 33774</td>
</tr>
<tr>
<td>Crotty - town and smelter area</td>
<td>42°12'S 145°38'E</td>
<td>200 m</td>
<td>G.C. Bratt, M.H. Bratt</td>
<td>01.04.1972</td>
<td>HO 33752</td>
</tr>
<tr>
<td>Crotty</td>
<td>42°12'S 145°38'E</td>
<td>200 m</td>
<td>G.C. Bratt</td>
<td>01.04.1972</td>
<td>MEL 1017341A</td>
</tr>
<tr>
<td>16 km north of Tullah, on Murchison Hwy</td>
<td>41°37'S 145°39'E</td>
<td>660 m</td>
<td>G.C. Bratt, M.H. Bratt</td>
<td>02.04.1972</td>
<td>HO 33753</td>
</tr>
<tr>
<td>Adamson's Peak Track, near end of Tramway</td>
<td>43°21'S 146°53'E</td>
<td>520 m</td>
<td>G.C. Bratt</td>
<td>21.05.1972</td>
<td>HO 33754</td>
</tr>
<tr>
<td>Mt Rufus Track, Myrtle Forest, hut</td>
<td>42°07'S 146°05'E</td>
<td>1120 m</td>
<td>G.C. Bratt</td>
<td>08.10.1972</td>
<td>HO 33733</td>
</tr>
<tr>
<td>Mt Rufus Track, Myrtle Forest - Hut.</td>
<td>42°07'S 146°05'E</td>
<td>340 m</td>
<td>G.C. Bratt</td>
<td>08.10.1972</td>
<td>MEL 1527066A</td>
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<tr>
<td>Mt Ossa: Arm River - Lake Ayr</td>
<td>41°49'S 146°07'E</td>
<td>1000 m</td>
<td>G.C. Bratt</td>
<td>12.1972</td>
<td>HO 33757</td>
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<tr>
<td>Mt Mueller Track - Forest near Hydro Road</td>
<td>42°47'S 146°30'E</td>
<td>400 m</td>
<td>G.C. Bratt</td>
<td>07.04.1973</td>
<td>HO 33776</td>
</tr>
<tr>
<td>Track to Lady Barron Falls</td>
<td>42°42'S 146°42'E</td>
<td>250 m</td>
<td>G.C. Bratt, J.A. Cashin, CO, HC, AB</td>
<td>19.05.1973</td>
<td>HO 33777</td>
</tr>
<tr>
<td>Mt Cameron</td>
<td>40°59'S 147°56'E</td>
<td>[&lt;551 m]</td>
<td>G.C. Bratt, J.A. Cashin</td>
<td>25.11.1973</td>
<td>HO 33720</td>
</tr>
<tr>
<td>Lookout near Franklin/Collingwood Junction</td>
<td>42°12'S 145°56'E</td>
<td>520 m</td>
<td>G.C. Bratt</td>
<td>02.1974</td>
<td>HO 33778</td>
</tr>
<tr>
<td>near Sawback Lookout, Gordon Road, south side of road (Boyd Lookout)</td>
<td>42°49'S 146°21'E</td>
<td>-</td>
<td>G.C. Bratt</td>
<td>24.11.1974</td>
<td>HO 33780</td>
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<tr>
<td>Location</td>
<td>Latitude/Longitude</td>
<td>Altitude</td>
<td>Collectors</td>
<td>Date</td>
<td>Collection No.</td>
</tr>
<tr>
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<td>--------------------</td>
<td>----------</td>
<td>-------------------------------------</td>
<td>------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Goshen - Pioneer Road</td>
<td>41°05'S 148°04'E</td>
<td>100 m</td>
<td>G.C. Bratt, K.M. Mackay</td>
<td>30.03.1975</td>
<td>HO 33759</td>
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<tr>
<td>Olga Camp, east to Gordon River</td>
<td>42°41'S 145°49'E</td>
<td>80 m</td>
<td>J.M. Gilbert</td>
<td>03.1975</td>
<td>HO 33758</td>
</tr>
<tr>
<td>Near Olga Camp and along Hydrology Track South</td>
<td>42°41'S 145°49'E</td>
<td>80 m</td>
<td>D.J. Thomas</td>
<td>03.1977</td>
<td>HO 33783</td>
</tr>
<tr>
<td>Picton (Swing) Bridge Track</td>
<td>43°07'S 146°42'E</td>
<td>-</td>
<td>G.C. Bratt, J.M. Gilbert, T. Gilbert</td>
<td>18.01.1976</td>
<td>HO 33760</td>
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<tr>
<td>Transect 11A, Hydrology Track (HEC Collection)</td>
<td>42°36'S 145°42'E</td>
<td>-</td>
<td>G.C. Bratt, J.A. Cashin, J.M. Gilbert</td>
<td>02.03.1976</td>
<td>HO 33762</td>
</tr>
<tr>
<td>Track to Leven Canyon</td>
<td>41°24'S 146°01'E</td>
<td>520 m</td>
<td>G.C. Bratt, K.M. Mackay, CF</td>
<td>14.06.1976</td>
<td>HO 33763</td>
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<tr>
<td>Hungry Flats</td>
<td>42°30'S 147°24'E</td>
<td>540 m</td>
<td>G.C. Bratt, J.M. Gilbert</td>
<td>03.1977</td>
<td>HO 33782</td>
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<td>Frenchmans Cap Track</td>
<td>42°16'S 145°52'E</td>
<td>960 m</td>
<td>G. Kantvilas</td>
<td>03.01.1981</td>
<td>HO 113233</td>
</tr>
<tr>
<td>Mt Wellington, Hunters Track</td>
<td>42°53'S 147°14'E</td>
<td>[&lt;1270 m]</td>
<td>A.V. Ratkowsky</td>
<td>25.03.1981</td>
<td>HO 114305</td>
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<tr>
<td>Mt Wellington, bank near Sphinx Rock</td>
<td>42°55'S 147°15'E</td>
<td>[&lt;1270 m]</td>
<td>A.V. Ratkowsky</td>
<td>04.10.1985</td>
<td>HO 114306</td>
</tr>
<tr>
<td>Mt Wellington, near Octopus Tree</td>
<td>42°55'S 147°15'E</td>
<td>[&lt;1270 m]</td>
<td>A.V. Ratkowsky</td>
<td>28.11.1985</td>
<td>HO 114304</td>
</tr>
<tr>
<td>Junction of Gordon Road and Five Road</td>
<td>42°44'S 146°25'E</td>
<td>440 m</td>
<td>G. Kantvilas</td>
<td>10.10.1981</td>
<td>HO 323141</td>
</tr>
<tr>
<td>Mt McCall</td>
<td>42°22'S 145°43'E</td>
<td>720 m</td>
<td>G. Kantvilas, P. James</td>
<td>08.02.1984</td>
<td>HO 323142</td>
</tr>
<tr>
<td>Raminea Plains</td>
<td>43°18'S 146°54'E</td>
<td>80 m</td>
<td>G. Kantvilas</td>
<td>30.03.1984</td>
<td>HO 113232</td>
</tr>
<tr>
<td>Raminea Plains</td>
<td>[cf. HO 113232]</td>
<td>80 m</td>
<td>G. Kantvilas</td>
<td>30.03.1984</td>
<td>S LS4785</td>
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<tr>
<td>Raminea Plains</td>
<td>[cf. HO 113232]</td>
<td>80 m</td>
<td>G. Kantvilas</td>
<td>30.03.1984</td>
<td>BG 63355</td>
</tr>
<tr>
<td>Raminea Plains</td>
<td>[cf. HO 113232]</td>
<td>80 m</td>
<td>G. Kantvilas</td>
<td>30.03.1984</td>
<td>BG 63356</td>
</tr>
<tr>
<td>Raminea Plains</td>
<td>[cf. HO 113232]</td>
<td>-</td>
<td>G. Kantvilas</td>
<td>NA</td>
<td>TSB 11820</td>
</tr>
<tr>
<td>Tasmania probably</td>
<td>-</td>
<td>-</td>
<td>G. Kantvilas</td>
<td>30.03.1984</td>
<td>UPS L-004554</td>
</tr>
<tr>
<td>Elliot Range</td>
<td>42°28'S 145°43'E</td>
<td>880 m</td>
<td>G. Kantvilas</td>
<td>12.01.1985</td>
<td>HO 89233</td>
</tr>
<tr>
<td>Standard Hill, 30 km WSW of Deloraine.</td>
<td>41°33'S 146°18'E</td>
<td>440 m</td>
<td>J.A. Curnow</td>
<td>28.11.1988</td>
<td>CBG 8807565</td>
</tr>
<tr>
<td>Argent Dam near Renison Bell</td>
<td>41°48'S 145°24'30&quot;E</td>
<td>240 m</td>
<td>G. Kantvilas</td>
<td>05.12.1989</td>
<td>HO 120069</td>
</tr>
<tr>
<td>Crater Lake trail, Cradle Mountain National Park, 37 km NE of Rosebery.</td>
<td>41°40'S 145°56'E</td>
<td>1060 m</td>
<td>J.A. Elix</td>
<td>9.01.1990</td>
<td>CBG 9708515</td>
</tr>
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<td>Timbs Track, between Gordon Road and Florentine River, 19 km WNW of Maydena.</td>
<td>42°44'S 146°24'E</td>
<td>480 m</td>
<td>J.A. Elix</td>
<td>16.01.1990</td>
<td>CBG 9708664</td>
</tr>
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<td>4 km N of Mt. Wedge</td>
<td>42°51'S 146°17'E</td>
<td>360 m</td>
<td>G. Kantvilas, P.W. James</td>
<td>13.10.1990</td>
<td>BG 32874</td>
</tr>
<tr>
<td>[no details]</td>
<td>-</td>
<td>-</td>
<td></td>
<td>??..??</td>
<td>CABI 401223</td>
</tr>
<tr>
<td>Mt Field National Park, c. 2 km SE of Lake Emmett</td>
<td>42°38'S 146°33'E</td>
<td>980 m</td>
<td>G. Kantvilas</td>
<td>29.03.1990</td>
<td>HO 123125</td>
</tr>
<tr>
<td>Green Head, c. 3 km SSE of Greystone Bluff</td>
<td>43°06'S 146°04'E</td>
<td>800 m</td>
<td>G. Kantvilas</td>
<td>08.03.1991</td>
<td>HO 129667</td>
</tr>
<tr>
<td>Near Williamsford, 6 km SSW of Rosebery.</td>
<td>41°50'S 145°31'E</td>
<td>400 m</td>
<td>J.A. Elix</td>
<td>22.04.1992</td>
<td>CANB 613597</td>
</tr>
<tr>
<td>Williamsford Road, 4 km SSW of Rosebery.</td>
<td>41°49'S 145°31'E</td>
<td>320 m</td>
<td>J.A. Elix</td>
<td>22.04.1992</td>
<td>CANB 613628</td>
</tr>
<tr>
<td>Localities</td>
<td>Coordinates</td>
<td>Elevation</td>
<td>Collector(s)</td>
<td>Date</td>
<td>Accession No.</td>
</tr>
<tr>
<td>---------------------------------------------------------------------------</td>
<td>-----------------------</td>
<td>-----------</td>
<td>----------------------------</td>
<td>---------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Summit of Mt Tim Shea, 13 km W of Maydena.</td>
<td>42°43'S 146°28'E</td>
<td>940 m</td>
<td>J.A. Elix</td>
<td>26.04.1992</td>
<td>CANB 613807</td>
</tr>
<tr>
<td>Summit of Mt Tim Shea, 13 km W of Maydena.</td>
<td>42°43'S 146°28'E</td>
<td>940 m</td>
<td>J.A. Elix</td>
<td>26.04.1992</td>
<td>CANB 613810</td>
</tr>
<tr>
<td>Summit of Mt Tim Shea, 13 km W of Maydena.</td>
<td>42°43'S 146°28'E</td>
<td>940 m</td>
<td>J.A. Elix</td>
<td>26.04.1992</td>
<td>CANB 613836</td>
</tr>
<tr>
<td>half way up Mount Tim Shea Road</td>
<td>42°42'40&quot;S 146°28'E</td>
<td>650 m</td>
<td>A.E. Wright</td>
<td>26.04.1992</td>
<td>AK 213510</td>
</tr>
<tr>
<td>Timbs Track, 27 km WSW of Maydena.</td>
<td>42°48'S 146°19'E</td>
<td>350 m</td>
<td>J.A. Elix</td>
<td>26.04.1992</td>
<td>CANB 613869</td>
</tr>
<tr>
<td>Weindorfers Forest</td>
<td>41°38'S 145°56'E</td>
<td>1040 m</td>
<td>G. Kantvilas, B. Fuhrer, J. Jarman</td>
<td>16.05.1992</td>
<td>HO 443947</td>
</tr>
<tr>
<td>Summit of Gog Range</td>
<td>41°31'S 146°26'E</td>
<td>720 m</td>
<td>G. Kantvilas</td>
<td>27.10.1996</td>
<td>HO 44433</td>
</tr>
<tr>
<td>near Monk Bay, Tasman Peninsula</td>
<td>42°58'S 147°43'E</td>
<td>45 m</td>
<td>G. Kantvilas</td>
<td>26.08.2001</td>
<td>HO 513720</td>
</tr>
<tr>
<td>Corinna Road</td>
<td>41°32'S 145°12'E</td>
<td>320 m</td>
<td>G. Kantvilas</td>
<td>14.10.2003</td>
<td>HO 523778</td>
</tr>
<tr>
<td>Florentine Road, c. 2.5 km S of northernmost bridge over Florentine River</td>
<td>42°28'S 146°30'E</td>
<td>240 m</td>
<td>G. Kantvilas</td>
<td>10.11.2005</td>
<td>HO 534001</td>
</tr>
<tr>
<td>Lake Cygnus</td>
<td>43°08'S 146°14'E</td>
<td>880 m</td>
<td>G. Kantvilas</td>
<td>06.12.2006</td>
<td>HO 543174</td>
</tr>
<tr>
<td>Hartz Mountains, near the old hut site</td>
<td>43°13'S 146°46'E</td>
<td>840 m</td>
<td>G. Kantvilas</td>
<td>25.07.2007</td>
<td>HO 544929</td>
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<tr>
<td>The Pine Forest, c. 2 km SE of Lake Emmett</td>
<td>42°37'S 146°33'E</td>
<td>960 m</td>
<td>G. Kantvilas</td>
<td>24.01.2010</td>
<td>HO 554139</td>
</tr>
<tr>
<td>Tahune Forest Reserve, along old Huon Track</td>
<td>43°06'S 146°42'E</td>
<td>60 m</td>
<td>G. Kantvilas</td>
<td>09.01.2013</td>
<td>HO 49117</td>
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<td>Lake Strahan Track</td>
<td>42°09'S 145°16'E</td>
<td>20 m</td>
<td>G. Kantvilas</td>
<td>14.05.2013</td>
<td>HO 569173</td>
</tr>
<tr>
<td>Near Churchill Spur, south west Tasmania</td>
<td>42°42'S 146°27'E</td>
<td>-</td>
<td>J.A. Elix, (&amp; G.C. Bratt?)</td>
<td>-</td>
<td>CANB 625301</td>
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<tr>
<td>Mt. Arrowsmith (70/1299)</td>
<td>[42°12'30&quot;S 146°04'30&quot;E]</td>
<td>&lt;957 m</td>
<td>[G.C. Bratt?] P</td>
<td>[1970?] Q</td>
<td>HO? P</td>
</tr>
<tr>
<td>Constock Mine (70/55)</td>
<td>[41°53'30&quot;S 145°17'00&quot;E]</td>
<td>-</td>
<td>[G.C. Bratt?] P</td>
<td>[1970?] Q</td>
<td>HO? P</td>
</tr>
<tr>
<td>Tinder Box Road (68/819)</td>
<td>[43°02'30&quot;S 147°20'00&quot;E]</td>
<td>-</td>
<td>[G.C. Bratt?] P</td>
<td>[1968?] Q</td>
<td>HO? P</td>
</tr>
</tbody>
</table>

M – The name 'Van Diemen's Land' was officially replaced with 'Tasmania' in 1856, and the likely collector Ronald Campbell Gunn lived from 1808 till 1881.
N – The Farlow Herbarium type packet contains a sheet with 3 specimens, each with separately barcoded; Galloway & Elix (1980) selected ‘right-hand side lower specimen’ as lectotype, which is now FH 00377336. BM... is a isolectotype as mentioned by Galloway & Elix (1980).
O – Coordinate precision of specimen HO 61962 is better.
P – Galloway & Elix (1980: 485) write “A collection of Tasmanian specimens (not seen) of K. splachnirima, made by the late Dr G. C. Bratt is now housed in HO. The following localities are noted, with collecting numbers in parentheses: (…)”, but these three collections were not found in the HO herbarium database.
Q – These year numbers are likely, as in all other HO collections by G.C. Bratt with this collecting number format, the year is coded before the ‘/’ of the coll. no.
R – Assuming the locality is ‘Comstock Mine’, 4 km West of Zeehan township.
4. Discussion

After several years of field work on *I. splachnirima* in New Zealand, the author is convinced that this species’ distribution is still much understudied. This is mainly due to a lack of collectors conducting targeted search for uncommon lichens, but also because the species tends to grow in sites, that an ‘ordinary’ tramper avoids, because he or she is likely to get very wet and dirty there.

The discovery numerous new localities all along the West-coast of the South Island is anticipated, and the species is most certainly present in various bogs of the Te Anau-Manapouri area (Southland), e.g. Kepler Mire just east of the Te Anau/Manapouri Aerodrome and Dome Mire 13 km NNE of Te Anau (see Burrows & Dobson 1972). Perhaps it also occurs in the higher altitude regions of Banks Peninsula near Christchurch, but it was not found during two trips there. The vast boggy tussock grasslands on Antipodes Island appear very suitable for this species as well, and it is likely to grow on Snares Island due to its proximity to Stewart Island. *I. splachnirima* may also be present on Macquarie Island, considering the presence there of various species, which are often associated with it, as well as peaty soils and tussock grasslands as potential habitats (Kantvilas & Seppelt 1992). Therefore, the author would urge visitors to Macquarie Island to watch out for this species. Bogs and still natural subalpine areas of the New Zealand North Island should also be searched for this species, e.g. Tararua and Ruahine Ranges or the Urewera National Park. Another promising and potentially suitable North Island locality could be the Kopuatai Peat Dome ca. 30 km South of Thames (37°25'S 175°34'E), an extensive lowland peat bog covering ca. 10,000 hectares (Clarkson & Peters 2010, 2012). Based on the maps by Newsome (1987), further potential localities could be identified by searching for the vegetation cover type of known localities.

Although the author believes that *I. splachnirima* is much more widespread in New Zealand, than current records show, it seems that the species’ conservation status as ‘Nationally Vulnerable’ issued by De Lange & Blanchon (2014) is still justified, because of the ongoing destruction of its primary habitat, peat bogs, especially in the lowlands.

Probably the species is more widespread in Victoria than records show, e.g. it should be searched for it in the Australian Alps, which might even extend its range into New South Wales. King, Flinders and Cape Barren Islands, the major islands in Bass Strait north of Tasmania, could also have suitable habitats, although they lack the altitudinal range in which the species occurs most often. Tasmania is certainly the species’ stronghold, and will be very important for this species’ survival in course of the climate change.

The presented comprehensive compilation of the species distribution will provide a valuable reference for future conservationists, when the species’ presence in these sites will be reassessed. The author’s difficulty to find *I. splachnirima* in a couple of previous collecting sites in New Zealand,
points toward the necessity of monitoring the species’ presence and abundance in regular intervals, e.g. every five or ten years.

Furthermore, the presented distribution data can be used for future modelling of the species’ macro-habitat and potential distribution as done for other lichens by McCune & Printzen (2011) and Cameron et al. (2011), by superimposing vegetation cover maps from Newsome (1987) and metrological data from the NIWA (National Institute of Water and Atmospheric Research) National Climate Database (http://cliflo.niwa.co.nz/).

5. Summary

This chapter provides a comprehensive compilation of the known global distribution of *Icmadophila splachnirima* in New Zealand, Tasmania and Victoria, based on public herbarium records, literature records, and personal observations. It appears likely that *I. splachnirima* has a much wider distribution within New Zealand than current records show.
CHAPTER 2

A molecular-genetic reassessment of the phylogeny of the lichen genus *Icmadophila* Trevis.

1. Introduction


Until 1993 *Icmadophila* was a monotypic genus with the single species *I. ericetorum*, which has a holarctic distribution but was also reported from New Zealand and South Africa (Galloway 1985, 2007). It grows mainly in heathland and bogs on damp peaty soil, plant debris, bryophytes or rotting bark and wood, and is characterised by a pale green, granular-crustose thallus with pink, sessile to shortly stalked, biatorine apothecia.

Rambold et al. (1993) have synonymised *Glossodium* Nyl. with *Icmadophila*, and therefore transferred *G. aversum* and *G. japonicum* into *Icmadophila*. This was justified by similarities of *I. ericetorum*, *I. aversa* and *I. japonica* regarding the thallus morphology, secondary chemistry, substrate preference and ecology as well as spore and ascus characters. The main difference between *I. ericetorum* and the former *Glossodium* is the obvious macro-morphology of the ascomata, which are bisymmetrically tongue-shaped and distinctly stalked in the latter but discoid and usually sessile to subpedicillate in *I. ericetorum*. *Icmadophila aversa* is endemic to Central and South America (Wilk 2010) and *I. japonica* is known from Far East Russia and Japan (Ohmura 2011), where it is sympatric with *I. ericetorum*.

The Australasian endemic *Knightiella splachnirima* (Hook. f. & Taylor) Gyeln. (see Chapter 1 for detailed distribution) was synonymised with and transferred to *Icmadophila* by Galloway (2000), a course of action previously suggested by Galloway (1992), Gierl & Kalb (1993) and Rambold et al. (1993), who pointed out their similarities. This view, however, was soon challenged and rejected by Stenroos et al. (2002), whose analysis of nuclear SSU rDNA sequences placed *K. splachnirima* in a sister relationship to all remaining members of Icmadophilaceae, several nodes apart from *I. ericetorum*. Nevertheless, the name *Icmadophila splachnirima* remains in use and has replaced *Knightiella* in the

*Icmadophila eucalypti* has recently been described from Tasmania (Kantvilas in Lumbsch et al. 2011), but its generic placement is provisional due to the absence of fertile material. The small-squamulose thallus, however, bears some superficial resemblance to that of *I. splachnirima*.

This study aims to re-evaluate and clarify the relationships among the five species of *Icmadophila* using a molecular phylogenetic approach. The study has arisen from work on the reproductive ecology of *Icmadophila splachnirima*, which includes a population genetic approach using nuclear ribosomal DNA sequences. From there, it was only a small step to expand the research efforts towards the inclusion of all five species of this small genus. Furthermore, there was an obvious need to settle the controversial systematic and taxonomic position of *I. splachnirima* (cf. Stenroos et al. 2002), and also for molecular confirmation of the placement of *I. eucalypti* (Kantvilas in Lumbsch et al. 2011), as well as a reappraisal of the transfer of *Glossodium* to *Icmadophila* (Rambold et al. 1993). The timely publication of recently gathered collections of *I. aversa*, *I. japonica* and *I. eucalypti* has offered a unique opportunity to realize such a phylogenetic reassessment, as the author had easy access to *I. splachnirima* and *I. ericetorum* already.

In the context of the author’s ecological work on the reproductive biology of *I. splachnirima*, it is beneficial to know the exact affiliations with other members of the family, when comparing various aspects of their biology.

### 2. Methods

#### 2.1 Examined specimens

The most crucial step of this study was to obtain specimens of all five *Icmadophila* species, as four of them have very restricted global distributions in parts of the world very distant from each other. Also specimens of additional species of Icmadophilaceae have been examined, to increase the number of potential nodes within phylogenetic trees. Fortunately, recent collections of *I. aversa*, *I. japonica* and *I. eucalypti* were mentioned in the literature (Wilk 2010, Ohmura 2011, Lumbsch et al. 2011). Specimens were provided by Yoshihito Ohmura (*I. japonica*), Karina Wilk (*I. aversa*), Regine Stordeur and Helmut Mayrhofer (*I. ericetorum*), and Gintaras Kantvilas (*I. eucalypti*, *Siphulella coralloidea*).
DNA extracts of the following specimens / species have been used to obtain new DNA sequences:

**OTA 062507, Icmadophila splachnirima**, Blue Mts. near Tapanui, Otago, NZ, 45°55'56.3"S 169°20'18.9"E, ~900 m, leg. Lars Ludwig, 26.01.2012.

**OTA 063988, Icmadophila splachnirima**, Swampy Summit, Dunedin, Otago, NZ, 45°48'03.7"S 170°28'57.5"E, 07.09.2012, ~720 m, fertile, on S-slope among Pentachondra.

**OTA 063991, Icmadophila ericetorum**, Kampenwand bei Grassau, Bayern, Deutschland, 47°45'25"N 12°22'01"E, ca. 1550 m, leg. Lars Ludwig 28.08.2009, verrottender Kiefernstubben, direkt an Wanderweg vor Begin der Wand, Nord exponiert.

**OTA 061856, Icmadophila ericetorum**, Breitlahnalm, ca. 0.2 km SE of Breitlahn hut, along road to Schwarzenssee, Schladminger Tauern, Steiermark, Austria, 47° 18'56"N 13°53'16"E, 1075 m, leg. P. Bilovitz & H. Mayrhofer 19222, 11.10.2012, rotting tree stump (willow) on road side.

**OTA 063995** (duplicate of K.Wilk 7685), *Icmadophila aversa*, South America, Bolivia: Dept. La Paz, Prov. Franz Tamayo, Madidi National Park, Cordillera Apolobamba, NE of Keara village, the timber line forest of *Polylepis pepei* by Tolca Cocha lake, 14°41'14"S 69°05'18"W, alt. 4056 m; much humid habitat, over bryophytes on rock, leg. K.Wilk 7685, 14 Oct. 2007. [same specimen as in Wilk 2010]

**OTA 063996** (duplicate HO 568315), *Icmadophila eucalypti*, Australia, Tasmania: Lake Dobson, 42°41'S, 146°35'E, 1040 m, on *Arthrotaxis cupressoides* in subalpine woodland, leg. G. Kantvilas, 6/13, 24 Jan 2013.

**OTA 063997** (duplicate of HO 559275), *Icmadophila eucalypti*, Australia, Tasmania: Road to Strathgordon, 42°46'S, 146°33'E, 300 m, on trunk of mature *Eucalyptus obliqua* in wet eucalypt forest, leg. G. Kantvilas 270/10, 9 Nov 2010.

**TNS YO-6762**, Japan, Hokkaido, Prov. Kushiro: Mt. O-akan, on decayed stump, 494 m, 10 August 2009, Y. Ohmura 6762. [Same specimen as in Ohmura 2011]

**OTA 063987, Dibaeis absoluta**, Grahams bush, Sawyers Bay, Dunedin, Otago, NZ, 45°48'33"S 170°35'00"E, 140 m, leg. Lars Ludwig, 09.02.2011, claybank alongthe track from Sawyers Bay to organ pipes, in regenerating native bush.

**OTA 063977, Dibaeis absoluta**, Kepler Track, Southland, NZ, 45°24'25"S 167°38'57"E, ~600 m, leg. Lars Ludwig, Feb 2009, claybank along track between Brod Bay and Luxmore Hut, in native forest.

**OTA 063978, Dibaeis arcuata**, Whareatea Mine area, Denniston Plateau near Westport, NZ, 41°45'55"S 171°47'08"E, leg. Lars Ludwig, 03.03.2012.

**OTA 063986, Dibaeis arcuata**, Swampy Summit, Dunedin, Otago, NZ, 48°48'20"S 170°29'00"E, 650 m, leg. Lars Ludwig, 11.06.2011.
OTA 063993 (duplicate of HO 569181), *Siphulella coralloidea*, Australia, Tasmania: Lake Burbury, eastern slopes of Thureau Hills, 42°08’S 145°39’E, 280 m, on steep gravelly road cutting in buttongrass moorland, leg. G. Kantvilas 93/13, 13 May 2013.

OTA 063984, *Siphula decumbens*, Whareatea Mine area, Denniston Plateau near Westport, NZ, 41°46'00”S 171°47'20”E, 640 m, leg. Lars Ludwig, 03.03.2012.

OTA 062495, *Siphula decumbens*, Pryse Peak, Stewart Island, NZ, 46° 56'23.1”S 168°00'20.9”E, 350 m, leg. Lars Ludwig, 25.11.2012, TLC: thamnolic acid, fertile!, duplicate in HO

OTA 062496, *Siphula fastigiata*, details as in OTA 062495.

OTA 063994 (duplicate of HO 564725), *Siphula fastigiata*, Australia, Tasmania: Skullbone Plains, 42°02'S 146°19’E, 1000 m, on skeletal soil over boulders in open heathland, leg. G. Kantvilas 88/12, 29 Feb 2012, tlc: baemynesic and squamatic acid.


OTA 064254, *Siphula fastigiata*, Kaipo River catchment, ca. 4 km N of Mt Thunder Pk., Fiordland, NZ, 44°28’55.7”S 168°00’38.8”E, 1090 m a.s.l., leg. Lars Ludwig, 14.04.2014, W-facing slope of ridge, in open subalpine vegetation with Hebe, bog pine, tussock.

OTA 062500, *Siphula fastigiata*, near start of Scott’s Track, Arthur’s Pass, Canterbury, NZ, 45°56’03”S 171°33’32”E, ~ 800 m, leg. Allison Knight, 02/01/2013, TLC: baemynesic + squamatic acids, fertile!, duplicates in HO and private herbarium A. Knight.


It was initially intended to include *I. ericetorum* specimens from New Zealand in this study, as this species has been reported for New Zealand (Galloway 1985, 2007). However, it soon turned out that several herbarium specimens labelled as *I. ericetorum* were misidentified *Dibaeis absoluta*. Therefore, doubts arose about the presence of this species in New Zealand, and a detailed literature search and re-examination of herbarium specimens was conducted. This topic is treated separately in Chapter 6.

### 2.2 A homemade DNA extraction and PCR purification kit
Two DNA extraction methods were used: initially, DNA extractions were conducted according to Summerfield (2003), who used the method described in Cubero *et al.* (1999) with slight modifications.
Modifications of this method to include the use of silica membrane spin columns resulted in a quick, robust, low cost method, equivalent to commercial DNA extraction kits.

This method included the first steps from Summerfield (2003) and Cubero et al. (1999), which includes sample preparation, cell disruption with liquid N₂, cell lysis in CTAB Extraction Buffer followed by only one chloroform extraction.

The subsequent steps are based on the approach of Ye et al. (2004), who have used a commercial PCR purification kit to purify raw genomic DNA extracts, however in place of a commercial kit the present study used separately purchased low-cost silica membrane spin columns (EconoSpin® All-In-One Mini Spin Columns, Epoch Life Sciences Inc.) and homemade reaction buffers, based on recipes provided by the column manufacturer.

EconoSpin® columns and homemade buffers (PEX binding buffer, WS wash buffer, EB elution buffer) were used according to the instruction manual of the PureLink® PCR Purification Kit (Invitrogen, Life Technologies™). Raw genomic DNA extract was used instead of un-purified PCR product, PEX binding buffer replaced B2 buffer, WS wash buffer replaced W1 buffer, EB elution buffer (or 1 x TE buffer) replaced E1 buffer. The only modification compared to the PureLink® manual, is the use of 5 volumes of PEX binding buffer instead of 4 volumes of B2, as this ratio has been suggested in the PEX recipe provided by Epoch Life Sciences.

The same homemade buffers and EconoSpin® columns were later used for purification of PCR products, as this is the purpose for which the manufacturer sells the columns and provides the buffer recipes.

### 2.2.1. Composition of reagents for DNA extraction

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Extraction Buffer</em></td>
<td>100 mM Tris-HCl, pH 8.0; 1 M NaCl; 20 mM EDTA; 1% CTAB (w/v).</td>
</tr>
<tr>
<td><em>PEX binding buffer</em></td>
<td>5.5 M guanidine hydrochloride (GuHCl); 20 mM Tris-HCl, pH 6.6 (25°C) (eventually an improved recipe with 25% v/v isopropanol was used).</td>
</tr>
<tr>
<td><em>WS wash buffer</em></td>
<td>10 mM Tris-HCl, pH 7.5 (25°C) with 80% ethanol [final concentration after addition of ethanol].</td>
</tr>
<tr>
<td><em>EB elution buffer</em></td>
<td>10 mM Tris-HCl, pH 8.5 (25°C).</td>
</tr>
<tr>
<td><em>1x TE buffer</em></td>
<td>10 mM Tris-HCl, pH 8.0; 1mM EDTA.</td>
</tr>
</tbody>
</table>

### 2.2.2 DNA Extraction Procedure Part I – DNA raw extracts

**Sample preparation.** Thallus fragments (dry weight 1-50 mg) were thoroughly cleaned by manual removal of adhering dirt, moss, etc. under a dissecting microscope using super-fine tweezers and rinsing in de-ionised H₂O.
**Chapter 2 – Phylogeny of the genus *Icmadophila***

*Tissue disruption.* Cleaned and air-dried thallus fragments were ground up to a fine powder inside a 1.5 ml microcentrifuge tube using a micro-pestle and liquid N₂.

**Cell lysis.** 500 µl of *Extraction Buffer* and approximately 5 mg (a spatula tip) of polyvinylpolypyrrolidone (PVPP) powder were added to the tube. The tube was briefly mixed by vortexing and incubated in a heat block at 70°C for 30 minutes. (Old or smaller samples were incubated for 1 hour to increase the yield.)

**Chloroform extraction.** Under a fume hood an equal volume of chloroform (i.e. 500 µl) was added, and thoroughly mixed by manual shaking. Tubes were centrifuged at maximum speed (> 10000 g) for 5 minutes at room temperature in a bench top centrifuge to separate phases.

The upper aqueous phase (DNA raw extract) was carefully transferred into a new tube, without touching the phase boundary. Usually it was possible to recover 400 µl of the originally 500 µl aqueous phase. The interphase cell debris and lower chloroform phase were discarded.

### 2.2.3 DNA Extraction Procedure Part II – Spin-column purification of DNA raw extracts (and PCR products)

**Binding.** 5 volumes *PEX* binding buffer were added to the DNA raw extract (or PCR product) and gently mixed by slowly pipetting up and down several times. The mixture was transferred to an *EconoSpin* column and centrifuged for 1 minute at maximum speed; the flow through was discarded. (For 400 µl of raw DNA extract, the volume was split into two tubes with 200 µl each before adding *PEX*, and the column was refilled and centrifuged several times).

**Wash.** 650 µl *WS* wash buffer were added to the column, and it was centrifuged for 1 minute at maximum speed. After discarding the flow through, the column was dried by centrifuging again for 3 minutes.

**Elution.** The column was placed in a new 1.5 ml microcentrifuge tube, and 50 µl *EB* elution buffer (or alternatively 1x *TE*) were added to the centre of the column. The column was incubated at room temperature for 2 minutes, and then centrifuged for 2 minutes at maximum speed.

The yield was measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific).

### 2.3 Loci and Primer Selection

A main criterion for the choice of the loci used for this study, was the availability of reference sequences of other *Icmadophilaceae* in GenBank. Ribosomal DNA sequences have been frequently used in molecular phylogenetic studies. In particular the Internal Transcribed Spacer region (*ITS*) of nuclear ribosomal DNA is the region of choice for fungal and lichen barcoding (*Kelly et al.* 2011, *Schoch et al.* 2012). However, there are certain weaknesses (*Kiss* 2012), such as intraspecific and intragenomic
variation due to its multi-copy nature, which require the use of direct sequencing rather than sequencing after cloning. Except for the highly conserved 5.8S nuclear rDNA in its centre, the ITS region contains non-coding sequence, which means mutations can accumulate at a high rate without adverse effects. This results in high phylogenetic resolution of potentially closely related species (White et al. 1990), which is desired for the present study. The coding sequence of nuclear Large Subunit rDNA (nuLSU) is subjected to selection pressure, which makes it more suitable as marker in genus rank phylogenies within a family. However, it is directly linked to the ITS region, and therefore both have not evolved independently from each other. The mitochondrial Large Subunit rDNA (mtLSU) is not physically linked to the ITS and nuLSU, which makes it an independent second measure of evolutionary change in this study. Furthermore mitochondrial rRNA genes evolve faster than their nuclear counterparts (White et al. 1990), therefore the mtLSU is expected to be a locus with intermediate rate of evolutionary change, between that of ITS and nuLSU.

Sequences and sources of primers used in this study are listed in Table 8. All primers were bought from Invitrogen, Auckland, New Zealand.

Table 8. Primers used in this study.

<table>
<thead>
<tr>
<th>locus</th>
<th>Primer name</th>
<th>sequence 5’ to 3’</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS</td>
<td>ITS1-F</td>
<td>CTTGGTCATTTAGAGGAAGTAA</td>
<td>Gardes &amp; Bruns (1993)</td>
</tr>
<tr>
<td>ITS</td>
<td>ITS4</td>
<td>TCTCCGCTTATTGATATGC</td>
<td>White et al. (1990)</td>
</tr>
<tr>
<td>ITS</td>
<td>ITS4A</td>
<td>ATTTGAGCTGTGGCTGCTTCA</td>
<td>D. L. Taylor in Kroken &amp; Taylor (2001)</td>
</tr>
<tr>
<td>nuLSU</td>
<td>LR0R</td>
<td>ACCCGCTGAACCTAAGC</td>
<td>Cubeta et al. (1991)</td>
</tr>
<tr>
<td>nuLSU</td>
<td>LR5</td>
<td>TCCTAGGGAAACTTCG</td>
<td>Vilgalys &amp; Hester (1990)¹</td>
</tr>
<tr>
<td>nuLSU</td>
<td>LR16</td>
<td>TTCCACCCAAACACTCG</td>
<td>Moncalvo et al. (1993)</td>
</tr>
<tr>
<td>mtLSU</td>
<td>ML4</td>
<td>GAGGATAATTTGGCGAGTTCC</td>
<td>White et al. (1990)</td>
</tr>
</tbody>
</table>

¹ This primer sequence is from http://biology.duke.edu/fungi/mycolab/primers.htm, but it differs slightly from the original LR5 sequence published in Vilgalys & Hester (1990), which is ATCCTGAGGGAAACTTC.

² The online supplementary data of Printzen (2002) containing the primer sequences is apparently not accessible anymore. However, the same sequences were also published in Printzen & Ekman (2002), although without primer names. M.P. Nelsen kindly assigned the primer name and sequence of ML3.C (pers. comm. M.P. Nelsen, Chicago, 2012), which he used in Nelsen & Gargas (2009a).

Primer maps showing the relative positions of these primers are shown on the following websites:
- https://sites.google.com/site/mpnelsen/primer-maps
- http://www.lutzonilab.net/primers/page244.shtml
2.4 PCR protocols and sequences

Two kinds of commercial ready-to-use PCR master mix were used: “1.1X ReddyMix PCR Master Mix (1.5 mM MgCl$_2$)” (Thermo Scientific, Cat#: AB-0575/LD) and “1.1X ReddyMix PCR Master Mix (2.0 mM MgCl$_2$)” (Thermo Scientific Cat#: AB-0608/LD).

Deviating from manufacturer’s instructions, reactions were down scaled to a final reaction volume of 20 µl (instead of 50 µl), made up using 18 µl of “1.1X ReddyMix PCR Master Mix” (instead of 45 µl) plus 0.5 µl of each primer (at 10 pmol/µl) plus 1 µl of genomic DNA (at 10-200 ng/µl).

According to the manufacturer’s concentration information each 20 µl reaction consisted of: 0.5 units “ThermoPrime Plus DNA Polymerase, 75 mM Tris-HCl (pH 8.8 at 25°C), 20 mM (NH$_4$)$_2$SO$_4$, 1.5 or 2.0 mM MgCl$_2$, 0.01% (v/v) Tween” 20, 0.2 mM each of dATP, dCTP, dGTP and dTTP, precipitant and red dye for electrophoresis”, 0.25 pmol/µl of each primer, and 10-200 ng of genomic DNA. [Note: a faulty protocol is provided in the manual of the “1.1X ReddyMix PCR Master Mix (2.0 mM MgCl$_2$)” (Thermo Scientific Cat#: AB-0608/LD), as it obviously describes the setup of PCR using a 2-times concentrated master mix rather than 1.1-times.]

In a few cases, when specimens older than 3 years were extracted, previously failed amplifications were successful when 0.3 µl (0.75 units) of “FailSafe™ PCR Enzyme Mix” (Epicentre) was added to the reaction.

All reactions were prepared on ice and PCR was performed using the *Eppendorf Mastercycler*° or *Eppendorf Mastercycler*° gradient. Cycler conditions for the different primer combinations are summarized in Table 9.

Table 9. PCR conditions for ITS, nuLSU and mtLSU amplifications.

<table>
<thead>
<tr>
<th>Primer fwd. vs. rev.1 or rev.2</th>
<th>ITS1-F vs ITS4 or ITS4A</th>
<th>LR0R vs LR16 or LR5</th>
<th>ML3.C vs ML4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C, 5 min</td>
<td>95°C, 5 min</td>
<td>95°C, 5 min</td>
</tr>
<tr>
<td>Cycle denaturation</td>
<td>94°C, 1 min (or 45 s)</td>
<td>94°C, 1 min</td>
<td>94°C, 1 min</td>
</tr>
<tr>
<td>Cycle annealing</td>
<td>45°C, 1 min (or 45 s)</td>
<td>42°C, 1 min</td>
<td>58°C, 1 min</td>
</tr>
<tr>
<td>Cycle extension</td>
<td>72°C, 1 min (or 45 s or 90 s)</td>
<td>72°C, 80 s</td>
<td>72°C, 1 min</td>
</tr>
<tr>
<td>Cycle number</td>
<td>36</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C, 10 min (or 5 min)</td>
<td>72°C, 5 min</td>
<td>72°C, 5 min</td>
</tr>
<tr>
<td>Hold</td>
<td>14°C</td>
<td>14°C</td>
<td>14°C</td>
</tr>
</tbody>
</table>

PCR products were visualised by running 5-8 µl of PCR products on a 1% agarose (TAE buffer, Tris-acetate-EDTA) gel together with size marker “1 Kb Plus DNA Ladder” (Invitrogen) or “GeneRuler 1kb DNA Ladder” (Thermo Scientific). The composition of 1 x TAE buffer for gel electrophoresis is: 40 mM Tris (pH 7.6), 20 mM acetic acid, 1 mM EDTA.
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*PCR product purification.* PCR products were initially purified using the PureLink® PCR Purification Kit (Invitrogen, Life Technologies™). Later, the more cost-effective use of EconoSpin® columns together with homemade buffers was established (see above section 2.2).

*Sequencing.* Direct Sanger sequencing of purified PCR products (i.e. without prior cloning) was performed by the University of Otago Genetic Analysis Service. For that purpose 200 µl reaction tubes were provided, filled with a total volume of 5 µl of premixed primer (3.2 pmol/ 5 µl) and purified PCR product (0.01 ng DNA / bp product size / 5 µl). According to their website ([http://gas.otago.ac.nz/](http://gas.otago.ac.nz/)), the service provider used BigDye® Terminator Version 3.1 Ready Reaction Cycle Sequencing Kit for carrying out sequencing reactions followed by capillary separation using the 3730xl DNA Analyzer (Applied Biosystems).

Raw sequences were manually edited and analysed using the software Geneious (Biomatters Limited, [http://www.geneious.com/](http://www.geneious.com/)). Sequence quality was assessed according to the guidelines of Nilsson *et al.* (2012).

The newly generated rDNA sequences have been deposited at GenBank under the accession numbers KP759319 to KP759347 and KP984795 to KP984800 (Table 10). Annotation of the rRNA subunit boundaries follows Wuyts *et al.* (2002) for the 18S 3’ end, De Rijk *et al.* (1994) for the 5.8S 5’ end and Keller *et al.* (2009) for the 5.8S 3’ end as well as the 28S 5’end. This approach was taken, because other annotated GenBank sequences show considerable variability regarding the exact location of the boundaries of rRNA coding regions and the ITS regions, respectively.
Table 10. Overview of species, voucher specimens and GenBank accessions produced for or used in this study. Accession numbers of newly generated sequences are given in **bold** starting with KP...; those in the same row were derived from the same voucher specimen. For full details of voucher specimens see section 2.1 Examined Specimens.

<table>
<thead>
<tr>
<th>Species</th>
<th>Herbarium voucher</th>
<th>ITS</th>
<th>nuLSU</th>
<th>mtLSU</th>
</tr>
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<tbody>
<tr>
<td>Icmadophila ericetorum</td>
<td>OTA 061856</td>
<td>KP759337</td>
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<td>Icmadophila aversa</td>
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<td>Siphula polyschides</td>
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<td>(incl. subuliformis)</td>
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<td>Ochrolechia balcanica</td>
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<td>AF329171</td>
<td>AY568011</td>
</tr>
</tbody>
</table>
2.5 Sequence alignments and phylogenetic analysis.

2.5.1 Sequence alignments

Sequences of each locus were aligned separately using MUSCLE (Edgar 2004) run as plug-in version 2.0.9 in Geneious R7 (Biomatters, http://www.geneious.com/). The alignments were manually adjusted and are attached as Electronic Appendix, they will be deposited on TreeBASE prior to publication of this work in a peer-reviewed journal. Large introns present only in single taxa (e.g. in *Icmadophila ericetorum* or the *Siphula decumbens* group) were removed before running the alignment, as they are not informative but interfere with the automatic alignment. In addition, a concatenated alignment was created for those taxa where data for all loci was available.

GenBank sequences of several *Ochrolechia* ssp. were used as outgroup, since Ochrolechiaceae and Icmadophilaceae are both placed in the order Pertusariales (Tehler & Wedin 2008), based on earlier molecular phylogenetic results (Miadlikowska & Lutzoni 2004, Wedin et al. 2005, Miadlikowska et al. 2006, Lumbsch et al. 2007).

*O. balcanica* is the outgroup of the combined dataset, as it was the only *Ochrolechia* species for which sequences of all three loci were available. However, *O. balcanica* sequences of the ITS region and nuLSU are considerably shorter than those of the ingroup taxa. Therefore an additional outgroup sequence was used in the combined dataset, which is a ‘chimaera’ of longer sequences of *O. tartarea* (mtLSU, ITS region) and *O. frigida* (nuLSU).

2.5.2 Phylogenetic analyses

Four different phylogenetic analyses (Bayesian Inference, Maximum Likelihood, Maximum Parsimony, Neighbour Joining) were conducted for each of the four datasets (mtLSU, ITS region, nuLSU and combined data of all three loci). The works by Harrison & Langdale (2006) and Hall (2011) were used as general guide lines.

Unreliably aligned positions of the alignments were excluded from analyses after they were identified using Gblocks 0.91b (Talavera & Castresana 2007) with all three ‘less stringent’ settings on the Gblocks web server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html). Remaining gaps within aligned sequences were not treated as missing data. Missing ends of shorter sequences were treated as missing data.

Bayesian Inference (BI) was carried out using Markov chain Monte Carlo (MCMC) sampling in MrBayes 3.2.1 (Ronquist & Huelsenbeck 2003). MrModeltest 2.3 (Nylander 2004) was used to select the ‘best-fit’ substitution models for each dataset based on the Akaike Information Criterion (AIC). Selected models were: GTR+G for mtLSU, SYM+I+G for the ITS region, GTR+I+G for nuLSU. The combined dataset was partitioned into the three loci (mtLSU, ITS region, nuLSU) and different ‘best-
fit’ substitution models were selected and applied to each partition. These models were essentially
the same as for the separate datasets but with slightly different parameters due to the different
number of taxa. Two parallel MCMC runs were made with 10,000,000 generations and 4 simultaneous
chains each, starting from random trees. Every 500th tree was sampled; the first 25% of all trees were
discarded as initial burn-in. Convergence of parallel runs was verified by an average standard deviation
of split frequencies below 0.01 as well as a potential scale reduction factor approaching or equal 1.0.

Maximum Likelihood analysis (ML) was conducted using PAUP* 4.0a142 (Swofford 1991). The
‘best-fit’ substitution models of the three separate loci were selected as done for BI. However, the
combined dataset was not partitioned, as PAUP* is not able of performing partitioned analyses;
instead a ‘best-fit’ GTR+I+G model was selected for and applied to the concatenated alignment. A
starting tree was obtained via ‘as-is’ stepwise addition of sequences. The ML tree was searched by a
tree-bisection-reconnection (TBR) branch-swapping algorithm and branch support was calculated by
100 bootstrap replicates.

Maximum Parsimony analysis (MP) was conducted using PAUP* 4.0a142 (Swofford 1991). A
heuristic search was performed with 1000 bootstrap replicates, each calculated with 10 random
addition sequence replications and otherwise default parameters.

Neighbour Joining analysis (NJ) was conducted using PAUP* 4.0a142 (Swofford 1991). One
thousand bootstrap replicates were calculated with random tie breaking and ‘uncorrected’ p-distance
as measure of pairwise sequence similarity.

Interpretation of the results considered the 50% majority-rule consensus trees of all four
analyses of all four datasets. Bayesian posterior probabilities ≥ 0.95 and bootstrap support values ≥
70% (for ML, MP, NJ) were considered statistically significant. If for the same dataset a poorly
supported clade of one analysis contrasts with the well-supported topology of other analyses, only
the well-supported results were considered authentic. Phylograms were drawn in FigTree v.1.4.2
(Rambaut 2006-2014) and further edited in GIMP 2.6.
3. Results

3.1 Phylogenetic results

Tree topologies of all analyses (Figures 5-8) are congruent with regard to the placement of current *Icmadophila* species relative to each other. The five members of the genus *Icmadophila* as currently circumscribed are not monophyletic. All analyses provide strong support for a core group that includes the type *Icmadophila ericetorum* together with *I. aversa* and *I. japonica*. Likewise, results are highly supportive of *I. eucalypti* and *I. splachnirima* forming two separate entities, without closer affinities to other species in the family Icmadophilaceae. However, the exact branching patterns for the relationships of *I. splachnirima* and *I. eucalypti* relative to *T. vermicularis* remain unclear in some of the 16 analyses; these are detailed below.

*Icmadophila splachnirima* has a well-supported sister relationship to all the remaining members of Icmadophilaceae. The position of *I. splachnirima* relative to *T. vermicularis* is not resolved by BI and ML using the mtLSU dataset (Figure 7). The BI tree shows *I. splachnirima* and *T. vermicularis* together in an unsupported clade that is sister to the remaining Icmadophila. In the corresponding ML analysis the same area of the tree is collapsed into a three-way polytomy (i.e. < 50% bootstrap support) from which both *I. splachnirima* and *T. vermicularis* emerge separately, along with the clade containing the remainder of the family. In contrast, MP and NJ analyses of the mtLSU dataset show good bootstrap support (> 70%) for *I. splachnirima* as most basal taxon within Icmadophila, while the next node separates Thamnolia form the bulk of the family (see dashed branches in Figure 7). This alternative mtLSU topology is considered to be the most plausible and it also resembles that of the other three datasets (Figures 5, 6 and 8).

*Icmadophila eucalypti* is also supported as a separate entity, which has *T. vermicularis* as its direct basal taxon and *I. eucalypti* is sister to the large clade comprising *Icmadophila* s. str., *Chirleja*, *Dibaeis*, *Siphulella*, and *Siphula*, which is split into the *S. ceratites* group and the *S. decumbens* group. The basal position of *T. vermicularis* relative to *I. eucalypti* remains somewhat doubtful, because NJ analysis of the combined dataset placed both together as sister taxa in an unsupported clade (bootstrap = 59), whereas MP analysis of the nuLSU dataset (Figure 6) placed them both in a three-way polytomy. However, altogether 9 of the 16 analyses are supporting (i.e. above significance thresholds) a separate placement of *I. eucalypti*, with *T. vermicularis* as direct basal taxon. Another 5 (of the remaining 7) analyses show the same topology in the 50% majority rule consensus trees, but their support values are below the significance thresholds.
Figure 5. Phylogram of Bayesian Inference analysis of the ITS region, including branch support values of all four analyses, and current members of the genus *Icmadophila* highlighted in bold font. The sequence of branch support values is: Bayesian Inference (BI) / Maximum Likelihood (ML) / Maximum Parsimony (MP) / Neighbour Joining (NJ). BI and ML values are usually above the branch, MP and NJ below the branch, unless this was not possible for practical reasons; in which case values may be written to the right side of the node. Support values of BI are posterior probabilities (range 0 to 1), and bootstrap values for the rest (range 1 to 100). ‘P’ indicates a polytomy in the respective analysis, i.e. no value. ‘-’ indicates absence of the respective branch in the analysis, such a discrepancy is explained in the main text when relevant to generic placement of any taxa. The ‘//’ symbol indicates a branch which has been reduced in length by the factor 2.
Figure 6. Phylogram of Bayesian Inference analysis of the nuLSU, including branch support values of all four analyses, and current members of the genus *Icmadophila* highlighted in bold font. The sequence of branch support values is: Bayesian Inference (BI) / Maximum Likelihood (ML) / Maximum Parsimony (MP) / Neighbour Joining (NJ). BI and ML values are usually above the branch, MP and NJ below the branch, unless this was not possible for practical reasons; in which case values may be written to the right side of the terminal node. Support values of BI are posterior probabilities (range 0 to 1), and bootstrap values for the rest (range 1 to 100). ‘P’ indicates a polytomy in the respective analysis, i.e. no value. ‘-’ indicates absence of the respective branch in the analysis, such a discrepancy is explained in the main text when relevant to generic placement of any taxa. The ‘//’ symbol indicates a branch which has been reduced in length by the factor 2.
Chapter 2 – Phylogeny of the genus *Icmadophila*

**Figure 7.** Phylogram of Bayesian Inference analysis of the mtLSU, including branch support values of all four analyses, and current members of the genus *Icmadophila* highlighted in bold font. The sequence of branch support values is: Bayesian Inference (BI) / Maximum Likelihood (ML) / Maximum Parsimony (MP) / Neighbour Joining (NJ). BI and ML values are usually above the branch, MP and NJ below the branch, unless this was not possible for practical reasons; in which case values may be written to the right side of the node. Support values of BI are posterior probabilities (range 0 to 1), and bootstrap values for the rest (range 1 to 100). ‘P’ indicates a polytomy in the respective analysis, i.e. no value. ‘-’ indicates absence of the respective branch in the analysis, such a discrepancy is explained in the main text when relevant to generic placement of any taxa. The ‘//' symbol indicates a branch which has been reduced in length by the factor 3. Dashed lines show an alternative topology to the unsupported BI clade, which combines *I. splachnirima* and *T. vermicularis* in a sister relationship. The dashed alternative topology with *I. splachnirima* as most basal taxon of the family is well-supported by MP and NJ bootstrapping. Therefore, based on this mtLSU dataset, the dashed topology is here considered most plausible due to failure of BI and ML to reliably resolve the relationship between *I. splachnirima* and *T. vermicularis*. This topology also resembles that of the other three datasets.
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**Figure 8.** Phylogram of Bayesian Inference analysis of the combined dataset, including branch support values of all four analyses, and current members of the genus *Icmadophila* highlighted in bold font. The sequence of branch support values is: Bayesian Inference (BI) / Maximum Likelihood (ML) / Maximum Parsimony (MP) / Neighbour Joining (NJ). BI and ML values are usually above the branch, MP and NJ below the branch, unless this was not possible for practical reasons; in which case values may be written to the right side of the node. Support values of BI are posterior probabilities (range 0 to 1), and bootstrap values for the rest (range 1 to 100). ‘P’ indicates a polytomy in the respective analysis, i.e. no value. ‘-’ indicates absence of the respective branch in the analysis, such a discrepancy is explained in the main text when relevant to generic placement of any taxa. The ‘//’ symbol indicates a branch which has been reduced in length by the factor 2.
4. Discussion

4.1 The Phylogeny of Icmadophila

The presented data show that only three of the five species currently placed in *Icmadophila* belong in this genus: the type *I. ericetorum*, *I.aversa* and *I. japonica*.

*Icmadophila splachnirima* is only very distantly related to *I. ericetorum*, having a sister relationship to all other Icmadophilaceae, as already shown in the nuclear SSU phylogeny of Stenroos *et al.* (2002). Therefore, the monotypic genus *Knightiella* Müll. Arg. with the species *K. splachnirima* (Hook.f. & Taylor) Gyelnik 1931 should be reinstated, as suggested by Stenroos *et al.* (2002). Taking into account the recent emendation of this species’ description by Ludwig (2011) due to the frequent occurrence of marginal soralia on mostly infertile thalli, the valid and fully emended species name should be:

*Knightiella splachnirima* (Hook.f. & Taylor) Gyelnik 1931 emend. L. Ludw. 2011.¹


For additional synonymy see Galloway (2007: 650–651).

¹ The formal emendation of the species’ description should be retained in its name, as it represents a considerable change of this species’ circumscription (Art. 47 ICBN Melbourne Code), which alternatively could have resulted in the erection of a separate species/taxon following the lichenological Species Pair concept as discussed in Ludwig (2011). Ludwig (2011) used the taxon author abbreviation “L.R. Ludwig” in ignorance of the recommendations of Art. 46 of the ICBN Vienna Code ([http://www.iapt-taxon.org/icbn/main.htm](http://www.iapt-taxon.org/icbn/main.htm)) and the conventions of Brummitt & Powell (1992) for authors of plant names ([http://www.kew.org/data/authors.html](http://www.kew.org/data/authors.html)). The abbreviation above conforms with customary abbreviations of similar names in Brummitt & Powell (1992), according to [http://www.ipni.org/ipni/authorsearchpage.do](http://www.ipni.org/ipni/authorsearchpage.do) and recommendations under Art. 46 of the more recent ICBN Melbourne Code.
Icmadophila eucalypti, which has been provisionally placed in Icmadophila by Kantvilas (2011; in Lumbsch et al. 2011: 73), is neither closely related to I. ericetorum nor to any other species or genus in Icmadophilaceae. Therefore, it should better be placed in a genus of its own. The new genus Knightiellastrum and the new combination Knightiellastrum eucalypti are proposed:

Knightiellastrum (Kantvilas) L. Ludw. & Kantvilas gen. nov.

Description: see description of Icmadophila eucalypti Kantvilas in Lumbsch et al. (2011), Phytotaxa 18: 72.

Typus generis: Knightiellastrum eucalypti (Kantvilas) L. Ludw. & Kantvilas.

Etymology: from Knightiella and the Latin suffix ‘-astrum’ indicating incomplete resemblance, because the thallus appearance reminds the author of a small and infertile version Knightiella splachnirima.

Knightiellastrum eucalypti (Kantvilas) L. Ludw. & Kantvilas, comb. nov.


It should be clarified that this dissertation is not meant to be the place of publication of any nomenclatorial changes. Here merely the necessity and intention to publish such changes is indicated in a manner resembling an article of a scientific journal. Proposed nomenclatorial changes will be validly published in a peer-reviewed international journal soon after this dissertation has been accepted.

4.2 Phylogenetic relationships among the remaining genera of Icmadophilaceae

Siphula decumbens group. The genus Siphula Fr. as currently circumscribed is consistently polyphyletic, with the S. decumbens group being separated from the generitype S. ceratites, confirming the results of the nuLSU phylogeny by Grube & Kantvilas (2006) and the nuSSU analysis of Stenroos et al. (2002). This is suggestive of a segregation of the S. decumbens group into a genus of its own, a matter which is treated separately in Chapter 6, along with reports on the discovery of ascomata in S. decumbens and S. fastigiata.

Siphulella coralloidea. The placement of Siphulella coralloidea in Icmadophilaceae by Rambold et al. (1993) is here confirmed and the nuLSU data strongly support a sister-relationship to the Siphula decumbens group. This, however, remains controversial considering the ITS analyses, in which only BI shows sufficient clade support. Therefore, for the time being, its status as a monotypic genus remains unchanged. Even if the sister relationship receives further support in the future, this would not
necessarily justify an inclusion of *S. coralloidea* in the *S. decumbens* group. It is noteworthy that in the nuSSU analysis of Stenroos *et al.* (2002) *Endocena informis* is monophyletic with the *S. decumbens* group, too. This could indicate that *E. informis* and *S. coralloidea* might be congeneric. Regrettably, no specimen of *E. informis* was available for inclusion in the present study, so its exact relationship with *S. coralloidea* or the *S. decumbens* group needs to be clarified later. It would be desirable to do this in a study comprising all members of Icmadophilaceae, i.e. including *Pseudobaeomyces* and further species of *Dibaeis* and *Siphula* s.lat..

5. Summary

Sequences of the Internal Transcribed Spacer region (ITS) and Large Subunits of nuclear and mitochondrial ribosomal DNA were generated for all five species currently placed in *Icmadophila*, and additional members of Icmadophilaceae. Results of phylogenetic analyses strongly indicate that *Icmadophila* consists of only three species: the type *I. ericetorum* plus *I. aversa* and *I. japonica*. For *I. splachnirima* the genus *Knightiella* Müll. Arg. has been resurrected, and *I. eucalypti* belongs in a genus of its own, for which the new genus *Knightiellastrum* has been proposed.
CHAPTER 3

Population-genetic analysis of Icmadophila splachnirima

1. Introduction

The previous Chapter 2 on the phylogeny of *Icmadophila* and the current one are tightly connect by the use the same molecular-genetic techniques and largely the same samples, and in fact the previous chapter was derived from this population genetic work, after specimens of the additional four *Icmadophila* species became available. The species’ biogeography in Chapter 1 was an essential prerequisite for the current chapter, in order find potential sampling sites and evaluate the final sample coverage relative to the species’ global distribution.

The term population genetics summarises different approaches to compare the genetic variability among populations, usually in order to quantify the gene flow among or within them (see Nielsen & Slatkin 2013) for a general introduction to the subject). Several different markers can be used to measure genetic variability, the most commonly used ones are micro-satellites and single nucleotide polymorphisms (SNPs). The use of microsatellites is essentially what is commonly known as ‘genetic finger-printing’ in forensics and paternity assignment. Microsatellites are amplified short sequence repeats which can vary in length (i.e. number of repeats) among individuals, and the simultaneous use of several different micro-satellite loci generates a distinct ‘genetic finger-print’ when the amplification products are run on an agarose gel. This technique is very sensitive and highly specific and allows one to distinguish closely related specimens even within a local population; the drawback is that the development of (i.e. search for) suitable micro-satellite loci requires the availability of whole genome sequences (WGS), or alternatively excessive trial-and-error screenings.

An alternative to the use of microsatellites is to use unspecific (universal) fragment length polymorphism markers like AFLP (Amplified Fragment Length Polymorphism), RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequence Repeats); however since these markers are unspecific, use of these would pick up random contaminations and can only be used with very pure DNA samples, e.g. from axenic tissue cultures. When no WGS information or pure tissue cultures are available, it is common practice to use single nucleotide polymorphisms (SNPs) instead; however, this technique is less sensitive and can usually only pick up differences between specimens from spatially wide spread populations. In contrast to microsatellites, only one or a few loci are examined here, and the actual sequence of the amplified product (not its length) contains the desired information. The loci employed are usually already known to exhibit some degree of sequence variability, because they are non-coding sequences or sequences
containing introns, which allows mutations (substitutions, insertions or deletions of individual nucleotides) to occur without selective disadvantage.

Population genetic studies on lichens have recently been thoroughly reviewed by Werth (2010a) and a few more have been published since (Itten & Honegger 2010, Fernandez-Mendoza et al. 2011, Francisco de Oliveira et al. 2012). Relevant studies are outlined and discussed as appropriate in the discussion section of this chapter. Werth (2010b) also provided valuable sampling guide lines for such studies. As lichens are symbiotic ‘dual-organisms’, these studies often involve genetic analyses of both organisms, which allows one to infer the relative importance of sexual versus vegetative reproduction in species capable of both reproductive modes; however, this usually does not take into account the possibility of horizontal gene transfer (Tunjic & Korac 2013). The symbiotic nature of lichens also creates the additional problem of interfering signals from the other symbiont, especially for developing specific microsatellites. SNP analyses, on the other hand, can easily ensure symbiont specificity by using generally fungal or algal specific primers or loci, which do not occur in the other symbiont, e.g. photosynthesis related genes of the alga or genes involved in chitin or ergosterol biosynthesis of the fungus.

The only population genetic studies dealing with fairly close relatives of *I. splachnirima* were those by Cassie & Piercey-Normore (2008) and Nelsen & Gargas (2009a+b) on Thamnolia vermicularis (Icmadophilaceae), an exclusively clonally reproducing species without known mode of sexual reproduction. Because Nelsen & Gargas (2009a+b) have successfully used SNP analysis, this approach has been adopted for the present study, and the sequences are also useful for phylogenetic analyses (Chapter 2).

The aim of this study is to examine whether gene flow occurs among the very disjunct known populations of this species (see Chapter 1 and Figure 9), and to assess the relative importance of either reproductive mode for long-distance dispersal between localities. This will be done by examining the among-population variability of rDNA sequences of both the mycobiont and photobiont of *I. splachnirima* specimens originating from all over its geographical range. The inclusion of sterile and sorediate specimens, which Ludwig (2011, 2012) considers conspecific with the long-known fertile and esorediate form, tests whether this view is correct, or if a Species Pair sensu Poelt (1970) and Tehler (1982) exists. Werth (2010a) states that “Extremely rare species with small local population sizes should show high differentiation among populations both at small and at large spatial scales; this pattern yet remains to be demonstrated for lichenforming fungi”. Considering the disjunct distribution of *I. splachnirima* and relative rarity in NZ, this may be one possible outcome of the study. The studies by Nelsen & Gargas (2009a+b) and Francisco de Oliveira et al. (2012) were used as guide lines for this study, as the approach and extent of sampling in these studies is similar.
Current knowledge on the identity of the *I. splachnirima* photobiont presumes that it belongs in the genus *Coccomyxa* (Galloway & Elix 1980). In an attempt to assess how common the *I. splachnirima* photobiont is, its ITS sequence was compared with that of photobionts of other New Zealand lichens known to be associated with *Coccomyxa* photobionts. This might allow one to infer whether the *I. splachnirima* photobiont is rather common or not; this is an important aspect of the species’ sexual reproduction, for which it depends on re-lichenization with free-living algae. The ITS sequence was also used to attempt a more precise identification of the *I. splachnirima* photobiont, by comparing it with GenBank sequences as well as algal sequences generated from other *Icmadophila* species.

2. Material and Methods

2.1 Origin of analysed specimens

This study endeavoured to include *I. splachnirima* specimens originating from localities covering the entire global range of the species. 28 specimens from 17 localities were examined, with vouchers deposited in the OTA Otago herbarium (OTA, and duplicated where appropriate):

**OTA 062513**, *Icmadophila splachnirima*, South East Harbour catchment, Campbell Island, NZ, 52.59124°S 169.16098°E, ~100 m, leg. S.King, K. Vincent, A. Whittaker, 19/11/2012, NE facing slope, low scrub +sedges, fertile + esorediate.


**OTA 062503**, *Icmadophila splachnirima*, Blue Mts. Near Tapanui, Otago, NZ, 45°55'43.8"S 169°21'07.2"E, ~900 m, leg. Lars Ludwig, 03.02.2012, site is totally sheltered but bone dry. >>F<<


**OTA 062505**, *Icmadophila splachnirima*, Denniston Plateau near Westport, NZ, 41°46'25.0"S 171°45’39.0"E, 800 m, leg. Lars Ludwig, 05.03.2012, on ground-dwelling moss in bush.

**OTA 063975**, *Icmadophila splachnirima*, Aparima Forks bog, Takitimu Mts., Southland, NZ, 45°41’25.2"S 167°57’34.1"E, 550 m, leg. Lars Ludwig, 26.10.2011, fertile, esorediate, species is abundant there.
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**OTA 063974**, *Icmadophila splachnirima*, Aparima Hut bog, Takitimu Mts., Southland, NZ, 45°42'17.5"S 167°58'58.5"E, 490 m, leg. Lars Ludwig, 26.10.2011, sterile + sorediate, among *Empodisma* and *Dracophyllum* on moss.

**OTA 063973**, *Icmadophila splachnirima*, Rakeahua flat, Stewart Island, NZ, 46°59'25.6"S 167°50'20.9"E, 18 m, leg. Lars Ludwig, 12.12.2011, large fertile colony on moss and debris on old rotting manuka stump directly on the track.

**OTA 063972**, *Icmadophila splachnirima*, Rakeahua flat, Stewart Island, NZ, 46°58'49.8"S 167°53'04.1"E, 4 m, leg. Lars Ludwig, 13.12.2011, very large sterile colony (ca. 50 x 25 cm) grown over a big dead stump surrounded by ± dense manuka shrub, in generally open and wet clearing, ca. 330 m E-NE of Rakeahua Hut.

**OTA 062506**, *Icmadophila splachnirima*, Blue Mts. near Tapanui, Otago, NZ, 45°55'56.3"S 169°20'18.9"E, ~900 m, leg. Lars Ludwig, 26.01.2012.

**OTA 062507**, *Icmadophila splachnirima*, Blue Mts. near Tapanui, Otago, NZ, 45°55'56.3"S 169°20'18.9"E, ~900 m, leg. Lars Ludwig, 26.01.2012.


**OTA 062510**, *Icmadophila splachnirima*, between “Organpipes” and Butter’s Peak, Mt. Cargill area, Dunedin, Otago, NZ, 45°48'40.2"S 170°33'57.8"E, 590 m, leg. Lars Ludwig, 12.05.2013, on soil on rotting log directly along the board walk track, S-facing, sterile.

**OTA 062509**, *Icmadophila splachnirima*, Swampy Summit, Dunedin, Otago, NZ, 45°47'46.3"S 170°29'00.2"E, ~720 m, leg. Lars Ludwig, 29.03.2012, fertile specimen in “Murray’s Domain”.

**OTA 062508**, *Icmadophila splachnirima*, Swampy Summit, Dunedin, Otago, NZ, 45°47'45.7"S 170°28'56"E, ~720m, leg. Lars Ludwig, 29.03.2012, sterile patches among tussock on decaying tussock mount.

**OTA 063976**, *Icmadophila splachnirima*, Oban, Stewart Island, NZ, 46°54'15.4"S 168°07'15.1"E, 5 m, leg. Lars Ludwig, 29.03.2013, small peninsula between Thule and Golden Bay, ca. 40 m, West of jetty in Golden Bay, on liverwort growing on soil on S-facing slope, just above coastal rock under old *Dracophyllum longifolium*. Few small fertile patches, with *Siphula* sp.

**OTA 063988**, *Icmadophila splachnirima*, Swampy Summit, Dunedin, Otago, NZ, 45°48'03.7"S 170°28'57.5"E, ~720 m, leg. Lars Ludwig, 07.09.2012, fertile, on S-slope among Pentachondra.
**AK 303501** (duplicate is CHR 548855) *Icmadophila splachnirima*, New Zealand, Chathams Ecological Region and District, Rekohu (Chatham Island), Tuko-a-Tamatea Nature Reserve, Map CI1 412428, c. 256 m, 44°3'S 176°36'W, P J de Lange CH1728, P B Heenan, 02 Jun 2008, in dense Tarahinau/Matipo forest. Growing on side of track on damp peat in a small, well lighted, canopy gap. Adaxial thallus surface bright green when fresh. Forming small circular patches on peat.

**CHR 548855** (duplicate of AK 303501), details as AK 303501.

**AK 304001** (duplicate is CHR 548847), *Icmadophila splachnirima*, New Zealand, Chathams Ecological Region and District, Rangiauria (Pitt Island), Hakapa Hill (Walkemup), Map CI1 762205, c. 220 m, 44°15'S 176°10'W, coll. P J de Lange CH1910, PB Heenan, 30 Nov 2008. Uncommon. Found in one place on damp, eastern facing, peat banks partially shaded by overhanging fronds of Blechnum montanum. Growing intermixed with Kurzia and Telaranea liverwort turf. Plants rather dried out at the time of collection.

**CHR 548847** (duplicate of AK 304001), details as AK 304001.

**OTA 064245**, *Icmadophila splachnirima*, Silver Peaks North of Dunedin, between Pulpit Rock and Devil’s Staircase, 45°44'32.1"S 170°27'04.6"E, 740 m a.s.l., leg. Lars Ludwig, 15.09.2013, fallen off (by erosion) from large fertile patch on + W-facing bank along tramping track to Jubilee Hut.

**OTA 064246**, *Icmadophila splachnirima*, Silver Peaks near Dunedin, between Green Hill and Pulpit Rock, 45°44'48.0"S 170°27'27.7"E, 675 m a.s.l., leg. Lars Ludwig, 15.09.2013, from huge sterile colony immediately along track close to Rosella Ridge ‘intersection’ when walking up to Pulpit Rock. Colony still sterile + sorediate since 2008!

**OTA 064242**, *Icmadophila splachnirima*, Heaphy Track, near Perry Saddle Hut, 32 km SW of Collingwood, NZ, 40°53'59.2"S 172°24'20.4"E, 885 m a.s.l., leg. Lars Ludwig, 01.01.2014, along track, several small fertile thalli there.

**OTA 064241**, *Icmadophila splachnirima*, Heaphy Track, near James Mackay Hut, ca. 40 km NEN of Karamea, Westcoast of South Isl., NZ, 40°53'11.8"S 172°13'01.8"E, 695 m a.s.l., leg. Lars Ludwig, 04.01.2014, sterile.

**OTA 064240**, *Icmadophila splachnirima*, Croesus Track, Paparoa Range near Greymouth, West coast of Soth Island, NZ, 42°20'05"S 171°23'48"E, ~330 m a.s.l., leg. Lars Ludwig, 07.01.2014, less than 5 min from Blackball end of the track; on peatbank by the track, and on coal seam that is exposed there, also growing abundantly in last bend of road before carpark.

**DoC collections**, *Icmadophila splachnirima*, Coalburn catchment, ca. 48 km West of Hump Ridge range, Southland, NZ, 46°10'42.6"S 166°42'10.8"E, ~250 m a.s.l, leg. Morgan McLean, 26.03.2013, two small specimens were collected during the Department of Conservation TIER1 biodiversity monitoring
program and are held with the whole collection, plot G171, specimen IDs NV201300029 and NV201300189.

Photobionts of the following additional species/specimens were examined:

**OTA 061856**, *Icmadophila ericetorum*, Breitlahnalm, ca. 0.2 km SE of Breitlahn hut, along road to Schwarzensee, Schladminger Tauern, Steiermark, Austria, 47° 18'56"N 13°53'16"E, 1075 m, leg. P. Bilovitz & H. Mayrhofer 19222, 11.10.2012, rotting tree stump (willow) on road side.

**OTA 063995** (duplicate of K.Wilk 7685), *Icmadophila aversa*, South America, Bolivia: Dept. La Paz, Prov. Franz Tamayo, Madidi National Park, Cordillera Apolobamba, NE of Keara village, the timber line forest of *Polylepis pepei* by Tolca Cocha lake, 14°41’14"S 69°05’18"W, alt. 4056 m; much humid habitat, over bryophytes on rock, leg. K.Wilk 7685, 14 Oct. 2007. [same specimen as in Wilk 2010]

**TNS YO-6762**, Japan, Hokkaido, Prov. Kushiro: Mt. O-akan, on decayed stump, 494 m, 10 August 2009, Y. Ohmura 6762. [Same specimen as in Ohmura 2011]

**OTA 063987**, *Dibaeis absoluta*, Graham’s Bush, Sawyers Bay, Dunedin, Otago NZ, 45°48’33"S 170°35’00"E, ~140 m, leg. Lars Ludwig, 09.02.2011, claybank along the track from Sawyers Bay to Organ Pipes, in regenerating native bush.

**OTA 063978**, *Dibaeis arcuata*, Whareatea Mine area, Denniston Plateau near Westport, NZ, 41°45’55"S 171°47’08"E, leg. Lars Ludwig, 03.03.2012.

**OTA 063978**, *Dibaeis arcuata*, Swampy Summit, Dunedin, Otago, NZ, 45°48’20"S 170°29’00"E, 650 m, leg. Lars Ludwig, 11.06.2011.

**OTA 063983**, *Nephroma australe*, Knight’s bush, Tuapeka West, Otago, NZ, 45°54’51"S 169°29’31"E, 70 m, leg. Allison Knight, 19.06.2011.

**OTA 064305**, *Nephroma australe*, Denniston Plateau near Westport, NZ, 41°45’55"S 171°47’10"E, 640 m, leg. Lars Ludwig, 03.03.2014, Whareatea Mine area, boggy site near ‘car park’, < 100m from *I. splachnirima*.

**OTA 063980**, *Lichenomphalia cf. alpina*, Swampy Summit, Dunedin, Otago, NZ, 45°48’36"S 170°29’06"E, 580 m, leg. Lars Ludwig, 02.01.2011, stipe and hat same yellow colour.
Figure 9. Global distribution map of I. splachnirima including New Zealand, Tasmania and Victoria. Red dots represent known localities, blue squares are towns or cities, green circles indicate localities from which specimens have been included in this population genetic study, ‘+’ symbols are intersections of every 5th meridian and parallel.
2.2 DNA Extraction

DNA extraction methods were identical to those described in Chapter 2 on the phylogeny of the genus *Icmadophila*.

2.3 Examined loci

The available literature was screened for SNP loci and the respective primers commonly used in lichen population genetics studies (Werth 2010a and studies therein, Nelsen & Gargas 2009a+b, Fernandez-Mendoza et al. 2011, de Oliveira et al. 2012). A small set of loci appeared to be potentially suitable for this study, as these loci were found in other studies to exhibit a reasonable degree of variability. The respective primers mentioned in the literature were used to test for variability in a small sample of five specimens from distant localities. Only the fungal ITS region showed a moderate degree of variability and was subsequently used for analysis, while the other tested loci (nuLSU, nuSSU, IGS, mtLSU) were invariable, or in the case of RPB2 the PCR was unsuccessful. For the photobiont only the ITS region was tested and chosen for the analysis because of sufficient sequence variability in initial trials.

2.4 DNA sequence generation

Composition of PCRs was as described in Chapter 2 with primer combinations as given in Table 12 of the present chapter. PCR conditions were usually adopted or derived from previously published protocols (e.g. in references in Table 1 above). Cycler conditions for fungal PCRs were as given in Table 12.

---

**Table 11.** Sequences of PCR primers used for this study. The abbreviations (m) and (p) indicate whether the name refers to the locus of the mycobiont or photobiont.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Name</th>
<th>Sequence 5' to 3'</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS (m)</td>
<td>ITS1F</td>
<td>CTTGGTCATTAGAGGAAATGA</td>
<td>Gardes &amp; Bruns (1993), Dyer &amp; Murtagh (2001)</td>
</tr>
<tr>
<td>ITS (m/p)</td>
<td>ITS4</td>
<td>TCCTCGGCTTATGATGC</td>
<td>White et al. (1990)</td>
</tr>
<tr>
<td>ITS (m)</td>
<td>ITS4A</td>
<td>ATTTGAGCTGTTGGCGCTTCA</td>
<td>Taylor in Kroken &amp; Taylor (2001)</td>
</tr>
<tr>
<td>IGS (m)</td>
<td>ITG12a</td>
<td>AGTCTGTTGAATTGGCG</td>
<td>Carbone &amp; Kohn (1999)</td>
</tr>
<tr>
<td>IGS (m)</td>
<td>ITS4A</td>
<td>ATTTGAGCTGTTGGCGCTTCA</td>
<td>Taylor in Kroken &amp; Taylor (2001)</td>
</tr>
<tr>
<td>SSU (m)</td>
<td>nu-SSU-1203-5' (NS23UCB)</td>
<td>GACTCAACACGGGGAAACTC</td>
<td>Stenroos et al. (2002); Gargas &amp; Taylor (1992)</td>
</tr>
<tr>
<td>SSU (m)</td>
<td>nu-SSU-1750-3' (NS24UCB)</td>
<td>AAACCTGTGACGACCTTTT</td>
<td>Stenroos et al. (2002); Gargas &amp; Taylor (1992)</td>
</tr>
<tr>
<td>ITS (p)</td>
<td>KL-ITS1A</td>
<td>CGATGGGTTGCTGGTGGAAG</td>
<td>Lohtander et al. (2003)</td>
</tr>
<tr>
<td>ITS (p)</td>
<td>nr-SSU-1780-5'</td>
<td>CTGCAGGAAGATCGATTAC</td>
<td>Skaloud &amp; Peksa (2010); Piercey-Normore &amp; DePriest (2001)</td>
</tr>
<tr>
<td>ITS (p)</td>
<td>ITS4</td>
<td>TCCTCGGCTTATGATGC</td>
<td>White et al. (1990)</td>
</tr>
</tbody>
</table>
Table 12. PCR protocols used in this study, primer sequences and references are given in Table 16. Abbreviations are: ITS (Internal Transcribed Spacer), IGS (Inter-genic Spacer), nuSSU (nuclear Small Sununit rDNA), nuLSU (nuclear Large Subunit rDNA), mtLSU (mitochondrial Large Subunit rDNA), min (minutes), s (seconds).

<table>
<thead>
<tr>
<th>Locus</th>
<th>ITS (fungal)</th>
<th>IGS</th>
<th>nuSSU (18S)</th>
<th>nuLSU (28S)</th>
<th>mtLSU (23S)</th>
<th>ITS (algal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>ITS1-F</td>
<td>IGS12a</td>
<td>nuSSU-1203-5'</td>
<td>LROR</td>
<td>ML3.C</td>
<td>KL-ITS1A2</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>ITS4 / ITS4A</td>
<td>NS1R</td>
<td>nuSSU-1750-3'</td>
<td>LR16 / LR5</td>
<td>ML4</td>
<td>ITS4</td>
</tr>
<tr>
<td>Initial denaturation</td>
<td>95°C, 5 min</td>
<td>95°C, 5 min</td>
<td>95°C, 5 min</td>
<td>95°C, 5 min</td>
<td>95°C, 5 min</td>
<td>95°C, 5 min</td>
</tr>
<tr>
<td>Cycle denaturation</td>
<td>94°C, 1 min</td>
<td>95°C, 1 min</td>
<td>94°C, 1 min</td>
<td>94°C, 1 min</td>
<td>94°C, 1 min</td>
<td>95°C, 1 min</td>
</tr>
<tr>
<td>Cycle annealing</td>
<td>45°C, 1 min (or 45 s)</td>
<td>53°C, 1 min</td>
<td>53°C, 1 min</td>
<td>53°C, 1 min</td>
<td>58°C, 1 min</td>
<td>58°C, 1 min</td>
</tr>
<tr>
<td>Cycle extension</td>
<td>72°C, 1 min (45 or 90 s)</td>
<td>72°C, 1 min</td>
<td>72°C, 1 min</td>
<td>72°C, 80 s</td>
<td>72°C, 1 min</td>
<td>72°C, 1 min</td>
</tr>
<tr>
<td>Cycle number</td>
<td>36</td>
<td>36</td>
<td>36</td>
<td>36</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C, 10 min (or 5 min)</td>
<td>72°C, 10 min</td>
<td>72°C, 10 min</td>
<td>72°C, 5 min</td>
<td>72°C, 5 min</td>
<td>72°C, 10 min</td>
</tr>
<tr>
<td>Hold</td>
<td>14°C</td>
<td>14°C</td>
<td>14°C</td>
<td>14°C</td>
<td>14°C</td>
<td>14°C</td>
</tr>
</tbody>
</table>

Attempts to amplify a portion of the second largest subunit of RNA polymerase II gene (RPB2) were unsuccessful, although primers RPB2-A-MNTH / RPB2-B-MNTH were used, which have worked for Thamnolia vermicularis (Nelsen & Gargas 2009a); the PCR program was similar as for nuSSU, except with 55 °C annealing temperature as in Nelsen & Gargas (2009a).

Purification and sequencing of PCR products was conducted as described in Chapter 2.

2.5 Population Genetic Sequence Analysis

Sequencing chromatogram files were manually edited and aligned using the commercial software Geneious 7 (Biomatters Limited, http://www.geneious.com/). All nucleotide positions in the mycobiont ITS sequences were unambiguous, as expected for sequences derived from haploid thalline tissue; therefore the directly sequenced genotypes are simultaneously haplotypes, and no haplotype reconstruction was necessary.

Most genotypic photobiont ITS sequences included one or several ambiguous nucleotide positions, which is not surprising since green-algae are diploid organisms, which can be heterozygous. Algal haplotypes were inferred using the haplotype reconstruction software PHASE v2.1.1 (Stephens et al. 2001, Stephens & Donnelly 2003) along with the online tool seqPHASE (Flot 2010). The software was not able to resolve all ambiguities, but the pool of possible combinations for genotypes with several ambiguous positions was reduced in some cases.

Haplotype networks of both mycobionts and photobionts were generated from stripped alignments using the freeware R 2.10.1 (run via Tinn-R 1.17.2.4) with the slightly modified script TempNet v1.4 by Prost & Anderson (2011, http://www.stanford.edu/group/hadlylab/tempnet/). All
theoretically possible haplotypes were included for algal genotypes with unresolved ambiguities after haplotype reconstruction.

Illustrations of stripped alignments (i.e. without identical positions, comparing variable positions only) were generated with the freeware program CLC Sequence Viewer 6.8.1 and manually edited with the freeware GIMP 2.6.8.

2.6 Molecular-genetic Identification of photobionts

A BLAST search with selected representative haplotypes was conducted using the GenBank website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to get an initial idea about the *I. splachnirima* photobiont identity. Entries of lab strains without species level identification have been ignored. Similar sequences (of *Coccomyxa* s. lat.) from GenBank were aligned in Geneious and a Bayesian Inference analysis was performed as described in Chapter 2, except that only 1 million generations were used, as this was sufficient for convergence. The nucleotide substitution model selected by AIC in MrModeltest 2.3 was GTR+G. Sequences of *Elliptochloris* were used as outgroup, as this genus is closely related to *Coccomyxa* s. lat. according Blanc et al. (2012). An ITS sequence of the widely used lab-strain *Coccomyxa subellipsoidea* C-169 was not available; Blanc et al. (2012) have used such a sequence in their study, but without citing or depositing a reference sequence in GenBank or elsewhere.

3. Results

3.1 Mycobiont of *I. splachnirima*

The 528 bp mycobiont ITS alignment (between primer sites ITS1F and ITS4) contains only 4 variable positions and 5 haplotypes (Figure 10, Figure 11). Some regional differentiation into subpopulations is recognisable. All five samples from the northern West coast of the NZ South Island belong to haplotype #D, which is distinguished from all other haplotypes by a T in position 94 instead of a C. Haplotype #E occurred only in two of five specimens from the Dunedin region, and has a C in position 435 instead of an A as in all other sequences. Haplotype #A occurred only in one of the two samples from Campbell Island, and is unique in having a T in position 451 instead of a C.

The other two haplotypes are fairly widely distributed, and differ in position 378. Haplotype #B has a G in position 378, and occurs in 8 samples from the southern NZ South Island, Stewart Island (Rakeahua Flat) and the Chatham Islands. Haplotype #C has a T in position 378, and occurred in 12 samples from the southern NZ South Island (Dunedin area, Blue Mts., Takitimu Mts., Ajax Hill,…), Stewart Island (Oban, Rakeahua flat), Campbell Island as well as in both samples from Tasmania.
Figure 10. Variable positions of *I. splachnirima* mycobiont ITS haplotypes. Vertically written numbers above columns indicate the nucleotide position in the full alignment. The five different haplotypes are labelled as #A to #E in the right column. Sequence labels provide details of provenance, voucher specimens and reproductive mode (sexual or vegetative in parentheses) of the examined specimens.

Figure 11. Un-rooted haplotype network of *I. splachnirima* mycobiont ITS sequences. Haplotype names correspond to those in 10 (except the # symbol was omitted). The bubble area corresponds to the number of occurrences in the dataset. Directly connected bubbles differ by one mutational step.
3.2 Photobiont of *I. splachnirima*

The photobiont sequences represent at least two distinct species, which have a nucleotide similarity score of only ca. 89%. Apart from being members of *Coccomyxa* s. lat., the taxonomic rank of the taxa cannot be determined, and therefore both will be provisionally referred to as OTU (Operational Taxonomic Unit) number 1 and 2. This make-shift DNA taxonomy was justified by pairwise sequence similarities of > 95% within each OUT, which was the criterion used by Caron *et al.* (2009) for assigning OTUs of micro-algae sequences, although these authors used the better conserved 18S Small Subunit of nuclear ribosomal DNA.

OTU1 is the algal partner in 26 of the 28 examined samples, and pairwise nucleotide similarities are 98.7% or more with up to 15 different nucleotide positions (Figure 12). The 807 bp alignment contains 32 variable sites, and most genotypes are unique, often with (or because of) several ambiguities. Haplotype reconstruction yielded 153 different haplotypes (Table 13, Figure 13, Figure 14). This huge number is largely caused by numerous hypothetical haplotypes, due to several genotypes with multiple unresolved ambiguities, and the power-law relationship between number of binary ambiguous positions of a genotype and possible haplotypes (i.e. number of haplotypes = $2^{\text{number of ambiguous positions}}$). The numerous unresolved ambiguities mean that haplotype reconstruction was generally not very helpful. Only in a few complex genotypes were multiple ambiguities resolved to yield much simpler haplotypes. Of the 153 (largely hypothetical) haplotypes, only 22 occurred more than once (Table 13), with the remaining 131 being unique.

OTU2 was the photobiont of only two samples with 96.9% pairwise nucleotide similarity, i.e. 26 variable positions in 802 bp alignment (Figure 15). Notably, these two samples came from Tasmania and the West coast of the NZ South Island. Because OTU2 is represented by only two samples, which differ considerably in nucleotide composition, an analysis of haplotypes was considered redundant. Reconstructed haplotypes are shown in Figure 16.

A few re-occurring mycobiont-photobiont haplotype combinations (called ‘symbiotic haplotype’ hereafter) have been observed (Table 14); these might be indicative of vegetative dispersal among localities. However, many of these observations are not reliable, since they include hypothetical photobiont haplotypes, due to multiple unresolved ambiguities. Without doubt identical symbiotic haplotypes were found in two cases: first, central Stewart Island and Swampy Summit near Dunedin (both #C-49; distance ca. 240 km), and second, the Blue Mts. in Otago and Takitimu Mts. in Southland (both #C-51; distance 110 km). Symbiotic haplotype #C-49 (or rather the algal haplotype) may also have occurred in the two other sites from the Dunedin Region, as well as Ajax Hill (Catlins, between Dunedin and Invercargill) and the Chatham Islands.

Further potentially identical symbiotic haplotypes involving fungal haplotype #B and several different algal haplotypes (see Table 14 for details), may occur in the Silver Peaks and Ajax Hill (both southern South Island) and the Chatham Islands.
### Figure 12. Variable positions of *I. splachnirima* photobiont OTU1 ITS genotypes. Vertically written numbers above columns indicate the nucleotide position in the full alignment. Sequence labels provide details of provenance, voucher specimens and reproductive mode (sexual or vegetative in parentheses) of the examined specimens. IUPAC coding applies to ambiguous nucleotide positions: K=G/T; M=A/C; R=A/G; S=C/G; W=A/T; Y=C/T; indels are denoted as dash (−).
Table 13. Comparison of OTU1 photobiont ITS haplotypes, including haplotypes of both symbionts. This table may be considered an extension of Figure 12, with rows in the same order. Samples with more than two entries listed show hypothetically possible haplotypes, due to multiple ambiguous positions. For ease of comparability among samples, re-occurring haplotypes (ID nos. vertically written) are compiled in the right-hand presence/absence section (+/-) with ++ representing homozygous genotypes.

<table>
<thead>
<tr>
<th>Sample / provenance</th>
<th>Fungal haplotype #</th>
<th>Unique OTU1 HTs (excl. re-occurring ones)</th>
<th>Re-occurring OTU1 haplotypes #...</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE Harbour area, Campbell Island, NZ, OTA 062513 (sex)</td>
<td>C 1-28 excl. 6, 12,15,23</td>
<td>1 12 3 9 55 7 9 9</td>
<td>1 11 1 1 1 1 1 1 1 1 1 1</td>
</tr>
<tr>
<td>Beeman Hill, Campbell Island, NZ, OTA 063971 (sex + veg)</td>
<td>A -</td>
<td>62 5 3 9 0 1 5 6</td>
<td>6 8</td>
</tr>
<tr>
<td>Coalburn catchment, Southland, S. Isl., NZ, DoC collection (sex)</td>
<td>B 29-44</td>
<td>- - - - - - - -</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Rakeahua flat Stewart Island, NZ, OTA 063972 (veg)</td>
<td>B 45-48</td>
<td>- - - - - - - -</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Rakeahua flat Stewart Island, NZ, OTA 063973 (sex)</td>
<td>C -</td>
<td>- - - - ++ - - - -</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Blue Mts., Otago, S. Isl., NZ, OTA 062503 (veg)</td>
<td>C -</td>
<td>- - - - ++ - - - -</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Blue Mts., Otago, S. Isl., NZ, OTA 062507 (sex)</td>
<td>C -</td>
<td>- - - - ++ - - - -</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Swampy Summit, Dunedin, Otago, S. Isl. NZ, OTA 062508 (veg)</td>
<td>E 68</td>
<td>- - - - + - - - -</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Heaphy Track, N West coast, S. Isl., NZ OTA 064242 (sex)</td>
<td>D 69-76 excl. 75</td>
<td>- - - - + - - - -</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Heaphy Track, N West coast, S. Isl., NZ OTA 064281 (veg)</td>
<td>D 77-94</td>
<td>- - - - - - - -</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Swampy Summit, Otago, S. Isl. NZ, OTA 062509 (sex)</td>
<td>C -</td>
<td>- - - - ++ - - - -</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Takitimu Mts., Southland, S. Isl., NZ, OTA 063974 (veg)</td>
<td>C -</td>
<td>- - - - ++ - - - -</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Takitimu Mts., Southland, S. Isl., NZ, OTA 063974 (sex)</td>
<td>C -</td>
<td>- - - - ++ - - - -</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Denniston Plateau, West coast, S. Isl., NZ, OTA 062504 (sex)</td>
<td>D -</td>
<td>- - - - ++ - - - -</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Denniston Plateau, West coast, S. Isl., NZ, OTA 062505 (veg)</td>
<td>D -</td>
<td>- - - - ++ - - - -</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Mt. Cargill area, Dunedin, Otago, S. Isl., NZ OTA 062510 (veg)</td>
<td>C 98, 99, 100</td>
<td>- - - - + - - - -</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Oban, Stewart Island, NZ, OTA 063967 (sex)</td>
<td>C 101-106 excl. 105</td>
<td>- - - - - - - -</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Silver Peaks, Dunedin, Otago, NZ, OTA 064245 (sex)</td>
<td>E 108, 110, 113</td>
<td>- - - - ++ - - - -</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Silver Peaks, Dunedin, Otago, NZ, OTA 064246 (veg)</td>
<td>B 114-124 excl. 118</td>
<td>- - - - ++ - - - -</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Lake Strahan Track, Tasmania, AU, OTA 063990 (sex)</td>
<td>C 125-128</td>
<td>- - - - - - - -</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Chatham Island, NZ, AK 303501 (sex)</td>
<td>B -</td>
<td>- - - - ++ - - - -</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Chatham Island, NZ, CHR 548855 (sex)</td>
<td>B 131, 133, 136</td>
<td>- - - - ++ - - - -</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Pitt Isl., Chatham Islands, AK 304001 (veg)</td>
<td>B 137</td>
<td>- - - - ++ - - - -</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Pitt Isl., Chatham Islands, CHR 548847 (veg)</td>
<td>B 138-145</td>
<td>- - - - ++ - - - -</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>near Ajax Hill, Catlins region, S. Isl., NZ, OTA 062511 (veg)</td>
<td>C 146-153</td>
<td>- - - - ++ - - - -</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Ajax Hill, Catlins region, S. Isl., NZ, OTA 062512 (sex)</td>
<td>B -</td>
<td>- - - - ++ - - - -</td>
<td>- - - - - - - -</td>
</tr>
</tbody>
</table>
Figure 13. Haplotype network of photobiont OTU1 ITS data. Haplotypes are colour coded and (when possible) grouped by provenance. Also see caption of Figure 11.
Figure 14. OTU1 photobiont ITS haplotypes reconstructed with the software PHASE. Both haplotypes of the respective samples are grouped together with the specimen label placed between them. See caption of Figure 12 for details.
Table 14. Re-occurring combinations of mycobiont-photobiont haplotypes, indicative of vegetative dispersal among the respective localities. Fungal haplotypes are capital letters and algal haplotypes (all of OTU1) are numbers. A superscript "H" indicates samples where the algal haplotypes are hypothetical, due to multiple unresolved ambiguities in the genotype.

<table>
<thead>
<tr>
<th>Shared fungal-algal haplotype combination</th>
<th>Locality / sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>#C-49</td>
<td>Rakeahua flat Stewart Island, NZ, OTA 063973 (sex)</td>
</tr>
<tr>
<td></td>
<td>Swampy Summit, near Dunedin, Otago, S. Isl. NZ, OTA 062509 (sex)</td>
</tr>
<tr>
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<td>Mt. Cargill area, near Dunedin, Otago, S. Isl., NZ OTA 062510 (veg)</td>
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<td>Pitt Isl., Chatham Islands, AK 304001 (veg)</td>
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3.3 Photobiont identity of *I. splachnirima* and some other species

Haplotype #49 was chosen to represent *I. splachnirima* photobiont OTU1 for phylogenetic analysis. This choice was made because #49 is the most frequently occurring haplotype, and it occurred two times as homozygous genotype as well (see Table 13), i.e. it is a truly existing ITS sequence, not just a hypothetical one, in contrast to most other reconstructed haplotypes. In pairwise full coverage alignments #49 is most similar (93%) to the ITS sequence of *Coccomyxa peltigerae* (FN 597599) and the next closest entry (90.4%) is *Pseudococcomyxa simplex* (FN298926).

For OTU2 the Genbank search was conducted with the sample from Paparoa Range, NZ (OTA 064240), which has a genotype with one ambiguity, i.e. two haplotypes differing in one position. This single difference between both haplotypes is irrelevant for the taxonomic analysis. The closest full coverage GenBank matches of OTU2 are very similar to those of OTU1, although the pairwise identity of both OTUs is only 89%. With full sequence coverage and 92% identity, *Coccomyxa peltigerae* (FN597599) is the closest to OTU2, followed by *C. solorinae* varieties (AY29367, AY293966, AY293965) with 89-90% and *Pseudococcomyxa simplex* (Mainx) Fott (FN298926, HE586504) with 89%.

Figure 17 shows that Bayesian Inference placed both *I. splachnirima* photobiont OTUs together in a well-supported clade with the photobionts of *Dibaeis arcuata* and *Lichenomphalia* sp., which is sister in a triple polytomy to a clade consisting of photobionts of various lichens of the order Peltigerales (genera *Nephroma*, *Peltigera*, *Solorina*). This third highly supported clade of the polytomy consists of *Pseudococcomyxa simplex*, *Coccomyxa rayssiae*, and *Choricystis chodatii*. The latter is in a remote and non-monophyletic position relative to the type of the genus *Choricystis minor*.

The photobionts of *Icmadophila ericetorum*, *I. aversa* and *I. japonica* are in a separate strongly supported clade including *Coccomyxa subellipsoidea* SAG 216-13 (AY328523) and the photobiont of *Peltigera britannica*. In pairwise nucleotide comparisons, the photobionts of the three *Icmadophila*...
spp. are more than 97% identical to the *C. subellipsoidea* ITS sequence. This ‘*C. subellipsoidea*-clade’ is the highly supported sister-clade to the whole ‘*I.splachnirima*+*Pseudococcomyxa*+*Peltigerales*’-clade described in the previous paragraph.

The photobiont of *Dibaeis absoluta* is closest to the outgroup taxon *Elliptochloris subsphaerica*, although it has only a 78% pairwise nucleotide identity score.

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**Figure 17.** Phylogenetic tree of *Coccomyxa* s. lat ITS sequences. Posterior probabilities of Bayesian Inference analysis are indicated. The abbreviation ‘pb.’ means photobiont of the respective lichen.
4. Discussion

Three locally restricted mycobiont haplotypes were found (#A, #D, #E), indicating local differentiation of subpopulations. In contrast, two widespread fungal haplotypes (#B, #C) indicate recent gene flow among distant populations; #C even occurs in Tasmania and several New Zealand (NZ) localities including Campbell Island (2,100 km from the Tasmanian locality). Furthermore, the mycobiont data provides clear evidence that fertile, esorediate specimens and sterile, sorediate specimens are conspecific as previously assumed by Ludwig (2011, 2012) rather than a species-pair sensu Poelt (1970), which is also supported by the outcome of reciprocal transplants described in Chapter 5. This is in accord with the view of Printzen (2014), who concludes that the Species-Pair Theory is obsolete, based on evidence from a growing number of molecular studies.

The algal genotypes and haplotypes are much more diverse than those of the mycobiont, which is interpreted as evidence that medium to long distance dispersal among the sampled populations occurs mainly via sexual ascospores followed by re-lichenization. This is in line with the argumentation of Werth & Sork (2010), who concluded that horizontal photobiont transmission via relichenized ascospores predominates in Ramalina menziesii, based on very dissimilar patterns of genetic diversity in both symbionts. However, in at least two cases, re-occurring mycobiont-photobiont haplotype combinations of I. splachnirima indicate that medium range dispersal (100-250 km) could take place via soredia as well. Perhaps vegetative dispersal has even occurred between the South Island and the Chatham Islands (ca. 1,000 km distance), but evidence for this is vague, as it is inferred from hypothetical algal haplotypes, because of multiple unresolved ambiguities in the respective genotypes. In theory, however, Werth & Sork (2010) have also pointed out that vegetative dispersal might become undetectable, if the original photobionts of dispersed soredia get replaced/outcompeted by better adapted local algae in course of thallus development.

It is interesting to see that Nelsen & Gargas’ (2009a: 47, Figure 1) ITS haplotype network of the strictly asexual Thamnolia vermicularis (Icmadophilaceae) shows a very similar degree of variability and relatedness as found in the present study for I. splachnirima. These authors have used a similar samples size (n=26), however, originating from ‘a broad geographic range’ (Alaska, China, Costa Rica, Norway). Thus I. splachnirima shows a comparatively high degree of ITS variability relative to the geographic range covered by the examined samples. The lack of sexual reproduction in T. vermicularis seems to be the best explanation for this difference, as sexual recombination within geographically wide-spread and more or less isolated populations should favour the evolution of new haplotypes. The occurrence of the same ITS haplotypes in T. vermicularis samples from Norway and Alaska may be explained by long distance dispersal via asexual conidio-spores shed from pycnidia,
which were recently re-discovered for *T. vermicularis* (Lord *et al.* 2013). The 14 different *T. vermicularis* photobiont ITS sequences of Nelsen & Gargas (2009b) were at least 89.6 % identical in pair-wise comparisons. This finding is the same as for the *I. splachnirima* algae, but the *T. vermicularis* photobiont belongs in the genus *Trebouxia*. However, at virtually identical sample size but much smaller geographical coverage, *I. splachnirima* is associated with a distinctly greater diversity of algal genotypes, as would be expected for a frequently sexual species compared with a strictly asexual one.

A study by Printzen & Ekman (2002) examined trans-Atlantic populations of *Cavernularia hultenii* and compared 40 mycobiont ITS sequences and 45 mycobiont IGS (inter-genic spacer) sequences in samples from Europe (Norway, Scotland), Newfound and the North American Westcoast. *C. hultenii* propagates mainly by soredia and apothecia are very rarely formed. The authors identified 11 ITS haplotypes and shared haplotypes between North America and Europe indicate long distance dispersal. The authors concluded that diaspore dispersal between the three regions is ‘unlikely to be frequent even on an evolutionary time scale’, because the shared haplotypes occupy central positions in the un-rooted haplotype network, while the unique and locally restricted haplotypes are situated in terminal positions. Some samples from the North American West coast, however, are fairly distantly related to the rest; this points towards prolonged periods of reproductive isolation, which has also resulted in formation of several unique haplotypes in that region. A similar pattern can be seen in the much simpler un-rooted haplotype network of the *I. splachnirima* mycobiont (Figure 11). When applying the same reasoning as Printzen & Ekman (2002), it can be concluded that the two common and central haplotypes (#B, #C) would be ancestral and were widely dispersed in historic times, while the three peripherally situated and locally restricted haplotypes (#A, #D, #E) have in all probability formed as a result of more recent reproductive isolation. Printzen & Ekman (2002) also conclude for their species that ‘the current pattern of disjunction is due to fragmentation of a formerly continuous range’. This is plausible considering a past connection of North America and Eurasia via a Bering land bridge, which is now a shallow sea. For *I. splachnirima*, a formerly continuous geographic range can most certainly be ruled out as alternative to long-distance dispersal, if the current species distribution and high similarity of mycobiont ITS sequences across its range ought to be explained. Firstly, there was no continuous landmass since Zealandia (the largely submerged continent of which NZ is a part) got separated from Australia and Tasmania about 53-83 million years ago (Campbell *et al.* 2012), and the NZ offshore islands (Chatham, Campbell and Auckland Islands) are separated from the main land by a vast ocean several hundred meters deep. These offshore islands were formed by volcanic activity on the continental shelf at different times in the last 25 million years (Neall & Trewick 2008) and were therefore separately colonised via long-distance dispersal. Furthermore, Campbell and Auckland Islands were largely ice-covered during the Quaternary
glaciations and must have been re-colonised via long-distance dispersal (Quilty 2007, Hodgson et al. 2014), likely within the last 15,000 years after peat accumulation resumed. Secondly, the preferred habitat type of *I. splachnirima*, peat bogs, has a very scattered distribution in southern NZ, with wide expanses of unsuitable habitat types. It appears unlikely that this situation was much different in the geologically recent past, which would be the only way to explain the high genetic similarities observed among distant localities without long-distance dispersal.

The work of Högberg et al. (2002) is similar to that of Printzen & Ekman (2002), comparing SNPs of trans-Atlantic populations of *Letharia vulpina*, a species in which ‘all individuals produce abundant soredia and/or isidioid soredia, but ascomata are rare in western North America and exceedingly rare in Europe’. Högberg et al.’s study is also comparable to the present one with respect to sample size (n=47), but they used 8 different loci, none of which is ITS, and only of the mycobiont. A total of 10 haplotypes were found among the 47 samples. Each of the seven North American samples (from the same locality in California) had different haplotypes, while the 40 European samples (from Sweden and Italy) together had only three haplotypes. This study provides a good example of the degree of genetic variability existing in populations reproducing almost exclusively by vegetative propagules (Europe) versus a population where sexual and vegetative reproduction co-occur (North America).

The SNP study on *Cetraria aculeata* by Fernandez-Mendoza et al. (2011) examined both mycobiont and photobiont. However, comparability of their study with the present one is limited, as *C. aculeata* disperses mostly vegetatively. They used 3 loci per symbiont (though including fungal and algal ITS), and far more samples (n=203) covering a bipolar distribution (Svalbard, Iceland, Spain, Turkey, Kazakhstan, Chile, Falkland Isl., Antarctica). Compared to the small *I. splachnirima* data set, the *C. aculeata* mycobiont ITS haplotype network appears surprisingly simple (27 haplotypes, 13 of them unique) considering the large sample size and vast geographical range covered; this can be attributed to the predominantly vegetative reproduction of the species. Furthermore, all three mycobiont loci show haplotypes shared between boreal and austral populations, indicating long distance dispersal over more than 11,000 km. This puts into perspective the present findings of identical fungal *I. splachnirima* haplotype #C in Tasmania and various NZ localities (max. distance 2,100 km), and also the potential vegetative dispersal between the South Island and the Chatham Islands (ca. 1,000 km). The *C. aculeata* photobiont haplotype networks are comparatively simple as well, also with shared and closely related boreal and austral haplotypes, which again supports vegetative long distance dispersal in this species. All together, comparison of the present study with that of Fernandez-Mendoza et al. (2011) reinforces the initial conclusion that the *I. splachnirima* data indicate prevailing sexual reproduction with occasional long-distance dispersal of sexual and vegetative propagules.
In contrast, no indication for trans-Atlantic long-distance dispersal was found by Palice & Printzen (2004), who examined mycobiont ITS sequences of 31 Latin American and 38 European specimens of *Trapeliopsis glaucolepidea*, without finding any haplotypes shared between the continents. Their study differs from the present one in sample size and its maximum distance between sites is far beyond that of the present study. However, comparison with *T. glaucolepidea* is especially interesting, because the general ecology of this species is exceptionally similar to that of *I. splachnirima*. According to Palice & Printzen (2004) this small-squamulose species grows mainly on freshly exposed naked peat, soil and plant debris in open moorland and other humid habitats. Just as in *I. splachnirima*, *T. glaucolepidea* reproduces frequently by both apothecia and marginal soralia, but “richly fertile specimens are only sparsely sorediate or the soredia are missing completely” and the “morphological differences apparently reflect environmental conditions, such as light, humidity (...)” (see Chapter 5 of this dissertation to understand the potential implications of these ecological parallels). The *I. splachnirima* dataset has no parallel to the disjunction between Europe and Latin America, but the European *T. glaucolepidea* subset alone is comparable to the *I. splachnirima* data with respect to sample size and distribution. The European part of the haplotype network shows a low degree of complexity that is partly comparable to the *I. splachnirima* mycobiont network (Figure 11), except that three of the seven haplotypes of *T. glaucolepidea* are only remotely related to the remaining four. The authors provide only very speculative potential explanations for this observation, and these are irrelevant with respect to *I. spachnirima*, as no equivalent pattern was observed in the present study.

The study by Yahr *et al.* (2006) on the cushion-forming ‘reindeer-lichen’ *Cladonia subtenuis* deals with a species endemic to eastern North America capable of both sexual reproduction by apothecia and vegetative reproduction by fragments (Beard & DePriest 1996). Yahr *et al.* (2006) examined the fungal and algal ITS (plus two further mycobiont loci) of 79 thalli sampled from 11 sites throughout the species’ range. Comparability of Yahr *et al.*’s study with the present one is impaired by different sampling strategies. Yahr *et al.* have sampled 3 to 10 thalli per locality; this allowed them to perform extensive statistical analyses, which was not the case in the present study. Within localities the authors detected high diversity of fungal ITS genotypes, but compared to the algal partners the mycobiont ITS diversity was still relatively small; this finding is similar to the *I. splachnirima* data reported here. With reference to the example of Kroken & Taylor (2001), Yahr *et al.* (2006) point out the possibility that in this particular species the ITS region may be a less variable genetic marker than other loci like EF1α or RPB2. It cannot be ruled out that the measured low genetic variability of *I. splachnirima* may also be biased by the use of a less variable locus. Because *C. subtenuis* has no specialised vegetative propagules, Yahr *et al.* (2006) consider the observation of several putative
clones (i.e. specimens with identical fungal and algal haplotypes) in distant localities (average 160 km, maximum > 550 km) as “perhaps most likely, chance reassociation with identical genotypes”. This possibility is rejected as best explanation for the occurrence of identical ‘symbiotic haplotypes’ in *I. splachnirima* (Table 14), because this species does produce soredia for vegetative reproduction (Ludwig 2011, 2012). Furthermore, Yahr *et al.* (2006) argue that “the occurrence of seven different algal genotypes with a single fungal genotype (…) supports the hypothesis of horizontal transmission of symbionts” which is “also consistent with (…) de novo lichenization”. Since the *I. splachnirima* data shows that fungal haplotypes #B and #C are associated with 7 and 11 different algal genotypes (Table 14, Figure 10, Figure 12), respectively, it can be confidently deduced that sexual reproduction is generally its predominant reproductive mode. Likewise, the conclusion of Yahr *et al.* (2006) seems applicable to *I. splachnirima*; they state: “Although clonal propagation cannot be rejected as contributing to the patterns of association between fungal and algal symbionts, it does not appear to be a major factor in this species”.

Francisco de Oliveira *et al.* (2012) have studied photobiont ITS sequences of the exclusively sexually reproducing *Ramalina sinensis* in Canada and the USA, but compared them also with Chinese GenBank sequences (n=23, [26 with GenBank]). Twelve algal haplotype were found among the 23 North American samples, which did not correspond with geographical location. This is a fairly similar outcome as for the presented *I. splachnirima* photobiont data, in which the true haplotype diversity is obscured by multiple genotypic ambiguities, which likely creates the impression of a higher algal diversity than might actually exists. From this it can again be concluded that sexual reproduction shapes the large scale population genetic structure in *I. splachnirima*.

Micro-satellites as used for population genetic studies on *Lobaria pulmonaria* (Walser *et al.* 2003, 2004 a+b, 2005, Wagner *et al.* 2006, Werth *et al.* 2006 a+b, 2013, Dal Grande *et al.* 2010, 2012 a+b, Widmer *et al.* 2010, Scheidegger *et al.* 2012, Singh *et al.* 2012, Werth & Scheidegger 2012) would have been a much more sensitive and desirable marker for genetic variability. However, this would have required development of micro-satellites or the availability of the whole genome sequence, and the extent of such studies with several hundreds or thousands of samples (e.g. Werth *et al.* 2006, Dal Grande *et al.* 2012, Scheidegger & Werth 2012, Singh *et al.* 2012) is beyond the scope of this study. A future study using micro-satellites could show to what extent vegetative dispersal shapes the genetic structure within local populations of *I. splachnirima*. It is plausible to assume that soredia are fairly important for establishment and population maintenance based on current knowledge (Bailey 1966, Armstrong 1987, 1991, 1994; Seymour *et al.* 2005b), but for now this remains speculative for *I. splachnirima*. 
The identification of the *I. splachnirima* photobiont beyond the status *Coccomyxa* s. lat. is currently not possible. The taxonomy of the green-algal genus *Coccomyxa* s. lat. is in urgent need of a molecular-genetic revision, since two genera have been segregated from *Coccomyxa*, based on seemingly marginal morphological differences compared to the generitype *C. confluens* (Kützing) Fott (syn. *C. dispar* Schmidle; however, no molecular-genetic verification has yet been presented to show that these are distinct monophyletic entities relative to *Coccomyxa* s. str. Currently accepted segregates of *Coccomyxa* s. lat. are the genera *Pseudococcomyxa* Korshikov with the type species *P. simplex*, and *Choricystis* (Skuja) Fott with the type species *C. minor* (according to AlgaeBase (http://www.algaebase.org). Etlt & Gärtner (1999: 449) list 23 *Coccomyxa* species they consider ‘uncertain species’, among them also the closest known relatives of the *I. splachnirima* photobionts: *C. peltigerae* Jaag, *C. peltigerae-venosae* Jaag, *C. solorinae* Chodat, *C. solorinae-bisporae* Jaag, *C. solorinae-croceae* Chodat, *C. solorinae-saccatae* Chodat. Furthermore, no ITS sequence data of *C. confluens* is currently available at GenBank, which means any molecular-genetic attempt to test whether a sample is truly congeneric based on ITS sequences is rendered futile. Nuclear Small Subunit rDNA sequences assigned to *C. dispar* (a synonym of *C. confluens*, see Gärtner & Ernet 1993) have been deposited at GenBank (AB488787, AB488788, AB488794, AB488795). However, this is not helpful for the present study on the ITS region, and apparently these sequences have not yet been published in a peer-reviewed research article, so they should be used with caution. An additional problem for a taxonomic revision of *Coccomyxa* s. lat. stems from the fact that many important pieces of taxonomic literature on this genus have been published in German (Kützing 1845-1849, Schmidle 1901, Jaag 1933, Skuja 1948, Fott 1974, 1976, Komárek 1979, Fott 1981, Komárek & Fott 1983, Gärtner & Ernet 1993, Etlt & Gärtner 1995), Ukrainian/Russian (Korshikov 1953) and Czech (Nemjová 2009) languages, which makes them virtually inaccessible for most researchers lacking the respective language skills. The thesis by Nemjová (2009) on the ‘Molecular phylogenetics and geometric morphometrics of aerophytic green algae of *Coccomyxa/Pseudococcomyxa* s. l. complex’ using nuSSU and ITS sequences is a desirable first step towards the clarification of the confusing taxonomy of *Coccomyxa* s. lat., but regrettably this treatment has still not been published as an English research article and is currently available only as the original thesis in the Czech language. As a supplementary notice (pers. comm. Lydia Gustavs, Rostock, 03.05.2015), it should be mentioned that a molecular-genetic revision of the genus *Coccomyxa* will soon be published by Darienko et al. (2015, in press), which hopefully allows to specify the generic position of the *I. splachnirima* photobionts within *Coccomyxa* s. lat.
The extraordinarily small cell size (ca. 4 x 2 µm) of the *I. splachnirima* photobiont is only similar to that of *Choricystis minor* (Skuja) Fott, as are illustrations of *C. minor* var. *gallica* in Komárek & Fott (1983: 647) and Ettl & Gärtner (1999: 433). However, a GenBank ITS sequence (FN870434) assigned to *Choricystis minor* (not var. *gallica*) is very distant to the sequences of the *I. splachnirima* algae.

The fairly close affinity of the *I. splachnirima* photobionts with *Pseudococcomyxa simplex* is puzzling considering that the latter is nested within two clades containing *Coccomyxa* spp., and *Pseudococcomyxa* is currently placed in the family Oocystaceae (according to http://www.algaebase.org, September 2014) rather than Coccomyxaceae.

Photobionts of *I. ericetorum*, *I. aversa* and *I. japonica* group with *Coccomyxa subellipsoidea* SAG 216-13 (AY328523), and can with some confidence be considered conspecific due to pairwise nucleotide identity scores of over 97 %. However, the species level identification of the culture SAG 216-13 is not reliable, since the strain is classified as not authentic according to the SAG website (http://sagdb.uni-goettingen.de/showstrains.php?genus=Coccomyxa&species=subellipsoidea) and originates from Austria, while Acton’s (1909) original description of *C. subellipsoidea* is based on studies of British material. Furthermore, no type collection has been specified by Acton (1909), nor is it designated on the AlgaeBase website, which necessitates a proper typification, preferably from Acton’s collections.

The genetic comparison of the *I. splachnirima* photobiont with those of some other New Zealand lichens presumably associated with algae of the genus *Coccomyxa* showed that *Lichenomphalia cf. alpina* from Swampy Summit contained an alga that is genetically indistinguishable from *I. splachnirima* photobiont haplotype #49. The remaining examined lichen species (*Dibaeis absoluta*, *D. arcuata* and *Nephroma australe*) had very different photobionts. It is unclear to what extent this finding can be generalised, but it is possible that the presence of *Lichenomphalia* could be used in future vegetation surveys to locate potential unknown sites of *I. splachnirima*, since both species co-occur very frequently; especially as the bright yellow mushrooms of *L. cf. alpina* are much easier to spot than *I. splachnirima* thalli. A noteworthy parallel is that the Northern Hemisphere *Lichenomphalia hudsoniana* was reported to be associated with the same *Coccomyxa* alga as *Icmadophila ericetorum* (Honegger & Brunner 1981, Tschermak-Woess 1988). However, it should be kept in mind that it was shown in Chapter 2 of this dissertation that *I. ericetorum* and *I. splachnirima* are not congeneric, which means this note should not be overrated.

Knowledge about the mating system of *I. splachnirima*, i.e. whether it can self-fertilise or not, is desirable for further interpretation the population genetic results. This aspect of the species’ reproductive ecology was examined in the following Chapter 4.
5. Summary

Fungal and algal ITS sequences of *Icmadophila splachnirima* were compared from specimens originating from localities covering almost the entire geographical range of the species, in order to examine whether gene flow occurs between the disjunct populations and to assess the relative importance of sexual versus vegetative propagules for long distance dispersal. Very little variability was observed in fungal sequences, however evidence for differentiation of regional subpopulations was found, but also evidence for long-distance dispersal, which likely predates the regional differentiation. In contrast, almost every sample has a unique algal genotype. This suggests that fungal gene flow may still occur among the very disjunct known localities, and that long distance dispersal usually occurs via ascospores rather than soredia. Furthermore, an identical algal haplotype was found in *Lichenomphalia cf. alpina*, which points towards a relatively abundant availability of compatible photobionts for re-lichenization, since this is a wide-spread species, often abundantly occurring in similar habitats to *I. splachnirima*. The previous identification of the *I. splachnirima* photobiont as *Coccomyxa* s. lat. has been confirmed, but more accurate classification needs to be postponed until a molecular-genetic revision of this green-algal genus becomes available.
CHAPTER 4

Characterisation of the mating type locus (MAT) and mating type analysis of *Icmadophila splachnirima*

1. Introduction

Examination of the mating system of *Icmadophila splachnirima* is a crucial aspect of the species’ reproductive biology, as it has a restricted and very disjunct distribution in South-Eastern Australasia (see Chapter 1) and was issued the threat rating ‘Nationally Vulnerable’ for New Zealand (De Lange et al. 2012). The future survival of isolated populations of this species may depend on whether or not it can self-fertilise, and knowledge of its mating system would ideally influence the action to be taken by conservation authorities.

Sexual reproduction of lichenised ascomycetes is controlled by the mating type locus *MAT* (abbreviated nomenclature after Turgeon & Yoder 2000), which codes for transcription factors required for sexual morphogenesis (Debuche & Turgeon 2006, Honegger & Scherrer 2008). Whether a lichen species is capable of self-fertilisation (homothallism) or relies on outcrossing (heterothallism) depends on the content of the *MAT* locus. In a heterothallic species, each haploid individual possesses one of two different forms of the *MAT* locus (MAT1-1 and MAT1-2; nomenclature after Turgeon & Yoder 2000), which are referred to as idiomorphs. Therefore in heterothallic species individuals belong to one of two mating types (‘sexes’), and successful mating is only possible between individuals of opposite mating type. In contrast, in a homothallic (self-compatible) species, the genome of a haploid individual contains either a single *MAT* locus with both idiomorphs, or two *MAT* loci, one of each mating type, both these organisations allow self-fertilisation. The term ‘idiomorph’ was introduced instead of ‘allele’ because the different forms of the *MAT* locus contain very different genes, although they occupy the same physical location in the genome. The genomic location of the *MAT* locus is highly conserved within the subphylum Pezizomycotina (i.e. Ascomycota excluding yeasts), which includes all lichenized ascomycetes (Figure 18). Usually the *MAT* locus is flanked by a cyto-skeleton assembly gene called *SLA2* on one side and a DNA lyase gene (also known as *APN2*) on the other side (Butler 2007). The relative orientation of these flanking genes is conserved as well, as their transcriptional direction is always towards the *MAT* locus.

The *MAT* genes/loci are extremely variable in sequence, size and orientation even among closely related taxa, which creates a major issue for their study. Only putative DNA-binding sites known as α-box (*MAT1-1*) and HMG-domain (*MAT1-2*) contain fairly conserved motifs at amino acid level, which still allows for considerable variability at nucleotide level. These characteristic amino acid
motifs allow homology-based search and identification of the highly variable MAT genes in previously unstudied species.

Numerous studies examining the MAT locus and mating system have been published for non-lichenised filamentous ascomycetes, mainly because of their agro-economic significance as plant pathogens and food-moulds (e.g. species of Aspergillus, Botrytis, Cochliobolus, Coletotrichum, Fusarium, Gibberella, Magnaporthe, Penicillium and Rhynchosporium) or because they are traditional genetic model organisms such as Neurospora crassa or Saccharomyces cerevisiae. The wealth of information produced since 1990 on mating systems and MAT loci of non-lichenised ascomycetes has been compiled in textbook chapters by Debuchy & Turgeon (2006), Butler (2007), Lin & Heitman (2007), Debuchy et al. (2010), and with specific focus on lichens by Honegger & Scherrer (2008).

The mating system of a number of lichen mycobionts has been studied so far, including species of Anaptychia, Cladonia, Diploicia, Graphis, Lobaria, Melanelixia, Ochrolechia, Parmelia, Parmelina, Physcia, Physconia, Pseudevernia, Ramalina, Xanthoparmelia and Xanthoria (Murtagh et al. 2000, Honegger et al. 2004, Scherrer et al. 2005, Seymour et al. 2005, Honegger & Zippler 2007, Singh et al. 2012). The methodology in these studies varied. Earlier studies (Murtagh et al. 2000, Honegger et al. 2004, Seymour et al. 2005, Honegger & Zippler 2007) have applied DNA finger-printing techniques to single-spore cultures derived from the same ascus (i.e. the same meiotic event) to test whether the progeny descended from one or two parents. Later studies (Seymour et al. 2005, Scherrer et al. 2005, Singh et al. 2012) have targeted the mating-type locus directly by PCR with degenerate primers, and a subsequent mating-type screening of single-spore cultures or thalli with species-specific MAT primers to reveal the mating system. Currently, evidence for heterothallism prevails among the examined lichen species, as it was found in 26 species: Anaptychia ciliaris, A. runcinata, Cladonia floerkeana, C. galindezii, C. portentosa, Diploicia canescens, Lobaria pulmonaria, Melanelixia glabra, Parmelia sulcata, Parmelina tiliacea, P. carporrhizans, P. quercina, Physcia aipolia, P. stellaris, P. tenella, Physconia distorta, Pseudevernia furfuracea, Ramalina fastigiata, R. fraxinea, Xanthoparmelia conspersa, X. stenophylla, Xanthoria calcicola, X. capensis, X. ectaneoides, X. polycarpa and X. resendei. Evidence for homothallism was found in only four lichens: Graphis scripta, Ochrolechia parella, Xantoria elegans and X. parietina. The vast majority of lichen mating systems were analysed indirectly via DNA finger-printing of single-spore cultures, i.e. without direct characterisation of the mating-type genes of these species. Therefore, full or partial DNA sequence information of the MAT idiomorphs is available for only a few lichen species: Cladonia galindezii, C. grayi, Lobaria pulmonaria, Xanthoria elegans, X. flammeum, X. polycarpa, X. parietina (see Materials & Methods section).
Figure 18. Simplified general scheme of the mating type locus (MAT) including its flanking loci (SLA2 and APN2), showing the configurations required for self-incompatibility and self-compatibility. The position of the MAT locus between the same two flanking genes is highly conserved, as is the transcriptional direction (arrow heads) of flanking genes. The orientation of the MAT genes is variable (therefore no arrow heads), as is the size of the MAT locus (usually in the range of 7 ± 2 kb). Self-incompatible species have two alternative MAT loci in different haploid individuals, therefore they have to mate with the opposite mating type (heterothallism). In self-compatible species (homothallism) each individual possesses both MAT genes in its haploid genome, e.g. as depicted next to each other in the same MAT locus. Homothallic individuals can self-fertilize or mate with other individuals.
2. Material and Methods

2.1 Outline of the approach

The following approach was used to examine the MAT locus and mating system of *I. splachnirima*:

**Step 1 - design of degenerate primers:** for more or less conserved sections of the α-box (*MAT1-1*) and HMG-domain (*MAT1-2*), as well as the potentially flanking loci *SLA2* and *APN2*. Alignments of sequences of known MAT idiomorphs and flanking genes of other lichens and ascomycetes were used to design these primers.

**Step 2 - degenerate primer screening:** attempt to amplify short ± conserved sections of the α-box (*MAT1-1*) and HMG-domain (*MAT1-2*), as well as the potentially flanking loci *SLA2* and *APN2*.

**Step 3 - inverse PCR (part I):** extend initial sequences with the aim to obtain full coding sequences of both MAT idiomorphs, to allow (initial) verification of their identity by amino acid comparison with homologs of other lichens and ascomycetes.

**Step 4 - inverse PCR (part II):** proceed with inverse PCR, trying to bridge the gaps between MAT idiomorphs and the putative flanking loci. Due to the poor phylogenetic sequence conservation of MAT idiomorphs and genes, only the genomic context/placement of putative MAT sequences between or near the *SLA2* and *APN2* loci provides unequivocal proof of their identity/function as MAT genes.

**Step 5 – MAT screening:** use specific primers for both MAT idiomorphs, to assess whether one or both MAT idiomorphs are present in a haploid genome, i.e. whether the species is hetero- or homothallic. That requires the use of DNA extracts of extremely small samples (ca. 2 mm²) of thalline, i.e. haploid, tissues. Large tissue samples are more likely to contain still invisible, fertilised apothecium initials, which would result in false proof of homothallism of a heterothallic species.

Ideally single spore derived cultures should be used, but here extracts of very small tissue fragments were used, as they should be haploid. This, however, makes the screening theoretically susceptible to false-positive results indicative of homothallism, if the tissue comprised cells of a second individual in a heterothallis species.
2.2 Design of degenerate primers for conserved motifs of MAT idiomorphs and potentially flanking loci

Degenerate primers to the α-box (MAT1-1) and HMG-domain (MAT1-2), SLA2 and APN2 were manually designed based on alignments of the corresponding sequences (orthologs) of the lichens, Cladonia grayi, Cladonia galindezii, Lobaria pulmonaria, Xanthoria parietina, Xanthoria polycarpa, Xanthoria elegans and Xanthoria [Xanthodactylon] flammeum. Sequences of non-lichenized ascomycetes were also included in the alignments (see below and Table 15), in order to identify regions highly conserved among a wider range of ascomycota. Aligned sequences were either downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) or from the websites of the respective whole genome sequencing projects of Xanthoria parietina ‘Xanpa1’ (http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Xanpa1), Cladonia grayi ‘Clagr2’ (http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Clagr2), Neurospora crassa ‘OR74A’ (http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html) as well as Aspergillus fumigatus ‘Af293’ and Aspergillus nidulans ‘FGSC A4’ (http://aspergillusgenome.org). The Lobaria pulmonaria MAT sequences were kindly supplied by Ms. Garima Singh prior to publication in Singh et al. (2012). Details of these sequences are listed in Table 15. Alignments of the respective MAT regions including successfully used primer sites are illustrated in Figure 20 (as part of the results). To limit the degree of primer degeneracy, only ambiguous positions of lichens were taken into account for design of degenerate primers.
Table 15. Sequences which were aligned to design degenerate primers of the MAT and putative flanking loci. 8-digit codes refer to GenBank accessions.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Source of aligned sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAT1-1 α-box</td>
<td>Cladonia grayi ‘Clagr2’ WGS, Scaffold_52: 173041-174424 (genesh1_pg.52_#_48)*</td>
</tr>
<tr>
<td></td>
<td>Lobaria pulmonaria JX520967</td>
</tr>
<tr>
<td></td>
<td>Xanthoria polycarpa AJ884599</td>
</tr>
<tr>
<td>MAT1-2 HMG domain</td>
<td>Cladonia galindezii AJ634274</td>
</tr>
<tr>
<td></td>
<td>Lobaria pulmonaria JX520966</td>
</tr>
<tr>
<td></td>
<td>Xanthodactylon flameum AJ888227</td>
</tr>
<tr>
<td></td>
<td>Xanthoria elegans AJ888226</td>
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<tr>
<td></td>
<td>Xanthoria parietina AJ884600</td>
</tr>
<tr>
<td></td>
<td>Xanthoria polycarpa AJ884598</td>
</tr>
<tr>
<td>SLA2</td>
<td>Cladonia grayi ‘Clagr2’ WGS, Scaffold_52: 171497-172456 (fgenesh1_kg.52_#<em>39</em>#_isotig06959)*</td>
</tr>
<tr>
<td></td>
<td>Xanthoria parietina AJ884600</td>
</tr>
<tr>
<td></td>
<td>Xanthoria parietina ‘Xanpa1’ WGS Scaffold_3: 898418-899433 (fgenesh1_pm.3_#_169)*</td>
</tr>
<tr>
<td></td>
<td>Xanthoria polycarpa AJ884598</td>
</tr>
<tr>
<td></td>
<td>Aspergillus fumigatus ‘Af293’ WGS, Chr3: 151671-1520611 (Afu3g06140)*</td>
</tr>
<tr>
<td></td>
<td>Aspergillus nidulans ‘FGSC A4’ WGS, Chr6: 2903571-2907341 (AN2756)*</td>
</tr>
<tr>
<td>APN2 (DNA Lyase)</td>
<td>Cladonia grayi ‘Clagr2’ WGS, Scaffold_52: 178035-180430*</td>
</tr>
<tr>
<td></td>
<td>Xanthoria parietina ‘Xanpa1’ WGS, Scaffold_3: 904836-907188*</td>
</tr>
<tr>
<td></td>
<td>Aspergillus fumigatus ‘Af293’ WGS, Chr3: 1526518-1528706 (Afu3g06180)*</td>
</tr>
<tr>
<td></td>
<td>Neurospora crassa ‘OR74A’ WGS, Supercontig 1: 1853476-1855571 (NCU01961)*</td>
</tr>
</tbody>
</table>

*Portions of whole genome sequences (WGS) are cited as the range of nucleotide positions of the respective scaffold, supercontig or chromosome number, with the systematic gene or locus name in brackets. See main text for access to WGSs.

Table 16. Sequences and experimental use of the primers that were successfully used for amplification of partial sequences of I. splachnirima MAT, SLA2 and APN2 loci. IUPAC coding applies to ambiguous nucleotide positions of degenerate primers: B=C/T/G; D=A/T/G; H=A/T/C; K=T/G; M=C/A; N=A/T/C/G; R=A/G; S=C/G; V=A/C/G; W=T/A; Y=C/T.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence 5’ to 3’</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALFAfwd01</td>
<td>TCBTKSATGRYRTYCYGKTAAGT</td>
<td>α-box degenerate PCR</td>
</tr>
<tr>
<td>ALFArev01</td>
<td>CMAADRKVSYCAATTCGGTYTTG</td>
<td>α-box degenerate PCR</td>
</tr>
<tr>
<td>HMGfwd01</td>
<td>CGBCBBCCAAYGCVTTCAT</td>
<td>HMG domain degenerate PCR</td>
</tr>
<tr>
<td>HMGfwd03</td>
<td>CGBCBBCCAAYGCVTTCATHTCBYACG</td>
<td>HMG domain degenerate PCR</td>
</tr>
<tr>
<td>HMGrvd02</td>
<td>CGACGCTTCTTATCGWGGCCTT</td>
<td>HMG domain degenerate PCR</td>
</tr>
<tr>
<td>HMGrvd03</td>
<td>CGRGTCATDCGAAGCTTCCTT</td>
<td>HMG domain degenerate PCR</td>
</tr>
<tr>
<td>SLA2fwd01</td>
<td>GATGTWGTSAACACARCAAC</td>
<td>SLA2 degenerate PCR</td>
</tr>
<tr>
<td>SLA2fwd02</td>
<td>TACAARAGAAYAAYCGTGGAC</td>
<td>SLA2 degenerate PCR</td>
</tr>
<tr>
<td>SLA2rev01</td>
<td>TGGTCGTCCATCTCCGASACTTGA</td>
<td>SLA2 degenerate PCR</td>
</tr>
<tr>
<td>SLA2rev02</td>
<td>GTCCASGRTTRTTCTTTCGTGGA</td>
<td>SLA2 degenerate PCR</td>
</tr>
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<td>SLA2rev03</td>
<td>TCYCGACYTTGAAYCTRCGC</td>
<td>SLA2 degenerate PCR</td>
</tr>
<tr>
<td>SLA2rev04</td>
<td>CAAAGAAGAAACATACTGCTGGACG</td>
<td>SLA2, semi-degenerate PCR</td>
</tr>
<tr>
<td>Lyasefwd03</td>
<td>TCCARTRCRTSRHCADATRAAHGT</td>
<td>DNA Lyase degenerate PCR</td>
</tr>
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<td>Lyasefwd06</td>
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</tr>
<tr>
<td>Lyasefwd07</td>
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</tr>
<tr>
<td>LyaseRev04</td>
<td>AGGGNTCHGAYCAYCTGCY</td>
<td>DNA Lyase degenerate PCR</td>
</tr>
<tr>
<td>LyaseRev08</td>
<td>GGYATGTWYACSTGTYGGG</td>
<td>DNA Lyase degenerate PCR</td>
</tr>
<tr>
<td>IsALFA-f3</td>
<td>CCGCTATGTATTCCGTAACTC</td>
<td>Inverse PCR</td>
</tr>
<tr>
<td>Primer</td>
<td>Sequence</td>
<td>Method</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>IsALFA-r3</td>
<td>GCAGGAAATCAGTGTCGTTG</td>
<td>Inverse PCR</td>
</tr>
<tr>
<td>IsALFA-25R</td>
<td>TGTAGAGCCACAGACATACCAAAGA</td>
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</tr>
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<td>IsALFA-26F</td>
<td>GACCAAATCGCAACTACT</td>
<td>Inverse PCR</td>
</tr>
<tr>
<td>IsALFA-180R</td>
<td>ATCAGATTCTTCTGTCGGGGT</td>
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<td>IsALFA-238R</td>
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<td>IsALFA-488F</td>
<td>TCATGAACGTTGTCAAGCCA</td>
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<tr>
<td>IsALFA-712F</td>
<td>ATCCACCGTGTCTCTCATTTT</td>
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<td>IsALFA-714R</td>
<td>TGGTTGATCTTTTGGCATTTTCG</td>
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<td>IsALFA-725R</td>
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</tr>
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<td>IsALFA-749F</td>
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<td>Inverse PCR</td>
</tr>
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<td>IsALFA-785F</td>
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<td>Inverse PCR</td>
</tr>
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<td>IsALFA-806R</td>
<td>TTACTTCTCGGAGCCCTTAGAT</td>
<td>Direct PCR contig confirmation</td>
</tr>
<tr>
<td>IsHMG-r4</td>
<td>TGATGCAAGGTCTGTGCCTCT</td>
<td>Direct PCR contig confirmation</td>
</tr>
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<td>IsHMG-49R</td>
<td>GTAGTAGATTTTGTTGCCCAGAAA</td>
<td>Inverse PCR</td>
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<td>IsHMG-103R</td>
<td>CAGTTCCTTTGTTGTCAAGCCA</td>
<td>Inverse PCR</td>
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<td>IsHMG-119F</td>
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<td>IsHMG-185F</td>
<td>ATAACTCAACACAGCAGCTAGC</td>
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<td>IsHMG-197F</td>
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<td>IsHMG-265R</td>
<td>CTCTCCATTGAGCAGCAAGTAT</td>
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<td>IsHMG-338F</td>
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<td>IsHMG-1888R</td>
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<td>IsHMG-2397R</td>
<td>CAAGCTCAAGTACCAACCTACA</td>
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<td>IsLy-f4</td>
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<td>IsLy-r9</td>
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<td>IsLy-1305R</td>
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<td>IsSLA2-f2</td>
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<td>IsSLA2-f8</td>
<td>TCAGCCAGCGCAACAAAGGAC</td>
<td>Direct PCR</td>
</tr>
</tbody>
</table>
2.3 Amplification of short conserved portions of the MAT idiomorphs and potential flanking loci using degenerate primers

Degenerate PCR using the following primer combinations and genomic DNA of *I. splachnirima* as template, yielded the following products:

**MAT1-1 α-box.** Primer pair ALFAfwd01 / ALFArev01 yielded a product of 132 bp (excl. primers), corresponding to positions 2904-3033 of *X. polycarpa* sequence AJ884599.

**MAT1-2 HMG domain.** Primer pair HMGfwd01 / HMGrev03 yielded a product of 251 bp (excl. primers), corresponding to positions 3760-4015 of *X. polycarpa* sequence AJ884598. Primer pairs HMGfwd03 / HMGrev02 and HMGfwd03 / HMGrev03 also worked, but their products are nested within the product of HMGfwd01 / HMGrev03, and therefore irrelevant.

Later, an additional 124 bp upstream extension (to a total fragment length of 375 bp) was generated by chance with the primer pair IsSLA2-f8 / IsHMG-r4, when trying to close the gap between IsHMG and IsSLA2.

**SLA2.** Primer pair SLA2fwd01 / SLA2rev03 yielded a product of 677 bp (excl. primers), corresponding to positions 898575-899254 in Scaffold_3 of WGS *Xanthoria parietina* ‘Xanpa1’.

Primer pair SLA2fwd01 / SLA2rev02 yielded a product of 336 bp (excl. primers), corresponding to positions 898575-898913 in Scaffold_3 of WGS *Xanthoria parietina* ‘Xanpa1’.

Primer pair SLA2fwd02 / SLA2rev01 yielded a product of 325 bp (excl. primers), corresponding to positions 22-346 in *Xanthoria parietina* sequence AJ884600, and positions 898937-899261 in Scaffold_3 of WGS *Xanthoria parietina* ‘Xanpa1’, respectively.

**APN2** (DNA Lyase). Primer pair LyaseFwd03 / LyaseRev08 yielded a product of 1151 bp (excl. primers), corresponding to positions 904908-906070 in Scaffold_3 of WGS *Xanthoria parietina* ‘Xanpa1’.

Primer pair LyaseFwd03 / LyaseRev04 yielded a product of 970 bp (excl. primers), corresponding to positions 904908-905892 in Scaffold_3 of WGS *Xanthoria parietina* ‘Xanpa1’.

Primer pair LyaseFwd06 / LyaseRev08 yielded a product of 242 bp (excl. primers), corresponding to positions 905832-906070 in Scaffold_3 of WGS *Xanthoria parietina* ‘Xanpa1’.

Primer pair LyaseFwd07 / LyaseRev08 yielded a product of 161 bp (excl. primers), corresponding to positions 905913-906070 in Scaffold_3 of WGS *Xanthoria parietina* ‘Xanpa1’.
Each PCR consisted of 0.5 µl of each primer (at 10 pmol/µl), 1 µl of genomic DNA and 18 µl of commercial ready-to-use PCR master mix (“1.1X ReddyMix PCR Master Mix (1.5 mM MgCl₂”, Thermo Scientific, Cat#: AB-0575/LD and “1.1X ReddyMix PCR Master Mix (2.0 mM MgCl₂)”, Thermo Scientific Cat#: AB-0608/LD).

Cycler conditions were as follows: initial denaturation at 95°C for 5 minutes; then 42 cycles with denaturation at 95°C for 45 or 60 seconds, annealing at 45°C, 50°C or 52°C for 45 or 60 seconds, elongation at 72°C for 40 to 120 seconds; followed by a final elongation at 72°C for 10 minutes and hold at 14°C.

The genomic template DNA was extracted as described in Chapter 2 from numerous (ca. 10-15) I. splachnirima apothecia together, which originated from at least 10 different thalli, in order to ensure the presence of both MAT idiromorphs, in case of potential heterothallism of the species.

Purification of PCR products and sequencing have been performed as described in Chapter 2. The resulting partial sequences or contigs of the IsMAT1-1 α-box (132 bp), IsMAT1-2 HMG-domain (375 bp contig), IsSLA2 (836 bp contig) and IsAPN2 (1151 bp) were used for subsequent inverse PCR as described below; this is why these preliminary results are mentioned in the methods section already.

2.4 Inverse PCR extension of initial MAT fragments and potentially flanking loci

Based on the initial fragments of the I. splachnirima α-box, HMG-domain, SLA2 and APN2, specific primer pairs were designed to extend these sequences via inverse PCR (Ochman et al. 1988), a strategy previously used by e.g. Scherrer et al. (2005), Singh et al. (2012), and Pöggeler et al. (2011) for the same purpose.

Previous attempts to extend and connect the initial fragments via TAIL-PCR (as described by Singh et al. 2012) or by direct long-range PCR (as described by Scherrer et al. 2005) with the ‘FailSafe PCR System’ (Epicentre), were unsuccessful.

Primer pairs for inverse PCR (see Table 16) were designed for an annealing temperature of 52°C (initially) or 58°C (later) using the Primer 3 plug-in of Geneious.

Each inverse PCR consisted of 0.5 µl of each primer (at 10 pmol/µl), 1 µl of circularised genomic DNA (see below) and 18 µl a commercial ready-to-use PCR master mix (see above under 2.3). Inverse PCR cycler conditions were: initial denaturation at 95°C for 5 minutes; then 35 to 42 cycles with denaturation at 95°C for 1 minute, annealing at 52°C or 58°C for 1 minute, elongation at 72°C for 1 minute; followed by a final elongation at 72°C for 10 minute and hold at 14°C.
2.4.1 Preparation of template DNA for inverse PCR

Template DNA was extracted from several (ca. 10-15) apothecia and digested using the restriction enzymes EcoRI, HindIII, and BamHI (Roche), resultant fragments were then circularised by self-ligation using T4 Ligase (Roche). Each DNA digestion reaction consisted of 5 μl enzyme-specific Enzyme Buffer, 2400 ng genomic DNA (ca. 10 μl), 2 μl of the restriction enzyme (equivalent to 20 units) and de-ionised water to a final volume of 50 μl. Reactions were prepared on ice according to manufacturer’s instructions prior to incubation at 37°C for 90 minutes. The digestion reaction was stopped by adding 4 volumes of B2 buffer (from Invitrogen ‘Pure Link QuickPCR Purification Kit’), which was also the first purification step. Before self-ligation, the digested genomic DNA was purified with the ‘Pure Link QuickPCR Purification Kit’ (Invitrogen) according to the manufacturer’s instructions.

Ligation reactions contained 3 μl of 10× ligation buffer, 3 μl (=3 units) of T4 DNA Ligase (Roche), 30 ng (c = 1 ng/μl) or 150 ng (c = 5 ng/μl) digested genomic DNA and de-ionised water to a final volume of 30 μl. Reactions were incubated at 4°C for 24 hours followed by 30 minutes at room temperature. Ligase was heat inactivated by incubation at 65°C for 10 minutes. DNA was purified using buffers of the ‘Pure Link QuickPCR Purification Kit’ (Invitrogen) together with EconoSpin® All-In-One Mini Spin Columns (Epoch Life Sciences Inc.). All steps of the standard Invitrogen instructions manual were performed twice, in order to increase yield and purity.

2.4.2 IsMAT1-1 inverse PCR (alpha box gene)

The first inverse PCR with primer pair IsALFA-f3 / IsALFA-r3 and EcoRI-digested and self-ligated template yielded extensions in both directions, a 251 bp upstream extension and a 454 bp downstream extension.

An additional upstream extension of 1543 bp was generated with primer pair IsALFA-26F / IsALFA-25R and BamHI-digested and self-ligated template. The latter extension was supported by a shorter matching extension with 127 bp overlap of the primers IsALFA-488F / IsALFA-238R and HindIII-digested and self-ligated template.

A first additional downstream extension of 1138bp was generated with primer pair IsALFA-749F / IsALFA-714R and HindIII digested self-ligated template, which was supported by a 583 bp overlap with the products of primer pair IsALFA-488F / IsALFA-238R and IsALFA-749F / IsALFA-180R with BamHI digested and self-ligated template.

Another downstream extension of 691 bp was generated with primer pair IsALFA-785F / IsALFA-725R with HindIII-digested and self-ligated template.
Continuity of the putative coding sequence of the IsMAT1-1 contig was tested and confirmed using the primer pairs IsALFA-712F / IsALFA-714R (product size 1546 bp) and IsALFA-749F / IsALFA-806R (product size 1595 bp). For primer sequences see Table 16.

2.4.3 IsMAT1-2 inverse PCR (HMG domain gene)
The first inverse PCR with primer pair IsHMG-197F / IsHMG-49R and HindIII-digested and self-ligated template yielded extensions in both directions. The downstream extension was 679 bp long. The upstream extension added another 658 bp, and was supported by a 353 bp overlap with the product of the same primer pair and an EcoRI-digested and self-ligated template.

An additional upstream extension of 798 bp was generated with primer pair IsHMG-119F / IsHMG-103R and EcoRI-digested and self-ligated template.

An additional 1107 bp downstream extension was generated with primer pair IsHMG-338F / IsHMG-265R and HindIII-digested and self-ligated template.

Continuity of the putative coding sequence of the IsMAT1-2 contig was tested and confirmed using direct PCR with the primer pairs IsHMG-185F / IsHMG-1888R (product size 1704 bp) and IsHMG-197F / IsHMG-2397R (product size 1543 bp) and undigested genomic DNA as template.

2.4.4 IsSLA2 inverse PCR
All attempts to extend the IsSLA2 sequence further by inverse PCR were unsuccessful. However, it was possible to extend it a little further downstream using semi-degenerate PCR with the primer pair IsSLA2-f2 / SLA2rev04, which yielded a product of 478 bp (excluding primers), corresponding to positions 21-485 in Xanthoria parietina sequence AJ884600, and positions 898936-89940 in Scaffold_3 of WGS Xanthoria parietina ‘Xanpa1’, respectively. This extended the IsSLA2 contig to a total length of 836 bp, corresponding to positions 898575-899400 in Scaffold_3 of WGS Xanthoria parietina ‘Xanpa1’.

2.4.5 IsAPN2 (DNA-Lyase) inverse PCR
The first unidirectional inverse PCR with primer pair IsLy-f4 / IsLy-r9 and EcoRI-digested and self-ligated template yielded a 1568 bp downstream extension, which was supported by a 175 bp overlap with the product of primer pair Isly-f4 / IsLy-r4 and a HindIII-digested and self-ligated template. A string of about 12 successive guanines (located about 404 bp downstream of primer site IsLy-r9, or 241 bp downstream of the putative stop codon) interfered with sequencing attempts across it, as it introduced double-peaks. Direct PCR using the primer pair IsLy-40F / IsLy-232R (product size 1077 bp) with the above mentioned ca. 1.5 kb product as template, made it possible to sequence this region from either side.
Another 880 bp downstream extension was generated with primer pair IsLy-348F / IsLy-97R and HindIII-digested and self-ligated template. The latter was supported by a 124 bp overlap with the product of primer pair IsLy-40F / IsLy-232R and BamHI-digested and self-ligated template (note: this was an unspecific star-activity cut).

The continuity of the IsAPN2 contig was tested and confirmed using direct PCR with the primer pairs IsLy-348F / Isly-1305R (967 bp product) and IsLy-40F / IsLy-232R (1077 bp product) with undigested genomic DNA as template.

2.5 Mating system analysis of *I. splachnirima*

DNA extracts of 27 miniscule samples of haploid tissue (ca. 2 mm² or smaller, from the thallus margin) were screened for the presence of both putative *IsMAT* genes. Samples were collected from several New Zealand localities, but 18 of the 27 samples represent the same locality near Dunedin (Swampy Summit and Silver Peaks region, 45°48’S 170°29’E), which should reveal the mating type ratio of a local population in case of heterothallism. Apothecial DNA extracts were used as positive control, since these should always contain both *MAT* idiomorphs.

The following primer pairs were used for mating type screening: IsALFA-712F / IsALFA-714R (product size 1547 bp incl. primers) and IsHMG-185F / IsHMG-1888R (product size 1704 bp incl. primers). The *MAT1*-2 primer binding sites (IsHMG primers) are located outside the putative coding region, so the PCR product contains the whole coding region of the putative *MAT1*-2-1 gene. The *MAT1*-1 primer binding sites (IsALFA primers) are located upstream of the putative *MAT1*-1-1 coding region and within the coding region, so they amplify the first ca. 750 bp of the coding sequence including the whole α-box domain with conserved intron.

Composition of PCRs was as described above in section 2.3, except with different primers. PCRs containing either primer pair were run together using the following step-down protocol: Initial denaturation at 95°C for 3 minutes. Then 10 step-down cycles with denaturation at 94°C for 30 seconds, annealing for 1 minute at temperatures dropping from 65°C to 55°C in increments of 1°C per cycle, elongation at 72°C for 3 minutes. This was followed by 35 cycles with denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute and elongation at 72°C for 3 minutes. After final elongation at 72°C for 10 minutes, the reactions were kept at 14°C.
3. Results

3.1 Putative MAT genes of *I. splachnirima*

Figure 19 shows an overview of the final contigs of the putative MAT idiomorphs as well as the potential flanking genes SLA2 and APN2 (DNA lyase) of *I. splachnirima*.

![Diagram showing the putative MAT genes and flanking genes SLA2 and APN2 of I. splachnirima](image)

Figure 19. Overview of the *I. splachnirima* mating type region contigs produced in this study. Putative coding sequences (exons) are depicted as wide, blue arrows pointing from 5’ to 3’; narrow blue sections within genes indicate introns. Black narrow lines are presumably non-coding sections of DNA. The coding sequences of IsMAT1-1 and IsMAT1-2 are complete, the start sections of IsSLA2 and IsAPN2 were not amplified and are therefore truncated. The grey terminal section attached to the IsSLA2 contig represents the last 33 bp of the coding sequence (10 amino acids plus stop codon) of the gene in *Cladonia grayi*, *Xanthoria parietina* and *Xanthoria polycarpa*. These last 33 bp were not amplified for *I. splachnirima*, but the corresponding section of the other lichens was included to illustrate the relative position if the IsSLA2 contig. Full contig lengths and GenBank assessments (in brackets) are: 4792 bp for IsMAT1-1 (KP984802), 3617 bp for IsMAT1-2 (KP984801), 836 bp for IsSLA2 (KP984804), 3486 bp for IsAPN2 (KP984803).

The putative *I. splachnirima* MAT1-1 contig is 4792 bp long, including a 1703 bp (548 aa) putative coding sequence (CDS), which has a 56 bp intron in a conserved position within the codon of cysteine C\(^{103}\). This cysteine residue itself is not conserved, but the flanking FR and YY residues are highly conserved among lichens (Figure 20) and also *Neurospora crassa* (Glass *et al.* 1990). The putative CDS is flanked by 1475 bp upstream of the putative start methionine and a further 1614 bp downstream of the putative stop codon. These are of unknown function.

The putative *I. splachnirima* MAT1-2 contig is 3617 bp long, including a 1280 bp (390 aa) putative CDS, which has a 61 bp intron between residues number 62 (leucine) and 63 (isoleucine), and a 46 bp intron within residue number 150 (serine). The putative CDS is flanked by 1139 bp upstream of the putative start methionine and another 1198 bp downstream of the stop codon. These are of unknown function.

The *I. splachnirima* SLA2 contig is 836 bp long, which represents a section close to the 3’ end of the CDS. The last 33 bp of the CDS (corresponding to 10 amino acids plus stop codon) are missing.
in the IsSLA2 contig, compared to *X. parietina, X. polycarpa, D. baeomyces* and *C. grayi*. The *I. splachnirima* SLA2 contig also contains a 71 bp intron, which is absent in *C. grayi* but present in the same location in the other three lichens listed above.

The *I. splachnirima* APN2 (DNA Lyase) contig is 3486 bp long, of which 1193 bp represent the downstream portion of the CDS with a 49 bp intron. This is at the same region of the gene in whole genome sequences of *X. parietina, C. grayi* and the non-lichenized *Aspergillus fumigatus* and *Coccoides immitis*. The remaining 2293 bp are downstream of the putative stop codon, and are of unknown function.

The apparently non-coding flanking regions of all contigs do not overlap with each other, nor do they contain homologous parts of MAT regions of other lichens. No identifiable open reading frames were detected as either.

The boundaries of putative introns in all contigs were manually identified by comparison with the splicing signals described in Schwartz et al. (2007) and Schneider-Poetsch et al. (2010).

Full sequences of all contigs were deposited at GenBank, accession numbers are: KP984801 (MAT1-2), KP984802 (MAT1-1), KP984803 (APN2), KP984804 (SLA2).

**Caption of Figure 20 (next page).** Comparison of putative *I. splachnirima* MAT1-1 α-box (above short caption) and MAT1-2 HMG domain (below short caption) with other species: *Xanthoria polycarpa, Lobaria pulmonaria, Cladonia grayi, Dibaeis baeomyces, Aspergillus fumigatus* (non-lichenised), and *Cladonia galindezii*. The MAT1-1 α-box alignment corresponds to positions 3079 to 2787 in Genbank sequence AJ884599 of *X. polycarpa*, and the MAT1-2 HMG domain alignment corresponds to position 3722 to 4045 in Genbank sequence AJ884598 of *X. polycarpa*. Amino acid sequences are depicted underneath corresponding DNA sequences, their orientation (5' to 3' and N to C terminal) is from left to right. Amino acids names are given in one-letter IUPAC nomenclature and were colour-coded according to polarity. Stretches of DNA sequence without translation are conserved introns; between 29 and 37 badly aligned nucleotides of the MAT1-2 intron were replaced by (...) to save space. Primer sites of the successfully used degenerate primers are indicated by green arrows below the alignments. Note that the sequences of the degenerate primers are based only on subsets of the illustrated alignment, which are *C. grayi, L. pulmonaria* and *X. polycarpa* for MAT1-1 α-box primers and *C. galindezii, L. pulmonaria, X. polycarpa, X. parietina* as well as *X. elegans* and *X. flammea* for MAT1-2 HMG domain primers. HMG sequences of *X. elegans* and *X. flammea* were omitted from this illustration, because they are very similar to the other *Xanthoria* and contribute no additional ambiguous positions to the degenerate primer sites. The alignment images were generated with Geneious R7 and manually edited in GIMP.
Figure 20. Comparison of putative I. splachnirima MAT1-1 α-box (above) and MAT1-2 HMG domain (below) with other species. See previous page for detailed caption.

[Note: This is a subsequently corrected version of the flawed original figure.]
3.2 The mating system of *I. splachnirima*

The mating type screening showed an alternating presence of either MAT1-1 or MAT1-2 in each sample (Figure 21). This pattern indicates that *I. splachnirima* is heterothallic, i.e. not capable of self-fertilisation. Of the total 27 examined samples, 13 contained only MAT1-1 and 14 contained only MAT1-2. The mating type ratio within samples of the Dunedin population is 8 to 10 (lanes 12 to 29 of Fig. 7).

![Figure 21. Results of the mating type screening. Top row: MAT1-1 screening (product size 1547 bp). Bottom row: MAT1-2 screening (product size 1704 bp). The sample order is identical in both rows, i.e. each column shows both screening results of the same DNA extract on top of each other. Lanes 12-29 show samples from the same local population near Dunedin, New Zealand. The size markers (M, lanes 1 and 30) show reference bands of 1650 bp and 2000 bp. Lane C+ is a positive control with apothecial DNA extract as template, which means it should show both products.](image)

4. Discussion

The mating type locus of *I. splachnirima* was sequenced using a combination of degenerate PCR and inverse PCR. The MAT-like sequences amplified from *I. splachnirima* in this study bear the expected degree of resemblance with MAT genes from other lichens and non-lichenized Pezizomycotina. Conservation at amino acid level in the α-box and HMG domain as well as the presence of conserved introns allows confident homology-based identification of the generated sequences as MAT genes, and the putative protein size is in the expected range. However, it was not possible to show that the *I. splachnirima* MAT genes are also flanked by the SLA2 and APN2 genes, as is the case in almost all previously studied Pezizomycotina (Butler 2007). At this stage, it cannot be ruled out that the amplified MAT-like genes might in fact represent different loci, due to the considerable inter-specific variability of MAT genes outside the conserved domains. Therefore, the final proof for the identity of these genes is still missing, and they should correctly be referred to as putative MAT genes of *I. splachnirima*. However, the result of the mating type screening does not only show that *I. splachnirima* is probably heterothallic, the observed alternating presence/absence pattern also provides strong support for the assumption that MAT genes have been amplified, as this pattern is only known for this locus (in case of heterothallism). Continued pursuit of the inverse PCR approach to connect the putative IsMAT genes with their flanking genes was considered not worthwhile, due to the uncertainty of success of this laborious and expensive procedure. When comparing the associated costs and benefits, the increasingly affordable whole genome sequencing services might be a more appropriate approach to fill the remaining gaps and validate the results in the near future. This would have the
additional benefit of obtaining a whole genome sequence, which could for example be used for microsatellite development.

Sequence comparison with further lichen MAT-loci could also provide further support for the identity of the putative IsMAT genes. In theory this is possible, since McDonald et al. (2013) have produced whole genome sequences of several additional lichens, including Dibaeis baemomycil (Icmadophilaceae), but regrettably those WGSs have apparently not yet been made publicly accessible, although their paper states that the ‘Data is housed in the Sequence Read Archive (SRA) accessible through the National Center for Biotechnology Information (NCBI) website’. Upon request, Dr Tami McDonald kindly supplied a sequence of the MAT1-1 idiomorph including the flanking SLA2 and APN2 genes of Dibaeis baemomycil (its WGS did not include the MAT1-2 idiomorph, pers. comm. T. McDonald, 30.04.2013). In this species, the orientation of the flanking genes is as in all other studied Pezizomycotina, and their stop codons are 5727 bp apart, with the MAT locus in between. This shows that at least this member of Icmadophilaceae ‘complies’ with the standard arrangement of the MAT region, and that a similar situation can be expected in I. splachnirima (and hopefully will soon be demonstrated). An ongoing PhD study by Ioana Onut Brännström (Uppsala, Sweden) is currently investigating the mating-type loci and mating systems of other members of Icmadophilaceae, which will allow comparison of her results with the I. splachnirima sequences in the near future.

Mating-type screening showed a consistently alternating presence of either MAT1-1 or MAT1-2 (Figure 21), which indicates heterothallism, i.e. that I. splachnirima is most likely not capable of self-fertilisation and relies on out-crossing. Mating of I. splachnirima is most likely mediated by pycnopores shed from pycnidia, which are considered to act as ‘male’ gametes (‘spermatia’) during fertilisation (Honegger & Scherrer 2008) and were first reported to exist in this species by Ludwig (2011) (see Figures 1-3). The use of single-spore cultures for the mating-type screening as in other studies (Scherrer et al. 2005, Seymour et al. 2005) is deemed not necessary, because the observed either/or-pattern indicative of heterothallism is already fully conclusive. In contrast, if the screening of thallus fragments would have indicated homothallism (both amplicons in each sample), the additional examination of single-spore cultures would have been advisable, in order to rule out the possibility of false positive test results. These could be caused by tissue ‘contaminated’ with cells of more than one heterothallic genetic individual, e.g. very young and still invisible apothecium initials. However, for the present study only tissue of the outermost thallus margin has been sampled, because apothecia (and visible apothecial initials) have only been observed to grow in the thallus centre and never right at the margin. However, it was also tried to obtain single-spore cultures of I. splachnirima. Fresh apothecia were sent to Prof Elfie Stocker-Wörgötter (Salzburg, Austria), a leading expert in the field of lichen culturing, who tried to establish single spore cultures, but without success.
A very balanced mating-type ratio was observed in the random sample of 27 specimens originating from various New Zealand localities as well as the 18 samples from the same local population near Dunedin; this is in accord with the very frequent occurrence of apothecia and apothecial initials. This is in strong contrast to the likewise heterothallic Lobaria pulmonaria, which can reproduce sexually and vegetatively as well, but was found by Singh et al. (2012) to have a highly skewed mating-type ratio in many populations. Therefore, these authors conclude that the generally rare occurrence of apothecia in L. pulmonaria could be attributed to a lack of suitable mating partners. The wide-spread Xanthoria polycarpa is also a heterothallic lichen-forming fungus (Scherrer et al. 2005), but unlike L. pulmonaria it is virtually always fertile, similar to I. splachnirima. The frequently fruiting I. splachnirima and X. polycarpa grow both in more open macro-habitats compared to the rarely fertile L. pulmonaria (based on light indicator values in Wirth 2010, and pers. observations for I. splachnirima), which is a characteristic species of denser old-growth forests, that provide shelter from excess light and heat (Gauslaa & Solhaug 1999, Jüriado & Liira 2009, Gauslaa 2013). Perhaps a more open habitat facilitates the transport of air-borne pycnospores (‘spermatia’) for fertilisation. Thus, a low frequency of apothecium formation in L. pulmonaria could be partly explained by low fertilisation success resulting from slow wind velocities in dense forest stands, even if sexually compatible thalli are not very far apart. This idea is supported by findings of Mikryukov et al. (2010), who report that the proportion of fertile L. pulmonaria sub-populations is 3 times greater in deciduous forests (open canopy during winter time) than in conifer forests (permanently closed canopy) of the Russian Ural region.

The finding that I. splachnirima does not fulfil the genetic requirements for self-fertilisation has major implications for its conservation. It means, that severe genetic bottle-ncks may cause isolated and small populations to loose the ability for sexual reproduction and therefore adaptation to a changing environment. In such a case, an affected population could probably survive temporarily via vegetative reproduction, but conservation authorities would be required to restore the mating-type ratio by transplantation of sexually compatible thalli, in order to assure long-term viability of the population.

One of the main achievements of this work is the design of I. splachnirima specific MAT1-1 and MAT1-2 primers and the development of a rapid PCR protocol for mating type identification, which allows to assess the mating type ratio in natural populations of I. splachnirima. Given the nationally vulnerable status of this species in New Zealand, the availability of such a protocol constitutes a significant conservational tool to infer the sexual reproductive potential especially of small and isolated populations.
5. Summary

The mating type locus (MAT) of *Knightiella splachnirima* (Icmadophilaceae) has been characterised using PCR with degenerate primers and inverse PCR. Screening for the presence of both MAT idiormorphs in DNA extracts of miniscule samples of haploid thalline tissue shows that only one of the two idiormorphs is present in each sample. This indicates that the species is most likely not self-fertile and requires the presence of compatible mating partners for sexual reproduction (heterothallic mating system). This new knowledge along with the presented protocol for mating type determination is an essential tool for the species’ conservation management.
CHAPTER 5
Micro-environmental effects on the reproductive mode
of Icmadophila splachnirima

1. Introduction
This chapter examines the reproductive ecology of I. splachnirima in situ, using field-experimental approaches.

1.1 Importance of micro-habitat conditions for lichens and their reproduction
As with higher plants and bryophytes, most lichen species are confined to certain habitats and substrates only, i.e. they are adapted to an ecological niche, characterized by special environmental conditions. The occurrence of many species is so reliably correlated with certain ranges of environmental factors that Ellenberg (1974) devised a system of so called ecological indicator values for vascular plants, which was subsequently expanded to cryptogams as well (Ellenberg et al. 1991), including Wirth’s account on Central European lichens, which has recently been expanded to over 500 species (Wirth 2010). Although this concept is based on the subjective assessment of environmental conditions related to a given lichen species, it has found statistical support in a range of studies correlating lichen species occurrence with environmental conditions. For example, in Kantvilas & Minchin (1989), Giordani (2006), Will-Wolf et al. (2006), Lichvar et al. (2009) and Temina & Kidron (2011) climate variables and species occurrence are consistently found to be correlated, especially at small spatial scales. Campbell & Coxon (2001) report microclimatic correlations with lichen growth rates on Canadian conifers, and Schoenwetter (2010) has also found strong correlations of micro-environmental factors and species composition of epiphytic lichen communities in New Zealand indigenous Nothofagus forest.

It is important to emphasise that, while often irrelevant to vascular plants, micro-environmental conditions are quite possibly more important to lichens, and can vary significantly on small spatial scales, even in the centimetre range (Jahns & Ott 1983, Canters et al. 1991). Not unexpectedly for a poikilohydric, photoautotroph ‘dual organism’, the most important environmental factors for lichens are thought to be light and moisture, but often the pH of the substrate is also important. However, a study by Hasse & Daniëls (2006) on terricolous lichens (mostly Cladonia spp.) subjected to experimental substrate changes indicates that substrate properties are relatively unimportant, at least for these terricolous species. Air temperature appears to be less important according to the study of Schöller & Jahns (1992) on the community structure of terricolous lichens, while light and especially moisture are the main determinants of small scale distribution of species. Various studies have examined the micro-climatic conditions associated with certain lichen species or communities, usually with a focus on the light and moisture regime (e.g. those mentioned above and
those detailed below). The relative importance of light versus moisture cannot be generalised. For example Ott (1989) found that light exposure is the most important factor for epiphytic lichen colonisation of deciduous phorophytes, i.e. some species prefer permanently exposed sites, while others grow preferentially in the canopy, where they are shaded during summer. In contrast, for generally exposed rock-dwelling lichen communities the water relations are the driving force shaping community structure (Ott et al. 1997). Another good example for differential importance of light versus moisture is given by Gauslaa et al. (2009). In their transplantation experiment dry matter gain was positively correlated with increasing light levels in Usnea longissima, while Lobaria pulmonaria grew best at medium light levels with depressed growth under high light conditions; growth of both species increased with increasing moisture.

The relative metabolic cost of sexual versus vegetative reproduction is central to this chapter. For several reasons it seems logical to assume that vegetative reproduction by soredia should be advantageous under suboptimal or stressful growth conditions. Formation of apothecia involves large investment in fixed carbon, a metabolic cost that is only affordable under optimum growth conditions. Firstly, an apothecium of I. splachnirima is a total sink of metabolic energy, since it lacks a thalline margin containing photobionts (biatorine margin). In contrast many other lichens do contain algal cells in the apothecial margin (lecanorine), which contribute to the fruiting body’s nutritional requirements or can even make it self-sufficient (Del-Prado et al. 2001). Secondly, fruiting bodies are formed at the expense of assimilative thalline tissue. In the case of I. splachnirima the apothecia grow very dense, relatively large and often they even fuse with each other, causing losses of assimilative thallus surface in fertile specimens of thirty percent or more. Thirdly, ascospores need to be provided with high-energy storage substances. In contrast, metabolic costs of vegetative reproduction are comparatively low. Soredia are at least to some extent self-sustaining, because they contain their own photobiont cells, and they do not reduce the assimilative thallus surface. Bearing these facts in mind, it is a logical and advantageous consequence to inhibit apothecial development during unfavourable periods, and to facilitate production of soredia instead. This would allow resource allocation towards physiological stress adaptation and repair mechanisms, while still maintaining reproductive function.

There is some evidence in the literature that suboptimal or stressful conditions have detrimental effects on the fertility and reproductive capacity of lichens. With regard to ascomycetes in general, Dyer et al. (1992) conclude that ‘environmental (…) factors must be correct before Ascomycetes may sexually reproduce’ and ‘suitable environmental conditions’ are among the factors ‘to trigger sexual morphogenesis in (…) Ascomycetes’.

Jahns et al. (1978) found a positive correlation between humidity and fertility of Cladonia furcata, and Ott (1987) also found indications for a correlation of apothecium formation and the moisture regime for Physcia tenella. Jahns et al. (2004) have observed varying numbers of apothecial discs in Cladonia rangiferina depending on micro-climatic condition, with abundantly fertile thalli in
well hydrated and humid sites, and reduced fecundity in drier sites. A very interesting correlation of environmental conditions and reproductive mode at community level was found by Monte (1993) for saxicolous lichens from Italy; this is in line with the considerations above. The author summarised: ‘as for reproductive strategies, it was found that reproduction by spores has a very high relative frequency (>80%) in intermediate microclimatic situations, whereas it has lower percentages under extreme conditions. Vegetative reproduction through soredia is maximum under extreme conditions: minimum luminosity and maximum humidity on one hand, maximum luminosity and minimum humidity on the other.’

Two tandem-studies of Gauslaa et al. (2006) and Gauslaa (2006) have highlighted the effect of micro-climatic conditions on vegetative growth and occurrence of Lobaria pulmonaria, and how this is related to the species’ capacity for vegetative reproduction (see discussion for more information). Gauslaa et al. (2006, 2009) have also used fish-eye photography to quantify light incidence, an approach adopted in the present study.

1.2 Light and moisture regime and their potentially negative physiological effects

When the microclimate becomes a limiting factor for the growth or reproduction of a lichen, this is likely a consequence of detrimental cell physiological changes, which are commonly referred to as stress. At cellular level, stress usually means damage of cellular structures caused by highly reactive oxygen species (ROS), and the processes involved are summarised as oxidative damage or oxidative stress or disruptive stress (Beckett et al. 2008). This is in contrast to limitation stress, which is e.g. the deprivation of essential nutrients (malnutrition, starvation), which is not the subject of this study and will not be discussed any further.

It is important to emphasize that it is not the anthropocentric definition of stress that applies here, but the definition based on the preferred growth conditions of the examined species! This is why the author considers it inappropriate to use the anthropocentric stress-concept for non-human organisms, as done by Fahselt et al. (1989), who report that the vast majority of lichen species growing in supposedly ‘stressful’ arctic environments reproduce sexually. It means that it is not important how hostile the conditions are for human, but how much they deviate from the optimal conditions for studied the species (Körner 2003), in the case of the present project I. splachnirima. The ecological definition of stress as in Cranston et al. (2012) shall be applied here as well, which is a reduction in the fitness of an individual to sub-optimal levels as a result of an external force.

Excess light particularly in dry or cold conditions, can lead to photoinhibition where the photosynthetic apparatus is over-saturated and cannot cope with the excess electrons liberated by photochemical water splitting. The free electrons cannot be passed through the photosynthetic electron transport chain quickly enough, which gives them opportunity to react with oxygen (which is also abundantly produced by photochemical water-splitting), thereby creating ROS. The ROS can (and
do) react with virtually all parts of the cell and photosynthetic apparatus, causing serious damage to it, which reduces the rate of photosynthetic electron transport further still. This negative feedback loop results in photoinhibition (Nishiyama *et al.* 2006).

Various methods exist to measure either the damage caused by stress or an organism’s physiological adaptations to it. High light stress in lichens has often been quantified by using the amounts and redox-state of glutathione and pigments of the xanthophyll cycle (e.g. Barták *et al.* 2004, Vrablikova *et al.* 2005, Štepigová *et al.* 2007). These are antioxidant compounds, which can reversibly react with ROS, thereby buffering the oxidative damage (Beckett *et al.* 2008). Another adaptation measured in lichen studies (e.g. Weissman *et al.* 2006) is the production of ROS scavenging enzymes like superoxide dismutase, catalase and peroxidases. A frequently used direct measure of cellular damage (rather than an adaptation to it) is lipid hydro-peroxidation (LHP). This is a very reliable and easily quantifiable measure of oxidative damage of cellular lipid membranes (Girotti 1998). Since lipid membranes are present in all organisms, this is a stress measure for both mycobiont and photobiont, and has therefore been used in the present study.

High light conditions can also be associated with desiccation, so light or drought stress are therefore often difficult to distinguish. Gauslaa & Solhaug (2004) have demonstrated that photoinhibition of lichens depends on their hydration status. The moisture regime is probably the most important stress factor of the lichen micro-habitat, due to their poikilohydric nature. Although desiccation tolerance is the characteristic feature of the poikilohydric life-style, “the extent to which lichens can tolerate drought stress is (...) also partly related to the moisture conditions to which they are adapted in their natural habitat” (Palmqvist *et al.* 2008: 194). The same authors (l.c.: 209) conclude that “lichen growth (...) may be primarily limited by water availability”, because “both photosynthesis and respiration are so strongly constrained by the water status of the thallus”.

Similar to light stress, desiccation stress is also manifest at the cellular level by a massive production of ROS (oxidative burst), especially during rehydration (Minibayeva & Beckett 2001, Kranner *et al.* 2008). Not only are the frequency, and duration of desiccation important, but also the rate of desiccation determines its severity, as slower drying allows the lichen to adapt more easily. Furthermore, sudden rehydration is known to result in leakage of important osmolytes (potassium, polyols, sugars), apparently due to damaged membrane proteins (Kranner 2008, and references in there), and more rehydration related nutrient leaching occurs in lichens from wetter habitats. Green (2009) mentions with regard to recovery of photosynthesis after desiccation: “Little data exist but it appears that lichens from habitats where they remain active for long periods once reactivated, recover more slowly than those from habitats like bare rock where activation is brief. This situation is also known from bryophytes.”

Optimal light and moisture conditions have not been experimentally determined for *I. splachnirima*, but the distribution and habitat preferences of the species indicate an adaptation to
permanently moist conditions. Also the absence of a lower cortex seems to support this view, because
the cortices of lichens essentially act as evaporation barrier (cf. Rundel 1982, Souza-Egipsy et al. 2000,
Barbosa 2010); therefore the lower cortex might be redundant in constantly moist wetland habitats
(cf. Poelt 1987), while the upper cortex still serves to protect the algal layer (Büdel & Scheidegger
2008: 50). Moreover, during supersaturation, the lack of a lower cortex facilitates gas exchange and
the loss of excess liquid water, which would otherwise cause a depression of carbon gain by restricted
CO₂ diffusion through water-filled intercellular spaces (Green et al. 1981, Jahns 1984, Valladares et al.
1998, Palmqvist et al. 2008, Büdel 2010). Also the lack of rhizinae and the loose archnoid medullary
structure facilitates fast drying (Richardson 1993: 284), i.e. the state of metabolic inactivity will be
quickly reached as soon as thallus hydration becomes insufficient. Many green algal lichens can meet
their water requirements exclusively by uptake of water vapour if the humidity is sufficiently high
leucophlebacia evidently shows this ability (Lange et al. 1986), and since this genus is often associated
with Coccomyxa, a photobiont related to that of I. splachnirima (see chapter 3), the latter is likely to
do so too. Considering this, the absence of a lower cortex together with the exposed archnoid medulla in I. splachnirima might not only facilitate evaporation of excess water, but in turn also
accelerate the uptake of water vapour (cf. Rundel 1982 and Richardson 1993), an abundantly available
water source for a terricolous wetland lichen even in the absence of precipitation.

Ludwig (2011) reported the occurrence of sterile and sorediate thalli of I. splachnirima for the
first time, and based on initial field observations the author interpreted the occasional production of
soredia as an adaptive response to seemingly unfavourable growing conditions. He hypothesised that
apothecia were produced only in favourable conditions, while in more stressful (or at least
suboptimal) micro-habitats the apothecial development is arrested at an early developmental stage
and sexual reproduction is replaced by vegetative reproduction via marginal soralia. It was
furthermore suggested that individual thalli might switch back and forth between both reproductive
modes over extended periods of time, when micro-climatic conditions are changing, for instance due
to plant succession or erosion. Galloway & Elix (1980) have already pointed out that I. splachnirima
“appears, in some areas at least, to be a successional plant”. Martínez-Sánchez et al. (1994) show that
sheltering vascular plants (tussock grasses) are important in providing suitable micro-habitats for
lichen communities. This situation can also be observed for I. splachnirima, which often grows in the
shade of tussock plants, where the numerous grass stems also supply additional moisture by combing
out misty air or clouds in the high country (Mark & Dickinson 2008, and author’s personal observation).
I. splachnirima often grows on decaying dead tussock mounds, and it can be assumed that shading by
aspect and the water retention of the tussock change during its decomposition.

Soon after the author discovered the sorediate form of I. splachnirima, he set up an initial field
experiment on Swampy Summit (Dunedin) in February 2009. This pilot study was a reciprocal
transplant experiment, which aimed to induce the alternative way of reproduction by swapping thalli of opposite reproductive mode. Changes in the reciprocally transplanted thalli of the pilot study were reassessed in December 2010 (22 months after the transplants), and the results were outlined in Ludwig (2012, appended). Results of the Swampy Summit pilot experiment were supportive of the hypothesis that the reproductive mode of *I. splachnirima* is affected by micro-climatic conditions. This gave the impetus for further testing of this hypothesis in the current PhD study started in March 2011.

1.3 Hypothesis, Objectives and Experimental Approaches

The working hypothesis of this chapter is that the reproductive plasticity (sexual versus vegetative) observed in *I. splachnirima* is a response to micro-environmental conditions, presumably stressful light or moisture levels. In order to test this hypothesis, four experimental approaches were taken.

The objective of the first approach was to test whether reproductive mode was a reversible, plastic response to the micro-habitat. Reciprocal transplants of thalli of each reproductive mode were conducted, in an attempt to induce the alternate mode of reproduction. A previous pilot study on Swampy Summit was successful and its results are largely in accord with the hypothesis. However, this pilot study used only 10 replicates and was not adequately controlled. This chapter presents a comprehensive transplant experiment based on the previous pilot study (Ludwig 2012).

The second experiment aimed at testing for a correlation of light incidence and reproductive mode. Fish-eye photography was used to quantify the light environment of thalli of each reproductive mode. This technique allows one to model the annual light incidence based on a single hemispherical fish-eye photo. This is a rather new methodology, but it was systematically tested by Jarčuška et al. 2010 and Promis et al. (2011), and also used in some lichenological studies (Gauslaa et al. 2006, 2009, Schoenwetter 2010, Gauslaa 2013).

The aim of the third experimental approach was to test for a correlation of the moisture regime and reproductive mode. Different methods were tested, to quantify the moisture regime of thalli of each reproductive mode.

The fourth experimental approach was designed to test for physiological indications for stress and different levels of stress-related cellular damage in sexual versus vegetative thalli. Reciprocal transplants similar to those described under the first experimental approach were conducted, followed by measurements of the physiological stress marker lipid hydro-peroxidation (LPH). LPH is a reliable and easily quantifiable measure of cellular damage caused by environmental stress like high light conditions or desiccation. It measures oxidative damage of cell membranes in all organisms, and therefore picks up cellular stress in both mycobiont and photobiont.
In summary, the objectives of this chapter were to answer the following questions:

1. Is reproductive mode a reversible, plastic response to environmental conditions?
2. Is light incidence correlated with reproductive mode?
3. Is the moisture regime correlated with reproductive mode?
4. Are sexually reproducing thalli showing more indicators of cellular stress in particular environmental conditions compared with vegetatively reproducing thalli?
2. Material & Methods

2.1 Study sites

Figure 22 and Figure 23 show the position of the two study sites Swampy Summit (just North of Dunedin) and the Blue Mountains (just East of Tapanui) in Otago, New Zealand.

Figure 22. Map of East Otago showing the two study areas Swampy Summit just North of Dunedin (D) and Blue Mountains just East of Tapanui village (T), G is the town of Gore. The distance between Dunedin and Tapanui is ca. 90 km.

Figure 23. Position of the two study areas (black frames) relative to the nearest settlements. Grid squares are 10 km (1: 250,000 Topo-map series, Land Information New Zealand [LINZ])
Figure 24. Google Earth overview of the Blue Mountains study area. Red dots represent known sites with *I. splachnirima*. Pink markers are sexual transplant sites and green markers are vegetative transplant sites. Paired transplant sites (replicates) are numbered as elsewhere below; f refers to fertile (sexual) sites and s indicates sterile (vegetative) sites. The scale bar in the bottom right corner is ca. 700m; some summits are indicated by blue cones with altitude in meter as reference for comparison with the 1:50000 topo-map.
Figure 25. Google Earth overview of the Swampy Summit study area near Dunedin. Red dots represent known sites of *I. splachnirima*, camera symbols with show spots where fish-eye photographs have been taken (FE...). Some FE sites are obscured because of the distant viewpoint. These are FE06 (just beside FE05), FE11 (next to FE12), FE04 and FE10 are between FE03, FE09 and FE22. The scale bar in the right bottom corner is ca. 200 m.
2.2 Micro-habitat

2.2.1 Approach, Experimental Setup and Expected Outcome

Twenty reciprocal transplants of fertile & esorediate (sexual) versus sterile & sorediate thallus (vegetative) pieces were set up in early February 2012 in the Blue Mountains summit plateau wetlands near Tapanui, Otago (45°56'S 169°21'E, ca. 900 m a.s.l., Figure 23). Each reciprocal transplant was conducted by removing and swapping circular thallus discs using a 2.5 cm cork borer from a site-pair of complementary reproductive mode. Discs were marked and held in place by four bamboo skewers (see Figures 31 and 32).

Immediately next to each transplanted disc, two controls were set up. The first control consisted of a 2.5 cm circular thallus disc being removed with the cork borer and returned in its original location after the actual transplants were completed. This was done to control for the effect of the transplantation procedure. The second control consisted of an untreated thallus area of the same size, which was marked with four skewers (see Figures 31 and 32). The exact placement of the controls relative to the transplanted discs depended on the micro-topography and size of the thalli, but was usually within centimetres from each other. The sites paired for a reciprocal transplant were between 2 m and 330 m meter apart. Exact coordinates of all sites are listed in Table 17.

If reproductive mode was a plastic response to environmental conditions, the prediction was to observe, over the course of one or two years, that vegetative (sterile/sorediate) thallus pieces transplanted to sites containing sexual thalli should show development/maturation of apothecial initials and a reduction of sorediate margins. In contrast, sexual thallus pieces (fertile/ esorediate) transplanted into the natural micro-habitat of vegetative thalli should develop sorediate margins. It would be intuitive to assume, that sexual thalli would also cease growth of already partly matured apothecia when transplanted into these sites; however, the pilot study on Swampy Summit (Ludwig 2012) already showed that this is not the case. Therefore, this aspect was not part of the expectation. All controls were expected not to change regarding their reproductive mode (qualitative difference), or to show only minor development compared to the transplants they control for (quantitative difference).
Table 17. Coordinates of reciprocal transplant sites in the Blue Mountains. Accuracy is ca. 3-5 m using the 1984 World Geodetic System (WGS84).

<table>
<thead>
<tr>
<th>Replicate no.</th>
<th>Sexual site (fertile &amp; esorediate)</th>
<th>Vegetative site (sterile &amp; sorediate)</th>
<th>Distance</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>45°55'56.4&quot;S 169°20'20.0&quot;E</td>
<td>45°55'57.6&quot;S 169°20'22.2&quot;E</td>
<td>60 m</td>
</tr>
<tr>
<td>02</td>
<td>45°55'55.5&quot;S 169°20'20.6&quot;E</td>
<td>45°55'56.4&quot;S 169°20'20.0&quot;E</td>
<td>20 m</td>
</tr>
<tr>
<td>03</td>
<td>45°55'56.3&quot;S 169°20'18.9&quot;E</td>
<td>45°56'03.6&quot;S 169°20'25.6&quot;E</td>
<td>250 m</td>
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<td>04</td>
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<td>45°56'04.2&quot;S 169°20'31.1&quot;E</td>
<td>160 m</td>
</tr>
<tr>
<td>05</td>
<td>45°56'15.4&quot;S 169°20'28.6&quot;E</td>
<td>45°56'13.2&quot;S 169°20'26.4&quot;E</td>
<td>80 m</td>
</tr>
<tr>
<td>06</td>
<td>45°56'27.0&quot;S 169°20'32.2&quot;E</td>
<td>45°56'27.0&quot;S 169°20'32.2&quot;E</td>
<td>2 m</td>
</tr>
<tr>
<td>07</td>
<td>45°55'46.9&quot;S 169°20'13.8&quot;E</td>
<td>45°55'46.9&quot;S 169°20'13.8&quot;E</td>
<td>10 m</td>
</tr>
<tr>
<td>08</td>
<td>45°55'50.7&quot;S 169°20'30.2&quot;E</td>
<td>45°55'51.1&quot;S 169°20'36.8&quot;E</td>
<td>140 m</td>
</tr>
<tr>
<td>09</td>
<td>45°55'38.5&quot;S 169°20'50.7&quot;E</td>
<td>45°55'33.5&quot;S 169°20'47.2&quot;E</td>
<td>155 m</td>
</tr>
<tr>
<td>10</td>
<td>45°55'46.2&quot;S 169°21'03.1&quot;E</td>
<td>45°55'48.5&quot;S 169°21'05.9&quot;E</td>
<td>100 m</td>
</tr>
<tr>
<td>11</td>
<td>45°55'37.0&quot;S 169°21'09.7&quot;E</td>
<td>45°55'44.1&quot;S 169°21'07.1&quot;E</td>
<td>230 m</td>
</tr>
<tr>
<td>12</td>
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<td>45°55'53.0&quot;S 169°20'37.7&quot;E</td>
<td>330 m</td>
</tr>
<tr>
<td>13</td>
<td>45°55'52.7&quot;S 169°20'37.0&quot;E</td>
<td>45°55'52.6&quot;S 169°20'36.1&quot;E</td>
<td>30 m</td>
</tr>
<tr>
<td>14</td>
<td>45°55'59.6&quot;S 169°20'33.8&quot;E</td>
<td>45°55'58.6&quot;S 169°20'30.6&quot;E</td>
<td>80 m</td>
</tr>
<tr>
<td>15</td>
<td>45°56'07.2&quot;S 169°21'03.7&quot;E</td>
<td>45°56'06.1&quot;S 169°21'02.8&quot;E</td>
<td>40 m</td>
</tr>
<tr>
<td>16</td>
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<td>45°56'07.2&quot;S 169°21'03.7&quot;E</td>
<td>60 m</td>
</tr>
<tr>
<td>17</td>
<td>45°56'05.4&quot;S 169°21'08.3&quot;E</td>
<td>45°56'06.9&quot;S 169°21'07.3&quot;E</td>
<td>60 m</td>
</tr>
<tr>
<td>18</td>
<td>45°56'15.9&quot;S 169°21'17.6&quot;E</td>
<td>45°56'19.1&quot;S 169°21'14.7&quot;E</td>
<td>110 m</td>
</tr>
<tr>
<td>19</td>
<td>45°56'18.4&quot;S 169°21'11.2&quot;E</td>
<td>45°56'19.0&quot;S 169°21'14.6&quot;E</td>
<td>80 m</td>
</tr>
<tr>
<td>20</td>
<td>45°55'38.8&quot;S 169°21'11.2&quot;E</td>
<td>45°55'43.8&quot;S 169°21'07.2&quot;E</td>
<td>180 m</td>
</tr>
</tbody>
</table>

2.2.2 Documentation and Reassessment

The transplant sites were photographed, to show the general setting and positions of transplants and controls relative to each other. Macro-photos were taken of all transplanted discs and controls, to record the initial state of the apothecial initials and the extent of sorediate margins of vegetative thalli and show the entire margins of fertile thalli.

In February 2013, i.e. 12 months after start of the experiment, all sites, transplants and controls were reassess and rephotographed. Photos were compared with those taken just after the experiment had been set up a year earlier. As an example, the before and after state of one replicate are illustrated in Figure 32. Photographs of all replicates are attached as Electronic Appendix.
2.3 Light environment

2.3.1 Approach and Field work

The methodology used identical equipment and analysing software as Jarčuška et al. (2010) and Promis et al. (2011), the latter only used a different camera model. Both studies have compared different analysing softwares as well. In lichenological studies this technique was previously used as well, e.g. Gauslaa et al. (2006, 2009), Schoenwetter (2010), Gauslaa (2013).

Fish-eye photography was used to quantify the light incidence immediately above thalli for lichens of both modes. The camera (Nikon Coolpix 5000) equipped with a fish eye lens (Nikon Fisheye Converter FC-E8 0.21x JAPAN, with mounting adapter Nikon UR-E6) was placed immediately above or next to thalli (when on a steep slope), and aligned perpendicularly to the ground with a bubble level, and a hemispherical fish-eye picture was taken to show the entire sky with a continuous horizon line and the zenith in its centre. Grid (geographic) North was tagged with a red-tipped bamboo skewer exactly North of the centre of the lens. Grid North was determined by placing a conventional compass on the flat lid of the still covered and aligned fish-eye lens and correcting for the deviation of Magnetic North to Grid (geographic) North, which is 22° or 23° East according to map sheets “NZTopo50-CE13 Tapanui” and “NZTopo50-CE1 Dunedin” from 2009. The camera was set to “fisheye 1 (F1)” and “normal” image quality at a resolution of 2560 by 1920 pixels, i.e. about 5 mega pixels. The aspect, slope, exact coordinates and altitude of the thalli were also recorded, as these parameters were used for subsequent modelling of the light environment using the software Gap Light Analyzer. This software was found to be well suited for such an approach by the software comparisons of Jarčuška et al. 2010 and Promis et al. (2011).
Figure 26. Example of a fisheye photography site (FE22). TOP: camera setup, the fisheye lens is directed towards the zenith, the red-tipped skewer indicates geographic North relative to the centre of the lens. The thallus is visible at the top right corner of the camera. BOTTOM: fisheye photo from the same site as original (left) and black/white (non-sky/sky) edited for analysis.
2.3.2 Image Editing and Analysis

Fish-eye images were analysed using the free software “Gap Light Analyzer version 2.0 (GLA v.2)” (Frazer et al. 1999) downloaded from http://www.ecostudies.org/gla/ (Note: program does not run on 64 bit operating systems).

The GLA v.2 software distinguishes between sky and non-sky areas based on a pixel brightness threshold for the entire image, therefore it was necessary to manually edit the images. The free image editing software GIMP version 2.6.8 was used as follows. The blue colour channel was extracted by dragging it into the ‘layer dialog’, there it was edited section by section mainly using ‘free select’ tool along with the ‘threshhold’ function. Where necessary, bright non-sky areas were painted black and dark sky areas were painted white. Consequently, the pixel brightness threshold value in GLA v.2 was left unchanged at 128 for analysis. Original and fully edited black/white fish-eye photos are attached as Electronic Appendix.

The following settings were used in GLA v.2 under “Configure”: Initial cursor point is geographic North; projection distortion is polar; solar time steps in 2 minute intervals; start date 1st January; end date 31st December; number of azimuth regions was 72 (i.e. 5° increments); number of zenith regions was 18 (i.e. 5° increments). Default radiation settings were used, i.e. data source was modelled, solar constant was 1367 Wm\(^{-2}\), cloudiness index was 0.5, spectral fraction was 0.5, units were mols m\(^{-2}\)d\(^{-1}\), beam fraction was 0.5, the UOC model for sky-region brightness, clear sky transmission coefficient was 0.65.

The following parameters were site-specific: latitude, longitude, elevation, slope (of thallus) and aspect (of thallus). To make the results reproducible, the site-specific parameters as well as pixel coordinates for geographic North (Initial Point) and geographic South (Final Point) are listed in Table 18.
Table 18. Site specific data used to model light environments based on hemispherical fish-eye photos. The 1984 World Geodetic System (WGS84) applies to geographic coordinates. Slope and aspect refer to the microtopographic slope of the thallus, and aspect is relative to geographic North. Initial and final points are the pixel coordinates (x,y) marking geographic North and South in the fish eye photos. Reproductive modes of the respective sites are given in Results Table 20.

<table>
<thead>
<tr>
<th>FE site</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Elevation [m]</th>
<th>Slope [°]</th>
<th>Aspect [°]</th>
<th>Initial Point coordinates</th>
<th>Final Point coordinates</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>45°47'46.5&quot;S</td>
<td>170°29'00.0&quot;E</td>
<td>715</td>
<td>0</td>
<td>0</td>
<td>1530, 868</td>
<td>24, 691</td>
</tr>
<tr>
<td>02</td>
<td>45°47'46.5&quot;S</td>
<td>170°28'59.2&quot;E</td>
<td>715</td>
<td>40</td>
<td>90</td>
<td>1473, 474</td>
<td>81, 1085</td>
</tr>
<tr>
<td>03</td>
<td>45°47'45.4&quot;S</td>
<td>170°28'52.2&quot;E</td>
<td>715</td>
<td>0</td>
<td>0</td>
<td>1527, 913</td>
<td>44, 641</td>
</tr>
<tr>
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<td>170°28'51.9&quot;E</td>
<td>715</td>
<td>15</td>
<td>180</td>
<td>1365, 318</td>
<td>179, 1241</td>
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<td>170°28'55.0&quot;E</td>
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<td>0</td>
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<td>0</td>
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<td>170°29'00.2&quot;E</td>
<td>715</td>
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<td>0</td>
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<td>17, 751</td>
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<td>170°28'51.8&quot;E</td>
<td>715</td>
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<td>89, 480</td>
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<td>170°28'51.2&quot;E</td>
<td>715</td>
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<td>90</td>
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<td>180</td>
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<td>715</td>
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<td>225</td>
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<td>60</td>
<td>150</td>
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<td>186, 1253</td>
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</table>

2.3.3 Statistical Analysis

Analysis of variance (ANOVA) was performed using the free software R version 2.10.1 and the command ‘aov()’. The GLA v.2 output data (Table 20) met the criteria necessary for ANOVA. The Shapiro-Wilk test for normal distribution was performed using the R-command ‘shapiro.test()’. Normality of data was assumed when the Shapiro-Wilk test yielded a p-value greater than 0.05 and a W-value greater than listed on page 605 of Shapiro & Wilk (1965) for the respective sample size and the significance level of 0.05. Levene’s test (R-command ‘leveneTest()’) was performed to test for homogeneity of variance, which was assumed when the p-value (confusingly designated as ‘Pr>(F)’ in) was greater than 0.05. The only site representing thalli with both apothecia and soralia (FE10) was included twice in the analysis, once as sexual and once as vegetative.
The data have been illustrated not only as box-whisker plots but also as dot plots, because dot plots show the detailed data distribution, which is hidden in box-whisker plots.

2.4 Moisture regime

2.4.1 Thallus fragment moisture measurements

Various trials to objectively quantify the moisture content of only the uppermost soil layer, on which lichen sits, failed. As the thalli are intimately connected to the substrate surface but lack penetrating roots, probably the ecologically most meaningful way to quantify the micro-climatic moisture regime relevant to a lichen thallus, would be to examine the water content of a thallus itself. Given that *I. splachnirima* is rare, it is inappropriate to collect large numbers of thalli for moisture measurements, so a new method was developed for this thesis involving small thallus transplants. These were pinned to the ground immediately next to a thallus in the field, for which they serve as ‘moisture dummies’ to measure in situ thallus hydration gravimetrically.

Numerous thallus pieces between 5 and 10 mm diameter were gathered, all adhering substrate (soil, detritus, bryophytes) was manually removed under a dissecting microscope using fine forceps and scalpel blades.

On 17.03.2013 the first experiment (or test run) was set up in 12 sites on Swampy Summit (in the same sites where fish-eye photos were taken and relative humidity and temperature data-logger were placed). Five ‘dummy’ thallus fragments were pinned next to a ‘native’ thallus at each site. On 20.03.2013, a partly sunny day, between 2.45 p.m. and 5.30 p.m. all pinned thalli were collected individually and placed in a universal glass vial with rubber sealed lid to prevent desiccation during transport.

The weight of the whole vial as well as only the thallus piece was determined before and after drying using a laboratory scale with 0.1 mg accuracy. Thallus fragments and vials were air dried for 3 days in an air conditioned office at ca. 20°C. It was tested whether the thalli could be further desiccated with silica gel beads, but these had no additional effect. Water content (%) of the thalli in percent was calculated based on the dry weight of the thallus as 100%, e.g. a thallus of 100 mg dry weight and a total amount of 150 mg water has a water content of 150%.

A second similar experiment was set up on 06.06.2013 and harvested on 07.06.2013 between 3 and 5 p.m. in course of the stress physiology experiment described in section 4 of this chapter. This second experiment differed from the first one described above only in the use of 10 ‘dummy’ thallus fragments in each of only four selected sites (FE06, FE15, FE20, FE24), which were used as treatment sites for the stress physiology experiment.

For statistical analysis of both ‘dummy’ thallus water measurements, the relative moisture content of the thalli was grouped according to reproductive mode of the sites, and was subjected to the Shapiro-Wilk’s and Lilliefors’s test of normality and Levene’s test for homogeneity of variance (using
the R commands shapiro.test(), lillie.test() and leveneTest()). These underlying assumptions of analysis of variance were met, and ANOVA was performed using the R command aov().

Data from the second set of measurements in course of the stress physiology experiment (see section 4) was additionally grouped by site and analysed as described in the paragraph above; the ANOVA result was post hoc checked with Tukey’s honestly significant difference test (using R commands TukeyHSD() and HSD.test()).

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**Figure 27.** Example of ‘dummy’ thalli pinned next to thallus in the field. White pin heads are ca. 3 mm in diameter.

### 2.4.2 Hydro-cloth discs moisture measurements

As the ‘dummy’ thalli described above had several draw-backs (discussed below), the approach was modified and the thallus fragments were replaced by circular discs of highly absorbent capillary watering mats, which are usually used for hydroponics. These were purchased from ‘Switched On Gardener’ (313 King Edward Street, Dunedin, NZ) as ‘Hydro Cloth’, the name which will be used hereafter. Discs of 2.5 cm diameter were punched out of the cloth sheet using cork borer. The weight of these uniformly sized cloth discs varied between 19 and 30 mg. As this would cause a considerable variation of relative moisture content, only discs weighing 25 ± 2 mg (23-27 mg) were selected for use in the experiment. Advantages of the hydro-cloth discs over the use of thallus fragments are that they are very robust, have a uniform thickness and shape, are easier and quicker to use, and their number is not limited by the availability of suitable material.

The hydro-cloth disc experiment was set up on Swampy Summit on 28.10.2013 in the sites used previously for fish-eye photography. Ten discs were pinned down around 23 naturally occurring thalli, (FE01-24, excluding FE14) using two pins per disc. Only site FE14 was not included in this experiment, because its immediate surrounding was densely covered by vegetation, while the thallus...
grew over a small, exposed chunk of peaty soil. Discs were collected again on 13.11.2013 between 4 p.m. and 8.30 p.m. They were placed in 2 ml microcentrifuge tubes, and weighed before and after drying as described above for the thallus fragments.

For statistical analysis, the relative moisture content of the hydro-cloth discs was grouped according to reproductive mode of the site, and was subjected to Lilliefors' test of normality and Levene's test for homogeneity of variance (using the R commands, lillie.test() and leveneTest()). The assumptions for analysis of variance were not met (nor for the log-transformed data). Therefore, the non-parametric Mann-Whitney-Wilcoxon rank-sum test was performed (R-command wilcox.test()) with relative water content as response variable, because the predictor variable reproductive mode has only two factor levels (for more factor levels the Kruskal-Wallis test would be appropriate). Site FE10 was included twice in the analysis, both as a sexual and a vegetative site.

Figure 28. Examples showing the setup of hydro-cloth disc moisture measurements in the field. Discs are 2.5 cm across, those on the left picture are wet and somewhat translucent, while those on the right picture are dry white.
2.4.3 Relative Humidity measurements

Relative humidity and temperature data-loggers (MadgeTech, model TransiTempII-RH, http://www.madgetech.com/data-loggers/product-applications/libraries-museums-archives-historical-sites/transitempii-rh.html) were placed in the following 12 sites on Swampy Summit (see Table 18 for coordinates and Table 20 or Table 21 for reproductive mode): FE01, FE02, FE06, FE07, FE08, FE15, FE18, FE19, FE20, FE22, FE23, FE24 (Figure 29). The sites provide an equal representation of both reproductive modes and subjectively appeared to provide contrasting light and moisture regimes. They also corresponded to the sites, where the moisture regime was quantified by ‘dummy thalli’ and hydro-cloth discs, as described above. Measurements were taken in the periods 26.01.2013 to 11.04.2013 and 25.05.2013 to 16.09.2013.

![Figure 29](image_url). Examples showing the setup of relative humidity and temperature data loggers in the field.
2.5 Stress Physiology

2.5.1 General introduction to the stress physiological experiment

This experiment addresses the question “Do vegetative specimens have a greater capacity to cope with stress than sexual specimens?” or in other words “Does production and maintenance of sexual fruiting bodies impair the capacity to cope with stress?”.

The approach to answer these questions consisted of reciprocal transplants in the field (outlined in detail below), and subsequent quantification of the physiological stress marker lipid hydroperoxidation, a very sensitive measure of oxidative damage.

2.5.2 Experimental setup in the field

2.5.2.1 Outline of experimental setup in the field

The field based reciprocal transplant experiment, conducted on Swampy Summit near Dunedin (45°47'50"S 170°29'00"E, ca. 715 m a.s.l.), consisted of a total of 96 samples, comprising 24 samples originating from each of 4 site categories (Figure 30). These represented naturally occurring combinations of both reproductive states, growing in (qualitatively) contrasting moisture and light conditions, referred to as ‘site categories’ for the remainder of this chapter:

1) sexual, wet, shade 2) sexual, wet, open 3) vegetative, dry, shade 4) vegetative, dry, open.

Notably, no specimens combining the character states sexual/dry or vegetative/wet have ever been observed in the field, and could therefore not be used. This means in turn, that sexual/wet and vegetative/dry are always combined.

Precise geographic coordinates of the four sites on Swampy Summit are given in Table 18 in the section this chapter dealing fish-eye photography, where they are called FE06 (sexual, wet, open), FE15 (vegetative, dry, open), FE20 (vegetative, dry, shade) and FE24 (sexual, wet, shade); see also Figure 25. These site name will also be used for this stress physiology experiment.

As the term reciprocal transplant implies, each of the four pre-transplant origin-sites, became a post-transplant treatment-site of the site category, i.e. same moisture and light conditions. The 24 samples representing the same origin site-categories, were evenly divided into four lots (of 6 samples each), which were reciprocally transplanted into the other three site categories or returned to their original site-category to act as controls. This means, after the reciprocal transplants each treatment-site (or former origin-site) harboured 6 samples originating from all four origin site-categories. Figure 30 illustrates the reciprocal transplantation scheme for better comprehension. After 25 days all samples were harvested and examined for their stress physiological response to the new habitat conditions.
Figure 30. Reciprocal transplantation scheme. Column headings and colour coding indicate reproductive mode and origin site-category (i.e. conditions). Lower case letters indicate different real origin sites of pooled samples representing the same origin site-category. The top row blocks ("before transplant") show the four artificially pooled populations consisting of specimens representing the same origin site-categories, which were placed together for acclimation. Within each column, the sites (and conditions) before and after transplants are the same, as indicated by the column heading. A lower case ‘z’ indicates samples sourced from Swampy Summit, all other samples were sourced from various sites in the Blue Mountains, which are indicated by different lower case letters.

2.5.2.2 Sample collection

Samples were collected between 10.-12. March 2013 in the wetlands in the Blue Mts. (900m asl) and on Swampy Summit (700m asl), respectively. Only samples representing the Sexual-Wet-Open origin site-category were collected in a single site on Swampy Summit (labelled ‘z’ in Figure 30), all others came from several sites in the Blue Mts. This inconsistency was unavoidable, because only one plentiful sexual-wet-open site was known from Swampy Summit, while it is absent from the Blue Mts., whereas the three other site categories are manifestly more abundant on the Blue Mts. than on Swampy Summit.

Circular thallus discs of 2.5 cm diameter were sampled using a cork borer, with a substrate layer ca. 1.0 cm thick still attached underneath. Fertile thalli were sampled in a manner ensuring a high proportion of thalline tissue relative to apothecia, because only thalline tissues should be used for physiological comparison with sterile thalli, so as to ensure that only ‘apples were being compared to apples’.
The experimental design required 24 samples per origin site-category. This fairly high number of samples, however, was rarely available from only one site representing a site category. Therefore, in three of the four site categories, samples from two or even four similar sites (representing the same origin site-category) had to be pooled to yield 24 samples. Again this inconsistency was unavoidable.

2.5.2.3 Pooling of artificial populations

Four artificial populations were pooled on 13. March 2013 in four new sites on Swampy Summit; these pooled samples were considered to represent the same site categories regarding light and moisture conditions as their original collection sites, and which were also the natural habitats of thalli exhibiting the same reproductive mode. All samples within a site category were arranged within an area of ca. 0.5 m diameter. Samples were left in the new ‘origin-sites’ to acclimatise for two months.

2.5.2.4 Reciprocal transplants

After the two months acclimation period, reciprocal transplants were conducted on 13. May 2013, i.e. three lots of 6 specimens (out of 24) were transplanted to the other three ‘origin-sites’ with different light-moisture regimes. The remaining 6 samples were left in the previous ‘origin site’ as controls, but were also lifted up and place in a slightly different spot to account for the potential handling-stress experienced by the 18 swapped samples. Samples from the same original collection sites (i.e. not just origin site-category) were evenly distributed among the four treatments, to avoid potential unintended provenance effects.

2.5.2.5 Harvest

All samples were harvested on 7 June 2013 between 2 and 4 pm, that is 25 days after transplanting. This day was chosen, as it was a sunny (though cool) day, following a week of rather clear and dry weather, which could presumably have emphasized the micro-climatic differences among the treatment site categories.

Individual samples were immediately stored on ice in sealable plastic bags in order to maintain the current moisture status. Until further processing, all sample bags were stored together in an ice-filled polystyrene box in a fridge set at 4°C.
2.5.3 Laboratory work

2.5.3.1 Preparation for lipid extraction

Adhering substrate (soil, plant debris, bryophytes, microbial slime) had to be manually removed from the fresh thalline tissue using forceps, scalpel blades and a dissecting microscope.

This cleaning procedure was extremely time consuming, and extended over an entire week. During all this time, specimens were kept on ice in a fridge in the dark. The order in which specimens were processed was recorded, to be able to test for an effect of the different waiting periods if results appeared anomalous or random. The order of processing was semi-randomized, in that four consecutively treated random samples always comprised all four treatment site categories.

Prior to cleaning, each specimen was fully hydrated by being placed in a Petri-dish lying on a water-saturated filter paper. The Petri-dish itself was placed in a larger ice-filled dish. Specimens were kept moist and cool in order to prevent additional desiccation or temperature stress and to facilitate cleaning. The aim of the cleaning procedure was to obtain 100 mg of clean, hydrated thalline tissue. Apothecial tissue was removed from fertile thalli. Cleaned pieces of thalline tissue were placed on water-saturated filter paper in another Petri-dish with lid, again sitting on ice. Placing thalline tissue in this moisture-saturated environment allowed for equilibration of the hydration status, in order to standardise the hydrated thallus weight. All thallus pieces were weighed (with 0.1 mg accuracy), the accurate hydrated weight (‘fresh weight’ or ‘FW’) was recorded. The sample was snap frozen in a 1.7 ml microcentrifuge tube in liquid nitrogen, and stored at -80°C until the lipid extraction.

The time required for cleaning a specimen, i.e. to obtain sufficient material, ranged from 15 to 30 minutes, but in several hard cases up to an hour was necessary. Therefore, one hour after starting the cleaning procedure, each sample was frozen in liquid nitrogen.

2.5.3.2 Lipid-extraction

Cleaned and weighed tissue samples were briefly ground with liquid nitrogen in the microcentrifuge tubes using a suitable tube pestle. The subsequent steps were performed in a fume cabinet. 0.6 ml of ice-chilled methanol: chloroform (2:1 v/v) were added to the pre-ground sample, and mixed by thorough vortexing. The tube was incubated at room temperature for 15 minutes and regularly vortexed. Grinding with the pestle continued during this period until all suspended fragments were smaller than 1 mm. This was necessary because initial liquid nitrogen grinding never resulted in sufficient tissue disruption, and an initial trial showed that extraction of larger fragments was very inefficient. 0.4 ml of ice-chilled chloroform was added, followed by vortexing for 60 seconds. Then 0.4 ml of ice-chilled deionised water was added, followed by vortexing for 60 seconds. Tubes were centrifuged for 30 seconds at full speed in an ordinary bench top centrifuge to facilitate phase separation. Two 70 µl aliquots were recovered from the lower chloroform phase, were briefly stored on ice and then frozen at -80°C.
2.5.3.3 Lipid hydroperoxidation assay

For each sample, 50 µl of lipid extract were used in a lipid hydroperoxidation assay, which was run by Dr. David J. Burritt, as outlined in Schweikert & Burritt (2012): “An aliquot of the lipid phase (50 µl) was transferred to a glass microtitre plate well and lipid hydroperoxides were determined using the ferric thiocyanate method of Mihaljevic et al. (1996) adapted for measurement in a microtitre plate reader. Levels were determined by measuring the absorbance at 500 nm. A calibration curve with t-butyl hydroperoxide was used and the lipid hydroperoxide content calculated as nmol of lipid hydroperoxide/g FW.”

Samples ‘below detection limit’ were excluded from subsequent analysis, as they were deemed to be the result of a failed extraction procedure, and using 0 values was considered to introduce an inappropriate bias. This was the case for 5 of the 96 samples. Fortunately, out of each origin-treatment-group consisting of 6 samples, never more than one fell ‘below detection limit’, which always left at least 5 samples for analysis.

2.5.4 Site conditions

In order to obtain objective data on the micro-climatic conditions in the four treatment sites, rather than the mere classification of ‘wet’, ‘dry’, ‘open’ and ‘shade’, the moisture and light conditions were quantified using ‘dummy’ thalli and fisheye photos as described in the respective sections of this chapter. Treatment site names were kept as used in the section on fish-eye photography, i.e. FE06, FE15, FE20 and FE24.

Arcsine-transformation was applied to the relative thallus water content dataset, in order to obtain a significant result. According to Leyer & Wesche (2007), who refer to Sokal & Rohlf (1995), arcsine-transformation is a recommended procedure for the analysis of percent data. Percent values were converted according to the equation given in Quinn & Keough (2002) as well as Whitlock & Schluter (2009): \( x = \arcsin(\sqrt{\text{percent} \_ \text{value}/100}) \).

Percent values were divided by 100 first, because arcsine calculation is possible only for proportion values ranging from 0 to 1 (1 being equivalent to 100% relative moisture content). For the same reason, all values above 100% were transformed by adding up arcsin(\( \sqrt{1} \)) and arcsin(\( \sqrt{\text{percent} \_ \text{value}/100} - 1 \)).
2.5.5 Statistical analysis
Analyses of variance (ANOVA), using the free software R version 2.10.1, were performed for the four data subsets comprising specimens of the same origin. The R-command `aov()` was used, not `anova(lm)`. To check whether the ANOVA assumptions were met, the Shapiro-Wilk-Test (R-command: `shapiro.test()`) was used to test for normality of the ANOVA model’s residuals as well as the data distribution within each origin-treatment-group, and Levene’s Test (R-command: `leveneTest()`) was used to test for homogeneity of variance. When necessary, log-transformed data were used to meet the normality criterion.

To interpret the calculated $W$-values of the Shapiro-Wilk-test, these were compared to the thresholds provided in Table 6 of Shapiro & Wilk (1965: 605) for the respective sample size at significance level 0.05. $W$-values above the thresholds were taken as evidence of sufficient normality, but only when the additional p-value given in the test’s output was greater than 0.05.

For statistical analysis, the dataset was divided according to origin site-category, and the treatment effect within each origin site-category was tested by pairwise comparison of the four treatment groups. Initially significant ANOVA results for an origin site-category were post-hoc subjected to Tukey’s honestly significant difference test (R-commands: `TukeyHSD()` and `HSD.test()`), to establish which treatments were significantly different from each other. In some cases it was necessary to use a log-transformed ($\log_{10}$(value)) or inverse-transformed ($1$/value) dataset to obtain significant results; both these ways of data transformation are commonly used according to Osborne (2002). Significances based on transformed data were marked as such in the results section.
3. Results

3.1 Micro-habitat

All except one of the vegetative thalli transplanted to sites supporting sexual thalli showed sexual development and in the majority the apothecia were strongly developed. Vegetative controls in their original sites usually showed no apothecial development or to a lesser extent than the respective transplants placed in sexual sites. Sexual thalli transplanted to sites consisting of vegetative thalli did not stop or abort apothecial development, but seemed to continue growth at a rate comparable to their respective controls; in two cases, however, sorediate margins had developed.

Outcomes of all 20 replicates in the Blue Mountains are summarised in Table 19. In 17 of 20 replicates the apothecial initials of previously vegetative transplants show clear signs of maturation after being placed in sexual sites. For these 17 replicates, the controls left in the vegetative sites show no signs of apothecial maturation (qualitative support) or to a distinctly lesser extent (quantitative support). Seven replicates showed a clear reduction of marginal soralia of the vegetative transplants placed in the sexual sites; two of them showed a complete loss of soralia.

Twelve replicates (nos. 02, 03, 04, 05, 07, 08, 11, 12, 15, 17, 18, 20) showed response predicted by the hypothesis. Another five replicates (nos. 06, 10, 13, 14, 16) have been scored as quantitatively supportive of the hypothesis. That means that a control in the vegetative site showed clear apothecial development, which, however, was either negligible compared to the apothecial development of the transplant in the sexual site and/or the second control lacked apothecial development.

In two replicates (nos. 09, 13) new development of small sorediate sections was evident in sexual transplants after placement in vegetative sites.

Three replicates gave different results. The first replicate (replicate 01) showed a high degree of apothecial maturation in the untouched control, which is comparable to that in the corresponding transplant, and also the moved control shows clear signs of apothecial maturation, although to a much lesser extent.

In the second (replicate 09), the transplant placed in the sexual site was completely decomposed, and the untouched control in the vegetative site was half decomposed. This is not considered to be a response to the treatment, because it occurred in the transplant and the untouched control, but it renders the replicate unsuitable for inclusion in further analysis and discussion.

In the third failed replicate (replicate 19) the transplant placed in the sexual site was surprisingly completely unchanged, i.e. no apothecial maturation has occurred at all. During the reassessment of this site, it was obvious that the setup of this transplant was faulty, in that the transplanted disc was virtually disconnected from the surrounding soil and host thallus. It was clearly dry while its surrounding was wet, which must have adversely affected the apothecial development.
Figure 31. Replicate 04 in February 2013, one year after transplantation. LEFT SIDE: sexual site. RIGHT SIDE: vegetative site. TOP: transplants. MIDDLE: moved controls. BOTTOM: untouched controls.
Figure 32. Replicate 04 February 2012, showing the condition immediately after transplantation. LEFT SIDE: sexual site. RIGHT SIDE: vegetative site. TOP: transplants. MIDDLE: moved controls. BOTTOM: untouched controls.
The transplant from the vegetative site has rotted away, the untouched control in the vegetative site is half decomposed, and the moved control in the vegetative site shows a lot of apothecial development. It is unclear why the transplant from the vegetative site and the untouched control have decomposed. BMT 09 should be omitted from analysis and discussion.

### Table 19
Summary of outcomes of all 20 reciprocal transplant replicates in the Blue Mountains after 12 months. Columns 2-7 are arranged according to the post-transplant state in the field, i.e. the transplant in the sexual site came originally from the vegetative site but its controls stayed in the vegetative site, and vice versa. According to the hypothesis, expected results were: (1) The originally vegetative (sterile and sorediate) transplant placed in the sexual site should show maturation of apothecial initials and possibly reduction of soralia, while its controls (both moved and untouched) remaining in the vegetative site should be unchanged. (2) The originally sexual (fertile and esorediate) transplant placed in the vegetative site should either stay unchanged or develop soralia along its lobe margins, and its controls left in the sexual site should show maturation of apothecial initials and possibly reduction of soralia, while its controls remaining in the vegetative site are strongly reduced, but still pronounced in the untouched vegetative control. Therefore it is considered quantitative support for the hypothesis.

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<td>C_UNTouched</td>
</tr>
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<td>≡</td>
<td>≡</td>
</tr>
<tr>
<td>08</td>
<td>A+, S≡</td>
<td>≡</td>
<td>≡</td>
</tr>
<tr>
<td>09</td>
<td>rotten^C</td>
<td>≡</td>
<td>≡</td>
</tr>
<tr>
<td>10</td>
<td>A++, S----</td>
<td>≡</td>
<td>≡</td>
</tr>
<tr>
<td>11</td>
<td>A++, S----</td>
<td>gone^E</td>
<td>≡</td>
</tr>
<tr>
<td>12</td>
<td>A+, S--</td>
<td>≡</td>
<td>≡</td>
</tr>
<tr>
<td>13</td>
<td>A+, S--</td>
<td>≡</td>
<td>≡</td>
</tr>
<tr>
<td>14</td>
<td>A++, S≡</td>
<td>≡</td>
<td>≡</td>
</tr>
<tr>
<td>15</td>
<td>A++, S≡</td>
<td>≡</td>
<td>≡</td>
</tr>
<tr>
<td>16</td>
<td>A++, S≡</td>
<td>≡</td>
<td>≡</td>
</tr>
<tr>
<td>17</td>
<td>A++, S≡</td>
<td>≡</td>
<td>≡</td>
</tr>
<tr>
<td>18</td>
<td>A++, S≡</td>
<td>≡</td>
<td>≡</td>
</tr>
<tr>
<td>19</td>
<td>A, S≡</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>20</td>
<td>A++, S≡</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Σ_EXPECTED</td>
<td>18</td>
<td>19</td>
<td>20</td>
</tr>
</tbody>
</table>

^A – Rep. 01: (compare photos P1320982 and P1330005) The untouched control in the vegetative site shows considerable apothecial development, similar in extent to that of the transplant placed in the sexual site. Perhaps the gap created by the transplant in the vegetative site just next to the untouched control allowed better access for water reaching bryophytes underneath, which improved water retention. However, the moved control in the vegetative site shows only very little apothecial development (negligible compared to the transplant), and as this control experienced a more similar treatment to the transplant placed in the sexual site, this could also be considered to be quantitative support of the hypothesis. As this might still be a controversial decision, I opt for the conclusion that BMT 01 does not support the hypothesis; however, it does not contradict it either, instead being inconclusive.

^B – Rep. 06: (compare photos P132405 and P1320415) The untouched control in the vegetative site shows apothecial development, but still much less than in the transplant from the same site; apothecia in the transplant are also generally larger. Soralia in the transplant are strongly reduced, but still pronounced in the untouched vegetative control. Therefore it is considered quantitative support for the hypothesis.

^C – Rep. 09: The transplant in the sexual site has rotted away, the untouched control in the vegetative site is half decomposed, and the moved control in the vegetative site shows a lot of apothecial development. It is unclear why the transplant from the vegetative site and the untouched control have decomposed. BMT 09 should be omitted from analysis and discussion.
D – Rep. 10: Both controls in the vegetative site show apothecial growth as well, but it is negligible compared to that of the transplant in the sexual site, in which all soralia have also disappeared. Therefore it is considered quantitative support of the hypothesis.

E – Rep. 11: the moved control in the sexual site has disappeared (see photos P1320651 and P1320653), it is unclear how/why. Since the untouched control worked, BMT 11 is considered to support the hypothesis.

F – Rep. 13: The untouched control in the vegetative site is unchanged regarding soralia, but one single large apothecium has grown, and another one grew just next to the same control, all other apothecial initials are unchanged. The two large apothecia arose from thallus sections partly overgrown with liverworts, which probably facilitated moisture retention there. The increase in size of these apothecia is remarkable and as the remaining apothecial initials stayed dormant, it could imply that once dormancy has been broken, resource availability is the only constraint to apothecial growth rate, but usually numerous maturing apothecia have to share them. (Similarly, BMT 12 also shows a large single apothecium just outside the untouched control in the vegetative site.) Nevertheless, BMT 13 is counted as quantitatively supportive of the hypothesis, because the moved control in the vegetative site is unchanged and the transplant in the sexual site shows apothecial maturation of more initials, as well as a reduction of soralia.

G – Rep. 14: untouched control in the vegetative site shows apothecial development, but still much less than in the transplant from the same site; apothecia in the transplant are also larger. Therefore it is considered quantitative support for the hypothesis.

H – Rep. 16: Both controls in the vegetative site show considerable of apothecial development, but still less than in the transplant from the same sit; apothecia in the transplant are also larger. Therefore it is considered quantitative support for the hypothesis.

I – Rep. 19: The transplant placed in the sexual site is unexpectedly unchanged, but as the photos (P1320487, P1320488) show, the transplant is obviously much dryer than the surrounding host thallus. Clearly the water flow towards the transplanted disc was severely impaired by the gap between the transplant and soil, and in hindsight it was a mistake to have picked this spot for the transplant. Due to the faulty setup it appear justified to omit BMT19 from the analysis and discussion.
3.2 Light environment

No support was found for a difference between sexual and vegetative sites regarding modelled canopy light transmission parameters (Figure 33). Analysis of variance probability values (p-values) of 0.441, 0.836 and 0.621 do not support a correlation of reproductive mode and percentages of direct, diffuse and total light transmission, respectively. Table 20 lists the modelled light incidence of all sites.

Table 20. Light Transmission parameters modelled using Gap Light Analyzer 2.0, based on hemispherical fish-eye photos from sites were either sexual or vegetative *I. splachnirima* thalli grow. Site numbers correspond to those in Table 18.

<table>
<thead>
<tr>
<th>FE site no.</th>
<th>Reproductive mode</th>
<th>% direct light transmission</th>
<th>% diffuse light transmission</th>
<th>% total light transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>sexual</td>
<td>0.87</td>
<td>36.33</td>
<td>22.54</td>
</tr>
<tr>
<td>02</td>
<td>vegetative</td>
<td>53.97</td>
<td>66.74</td>
<td>60.07</td>
</tr>
<tr>
<td>03</td>
<td>sexual</td>
<td>18.80</td>
<td>42.21</td>
<td>30.50</td>
</tr>
<tr>
<td>04</td>
<td>sexual</td>
<td>35.68</td>
<td>53.56</td>
<td>45.78</td>
</tr>
<tr>
<td>05</td>
<td>sexual</td>
<td>95.97</td>
<td>95.09</td>
<td>95.53</td>
</tr>
<tr>
<td>06</td>
<td>sexual</td>
<td>79.99</td>
<td>84.58</td>
<td>82.29</td>
</tr>
<tr>
<td>07</td>
<td>vegetative</td>
<td>53.76</td>
<td>73.12</td>
<td>63.44</td>
</tr>
<tr>
<td>08</td>
<td>sexual</td>
<td>29.12</td>
<td>62.25</td>
<td>45.68</td>
</tr>
<tr>
<td>09</td>
<td>vegetative</td>
<td>79.55</td>
<td>82.97</td>
<td>81.18</td>
</tr>
<tr>
<td>10</td>
<td>Both</td>
<td>0.87</td>
<td>20.92</td>
<td>17.86</td>
</tr>
<tr>
<td>11</td>
<td>vegetative</td>
<td>92.30</td>
<td>91.91</td>
<td>92.06</td>
</tr>
<tr>
<td>12</td>
<td>vegetative</td>
<td>95.17</td>
<td>84.03</td>
<td>85.71</td>
</tr>
<tr>
<td>13</td>
<td>sexual</td>
<td>15.09</td>
<td>31.54</td>
<td>23.31</td>
</tr>
<tr>
<td>14</td>
<td>vegetative</td>
<td>67.88</td>
<td>53.99</td>
<td>57.56</td>
</tr>
<tr>
<td>15</td>
<td>vegetative</td>
<td>52.24</td>
<td>72.63</td>
<td>64.12</td>
</tr>
<tr>
<td>16</td>
<td>vegetative</td>
<td>94.88</td>
<td>83.96</td>
<td>88.53</td>
</tr>
<tr>
<td>17</td>
<td>vegetative</td>
<td>81.49</td>
<td>81.40</td>
<td>81.45</td>
</tr>
<tr>
<td>18</td>
<td>vegetative</td>
<td>9.72</td>
<td>38.02</td>
<td>26.50</td>
</tr>
<tr>
<td>19</td>
<td>sexual</td>
<td>70.80</td>
<td>74.78</td>
<td>72.79</td>
</tr>
<tr>
<td>20</td>
<td>vegetative</td>
<td>14.44</td>
<td>33.98</td>
<td>27.21</td>
</tr>
<tr>
<td>21</td>
<td>sexual</td>
<td>18.85</td>
<td>37.05</td>
<td>33.14</td>
</tr>
<tr>
<td>22</td>
<td>vegetative</td>
<td>5.16</td>
<td>16.85</td>
<td>12.02</td>
</tr>
<tr>
<td>23</td>
<td>sexual</td>
<td>83.43</td>
<td>81.71</td>
<td>82.57</td>
</tr>
<tr>
<td>24</td>
<td>sexual</td>
<td>4.24</td>
<td>39.29</td>
<td>23.21</td>
</tr>
<tr>
<td>25</td>
<td>sexual</td>
<td>31.72</td>
<td>75.74</td>
<td>61.88</td>
</tr>
<tr>
<td>26</td>
<td>vegetative</td>
<td>49.40</td>
<td>71.35</td>
<td>60.37</td>
</tr>
<tr>
<td>27</td>
<td>vegetative</td>
<td>4.67</td>
<td>32.95</td>
<td>23.26</td>
</tr>
<tr>
<td>28</td>
<td>vegetative</td>
<td>18.61</td>
<td>41.53</td>
<td>32.69</td>
</tr>
<tr>
<td>29</td>
<td>Vegetative</td>
<td>35.33</td>
<td>46.35</td>
<td>43.14</td>
</tr>
</tbody>
</table>
Figure 33. Dot plots and box-plots illustrating the modelled direct, diffuse and total light transmission for sites harbouring sexual and vegetative thalli of I. splachnirima. Analysis of variance indicates no correlation of reproductive mode and the three light transmission parameters.
3.3 Moisture regime

3.3.1 Thallus fragment hydration measurements

The results of the first ‘dummy’ thallus water content measurements are summarised in Table 21 and Figure 34 and Figure 35. While the trend in mean % water content for dummy thallus fragments adjacent to vegetative thalli is lower than that of fragments adjacent to sexual thalli as expected, analysis of variance indicates no significant difference in relative thallus water content and the reproductive mode of thalli in the tested sites \((p = 0.195)\).

Table 21. Results summary of thallus fragment hydration experiment on Swampy Summit in March 2013, sample size per site is \(n = 5\). As indicated in the site number column, the sites correspond to those used previously for fish-eye (FE) photography in the section on light incidence.

<table>
<thead>
<tr>
<th>site no.</th>
<th>reproductive mode</th>
<th>mean (m(H_2O)) [mg] (\pm) standard deviation</th>
<th>mean % relative thallus water content (\pm) standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>FE01</td>
<td>Sexual</td>
<td>21.4 (\pm) 25.8</td>
<td>84.1 (\pm) 28.5</td>
</tr>
<tr>
<td>FE02</td>
<td>Vegetative</td>
<td>16.0 (\pm) 22.7</td>
<td>40.3 (\pm) 13.5</td>
</tr>
<tr>
<td>FE06</td>
<td>Sexual</td>
<td>7.7 (\pm) 7.0</td>
<td>47.5 (\pm) 14.2</td>
</tr>
<tr>
<td>FE07</td>
<td>vegetative</td>
<td>15.1 (\pm) 9.2</td>
<td>78.6 (\pm) 7.0</td>
</tr>
<tr>
<td>FE08</td>
<td>Sexual</td>
<td>33.5 (\pm) 47.5</td>
<td>100.4 (\pm) 6.8</td>
</tr>
<tr>
<td>FE15</td>
<td>vegetative</td>
<td>13.8 (\pm) 13.3</td>
<td>52.5 (\pm) 5.0</td>
</tr>
<tr>
<td>FE18</td>
<td>vegetative</td>
<td>20.4 (\pm) 14.9</td>
<td>106.1 (\pm) 26.1</td>
</tr>
<tr>
<td>FE19</td>
<td>Sexual</td>
<td>26.0 (\pm) 16.2</td>
<td>106.5 (\pm) 15.1</td>
</tr>
<tr>
<td>FE20</td>
<td>vegetative</td>
<td>15.8 (\pm) 9.2</td>
<td>93.8 (\pm) 10.4</td>
</tr>
<tr>
<td>FE22</td>
<td>vegetative</td>
<td>27.1 (\pm) 29.3</td>
<td>99.5 (\pm) 18.3</td>
</tr>
<tr>
<td>FE23</td>
<td>Sexual</td>
<td>14.3 (\pm) 4.4</td>
<td>85.6 (\pm) 28.2</td>
</tr>
<tr>
<td>FE24</td>
<td>Sexual</td>
<td>24.7 (\pm) 18.6</td>
<td>102.8 (\pm) 14.1</td>
</tr>
</tbody>
</table>

Figure 34. Results of the first ‘dummy’ thallus moisture measurements illustrated as dot-plot (left) and box-whisker-plot (right). Measurements are grouped by reproductive mode and expressed as percent relative water content. Analysis of variance indicates no significant correlation of reproductive mode and percent relative thallus water content.
Chapter 5 – Micro-environment and Reproductive Mode

Figure 35. Results of the first ‘dummy’ thallus moisture measurements illustrated as dot-plot (left) and box-whisker-plot (right). Measurements are grouped by site and expressed as percent relative water content. The reproductive mode of thalli growing in these sites is colour coded, red is sexual, blue is vegetative.

For the second ‘dummy’ thallus water content measurements analysis of variance indicates that ‘dummy’ thalli in sites harbouring vegetative thalli had significantly lower ($p < 0.0001$) thallus water content than those from sites with sexual thalli. Figure 36 illustrates the results grouped by reproductive mode of the tested sites. The detailed results for the individual sites are presented in Table 25 and Figure 41 section 3.4.2, as it is seems appropriate when describing the treatment site conditions of the stress physiology experiment.

Figure 36. Results of the second ‘dummy’ thallus moisture measurements illustrated as dot-plot (left) and box-whisker-plot (right). Measurements are grouped by reproductive mode and expressed as percent relative water content. Analysis of variance indicates that the two groups differ significantly ($p < 0.0001$).
3.3.2 Hydro-cloth discs moisture measurements

The results of the hydro-cloth disc moisture measurements are summarised in Table 22 and illustrated in Figure 37 and Figure 38. Moisture levels of sexual sites were found to be much higher than in vegetative sites. Five prominent outlier measurements in the vegetative sites 12, 16 and 17 show high moisture content as well. However, these are attributed to small-scale variation of soil moisture content in the centimetre range. They are not considered representative for the moisture regime of the respective thalli, because the remaining 8 or 9 out of 10 measurements in these three vegetative sites indicate much drier conditions. The Mann-Whitney-Wilcoxon rank-sum test result supports a correlation of reproductive mode and the moisture regime with $p < 0.0001$, which is also apparent by visual inspection of the data. Analysis of variance was not conducted, as the ANOVA assumptions are not met by the dataset.
Table 22. Results summary of hydro-cloth discs moisture measurements on Swampy Summit in October/November 2013, sample size per site is \( n = 10 \). In this experiment, the hydro-cloth disc water content was used as a proxy for the thallus hydration status.

<table>
<thead>
<tr>
<th>site no.</th>
<th>reproductive mode</th>
<th>mean ( m(\text{H}_2\text{O}) ) [mg] ± standard deviation</th>
<th>mean % relative cloth disc water content ± standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>FE01</td>
<td>Sexual</td>
<td>38.0 ± 13.4</td>
<td>151.8 ± 55.5</td>
</tr>
<tr>
<td>FE02</td>
<td>Vegetative</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.3</td>
</tr>
<tr>
<td>FE03</td>
<td>Sexual</td>
<td>24.2 ± 12.4</td>
<td>95.5 ± 49.9</td>
</tr>
<tr>
<td>FE04</td>
<td>Sexual</td>
<td>28.6 ± 20.3</td>
<td>114.3 ± 77.3</td>
</tr>
<tr>
<td>FE05</td>
<td>Sexual</td>
<td>27.8 ± 10.1</td>
<td>112.5 ± 42.9</td>
</tr>
<tr>
<td>FE06</td>
<td>Sexual</td>
<td>29.1 ± 11.1</td>
<td>120.0 ± 48.6</td>
</tr>
<tr>
<td>FE07</td>
<td>Vegetative</td>
<td>2.6 ± 2.2</td>
<td>10.3 ± 8.9</td>
</tr>
<tr>
<td>FE08</td>
<td>Sexual</td>
<td>16.5 ± 06.3</td>
<td>65.7 ± 23.8</td>
</tr>
<tr>
<td>FE09</td>
<td>Vegetative</td>
<td>0.4 ± 0.3</td>
<td>1.5 ± 1.3</td>
</tr>
<tr>
<td>FE10</td>
<td>Both</td>
<td>0.4 ± 0.3</td>
<td>1.5 ± 1.4</td>
</tr>
<tr>
<td>FE11</td>
<td>Vegetative</td>
<td>1.3 ± 1.8</td>
<td>5.3 ± 7.5</td>
</tr>
<tr>
<td>FE12</td>
<td>Vegetative</td>
<td>11.7 ± 23.7</td>
<td>46.3 ± 92.2</td>
</tr>
<tr>
<td>FE13</td>
<td>Sexual</td>
<td>42.6 ± 15.3</td>
<td>171.8 ± 62.1</td>
</tr>
<tr>
<td>FE15</td>
<td>Vegetative</td>
<td>0.2 ± 0.2</td>
<td>0.7 ± 0.6</td>
</tr>
<tr>
<td>FE16</td>
<td>Vegetative</td>
<td>3.0 ± 6.5</td>
<td>12.3 ± 26.6</td>
</tr>
<tr>
<td>FE17</td>
<td>Vegetative</td>
<td>6.6 ± 16.5</td>
<td>27.6 ± 69.3</td>
</tr>
<tr>
<td>FE18</td>
<td>Vegetative</td>
<td>0.5 ± 0.8</td>
<td>2.1 ± 3.1</td>
</tr>
<tr>
<td>FE19</td>
<td>Sexual</td>
<td>88.8 ± 3.4</td>
<td>353.7 ± 10.3</td>
</tr>
<tr>
<td>FE20</td>
<td>Vegetative</td>
<td>0.1 ± 0.2</td>
<td>0.4 ± 0.7</td>
</tr>
<tr>
<td>FE21</td>
<td>Sexual</td>
<td>31.3 ± 19.6</td>
<td>124.0 ± 75.0</td>
</tr>
<tr>
<td>FE22</td>
<td>Vegetative</td>
<td>0.7 ± 1.1</td>
<td>2.9 ± 4.7</td>
</tr>
<tr>
<td>FE23</td>
<td>Sexual</td>
<td>80.3 ± 21.8</td>
<td>313.3 ± 83.4</td>
</tr>
<tr>
<td>FE24</td>
<td>Sexual</td>
<td>50.6 ± 14.1</td>
<td>199.2 ± 53.2</td>
</tr>
</tbody>
</table>

Figure 37. Results of the hydro-cloth discs moisture measurements illustrated as dot-plot (left) and box-whisker-plot (right). Measurements are grouped by reproductive mode and expressed as percent relative water content. Mann-Whitney-Wilcoxon rank-sum test indicates that the correlation of reproductive mode and percent relative water content is significant (\( p < 2.2 \times 10^{-16} \)).
Figure 38. Results of the hydro-cloth discs moisture measurements illustrated as dot-plot (top) and box-whisker-plot (bottom). Measurements are grouped by site and expressed as percent relative water content, sample size in each group is n=10. The reproductive mode of thalli growing in these sites is colour coded, red is sexual, blue is vegetative.
3.3.3 Relative Humidity measurements

In a few test runs all 12 data-loggers were placed in the same location for extended periods (several days), to see how comparable their measurements were. Although the devices were meant to record relative humidity with a precision of 5%, the simultaneous measurements deviated up to 10% from one another. The inaccuracy would require acceptance of an error margin that renders the whole approach useless. Manual re-calibration of the devices is not possible, and returning them to the manufacturer is almost as expensive as buying new ones.

Furthermore, it appeared that relative humidity is a very bad proxy for thallus hydration in sites which are open and moist at the same time. On a clear, sunny day the openness reduces the relative humidity extremely, but soil-borne moisture in the bog keeps the thalli hydrated, irrespective of the low relative humidity.

The relative humidity data-logger approach was dropped for both reasons outlined above, i.e. the lack of precision and comparability of measurements, and because it is a very bad proxy for thallus hydration in open and moist sites.
3.4 Stress Physiology

3.4.1 Lipid hydroperoxide measurements

Table 23. Results summary of mean lipid-hydroperoxide measurements ± standard deviation in nmol/g fresh weight for all 16 Origin-Treatment categories. Regular sample size is n = 6, except when measurements were below detection limit, in which case faulty lipid extraction was assumed, and these measurements were omitted from analysis and sample size is n = 5, as annotated.

<table>
<thead>
<tr>
<th>Origin site category</th>
<th>Treatment</th>
<th>veg., dry, shade</th>
<th>sex., wet, shade</th>
<th>sex., wet, open</th>
<th>veg., dry, open</th>
</tr>
</thead>
<tbody>
<tr>
<td>wet, shade</td>
<td>3.204 ± 0.484</td>
<td>3.730 ± 0.588</td>
<td>3.594 ± 0.453</td>
<td>3.599 ± 0.592</td>
<td></td>
</tr>
<tr>
<td>dry, shade</td>
<td>3.602 ± 0.357</td>
<td>6.414 ± 0.944</td>
<td>7.188 ± 2.065</td>
<td>3.668 ± 0.479</td>
<td></td>
</tr>
<tr>
<td>wet, open</td>
<td>3.876 ± 0.323</td>
<td>3.669 ± 0.230</td>
<td>3.890 ± 0.339</td>
<td>3.480 ± 0.441</td>
<td></td>
</tr>
<tr>
<td>dry, open</td>
<td>3.889 ± 0.306</td>
<td>5.796 ± 1.041</td>
<td>4.976 ± 0.770</td>
<td>4.407 ± 2.643</td>
<td></td>
</tr>
</tbody>
</table>

Figure 39. Box-Whisker-plot summarizing the lipid hydroperoxide (LHP) measurements of all origin-treatment groups. Origin groups/characteristics are labelled above the graphs, and are separated by vertical lines. Treatments are labelled below the x-axis as two-capital-letter code. Abbreviations: Sex./Veg. - sexual or vegetative specimen; W/D - wet or dry origin/treatment site category; O/S - open or shady origin/treatment site category. Lower case letters above the x-axis show Tukey’s test results within origin groups, different letters within the same origin-group indicate statistically significantly different means of treatment groups, additional lower case letters in brackets are results based on inverse-transformed data.
**Figure 40.** Dot-plot of the lipid hydroperoxide measurements. Data is arranged as in Figure 39.
Table 24. Tukey’s honestly significant difference test results for pairwise comparisons of lipid hydroperoxide values of treatment categories grouped by origin site category. Significance values in brackets in origin site category Sexual, Wet, Open are based on inverse-transformed data.

<table>
<thead>
<tr>
<th>A) Origin site category: Sexual, Wet, Open</th>
<th>Treatment</th>
<th>dry, open</th>
<th>dry, shade</th>
<th>wet, open</th>
<th>wet, shade</th>
</tr>
</thead>
<tbody>
<tr>
<td>dry, open</td>
<td>0.019 (0.024)</td>
<td>0.432 (0.049)</td>
<td>0.202 (0.002)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dry, shade</td>
<td>0.432 (0.049)</td>
<td>0.001 (0.000)</td>
<td>0.000 (0.000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wet, open</td>
<td>0.432 (0.049)</td>
<td>0.001 (0.000)</td>
<td>0.974 (0.622)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wet, shade</td>
<td>0.202 (0.002)</td>
<td>0.000 (0.000)</td>
<td>0.974 (0.622)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B) Origin site category: sexual, wet, shade</th>
<th>Treatment</th>
<th>dry, open</th>
<th>dry, shade</th>
<th>wet, open</th>
<th>wet, shade</th>
</tr>
</thead>
<tbody>
<tr>
<td>dry, open</td>
<td>0.519</td>
<td>0.001</td>
<td>0.000</td>
<td>0.999</td>
<td></td>
</tr>
<tr>
<td>dry, shade</td>
<td>0.001</td>
<td>0.000</td>
<td>0.999</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wet, open</td>
<td>0.001</td>
<td>0.000</td>
<td>0.999</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wet, shade</td>
<td>0.001</td>
<td>0.000</td>
<td>0.999</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C) Origin site category: vegetative, dry, open</th>
<th>Treatment</th>
<th>dry, open</th>
<th>dry, shade</th>
<th>wet, open</th>
<th>wet, shade</th>
</tr>
</thead>
<tbody>
<tr>
<td>dry, open</td>
<td>0.951</td>
<td>0.998</td>
<td>0.995</td>
<td>0.974</td>
<td></td>
</tr>
<tr>
<td>dry, shade</td>
<td>0.992</td>
<td>0.995</td>
<td>0.974</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wet, open</td>
<td>0.992</td>
<td>0.995</td>
<td>0.974</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>D) Origin site category: vegetative, dry, shade</th>
<th>Treatment</th>
<th>dry, open</th>
<th>dry, shade</th>
<th>wet, open</th>
<th>wet, shade</th>
</tr>
</thead>
<tbody>
<tr>
<td>dry, open</td>
<td>0.580</td>
<td>0.999</td>
<td>0.999</td>
<td>0.030*</td>
<td></td>
</tr>
<tr>
<td>dry, shade</td>
<td>0.999</td>
<td>0.617</td>
<td>0.348</td>
<td>0.034*</td>
<td></td>
</tr>
<tr>
<td>wet, open</td>
<td>0.030*</td>
<td>0.348</td>
<td>0.034*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* these two significances are deemed ecologically meaningless due to the negligible range of values

Figure 39 and Figure 40 show, that the sixteen origin-treatment-groups (4 origins times 4 treatments each) fall into two range-categories according to their range of LHP values. Twelve origin-treatment groups, including all vegetative specimens, have more or less uniformly low individual measurements, never exceeding 4.7 nmol/g FW, with group means always below 4 nmol/ g FW. The other four origin-treatment groups have distinctly higher group means and individual measurements never below 4. It is noteworthy that those four groups with the highest values (means and individual measurements) share two characteristics: a sexual reproductive state (combined with wet origin) and a dry treatment.

In the Sexual-Wet-Open origin site-category, analysis of variance indicates a statistically significant difference between Wet-Shade-treatment and both Dry-treatments, but the significant difference to the Dry-Open-treatment applies to inverse-transformed (as well as log-transformed) data only. Also the Wet-Open-treatment has significantly lower values than the Dry-Shade-treatment, but the generally lower values of the Wet-Open-treatment compared to the Dry-Open-treatment do
only represent significant difference when inverse-transformed data are used. A smaller but also statistically significant difference was also found between the Dry-Open and Dry-shade treatments of the Sexual-Wet-Open origin-group, which however, is not consistent within its origin-group or within sexual specimens as a whole. Therefore, this result is dismissed as a potential artefact or as of lesser importance compared to the moisture effect described above.

The eight origin-treatment groups consisting of vegetative specimens show almost uniformly low LHP values. Within the Vegetative-Dry-Open origin-group no difference among the four treatments is detectable.

The Wet-Shade treatment of the Vegetative-Dry-Shade (VDS) origin-group, however, was found to have a statistically significantly lower mean than the corresponding Dry-Open and Wet-Open treatments. This indicates a light-effect, which, however, is neither consistent with the corresponding Dry-Shade treatment nor within vegetative specimens as a whole. Furthermore, the robustness and reliability of this apparent light effect is doubtful, as the significantly lower mean is maintained by the lowest value in the Wet-Shade treatment. When this extreme value is omitted from analysis, no statistical difference among the four treatments remains detectable. Moreover, given the very narrow range of values within the VDS origin-group, the apparent differences among treatments should be considered biologically meaningless, even though they are mathematically correct. Therefore this apparent light-effect is disregarded, based on the three reasons mentioned above, and subsequently the VDS origin-group shall be considered undifferentiated with respect to the four treatments as well.

Overall, the differential response of sexual specimens to the moisture treatment is the only consistent and largely statistically supported treatment-effect observed within the whole data set.
3.4.2 Site conditions

‘Dummy’ thallus moisture measurements from 07.06.2013, the day of harvest of all reciprocal transplant samples, and modelled light transmission values of the four treatment site are presented in Table 25 and Figure 41. The two sites classified as ‘dry’ have distinctly lower mean thallus moisture values than those previously classified as ‘wet’. The sites classified as ‘open’ have distinctly higher light transmission values than those classified as ‘shade’.

Analysis of variance of moisture content of the ‘dummy’ thalli in the four treatment sites (described in section 3.1.1) found, that when grouped according to reproductive mode, the treatment sites classified as wet (FE06 and FE24, which naturally harbour sexual thalli) were significantly (p < 0.0001) wetter than the treatment sites classified as dry (FE15 and FE20, which naturally harbour vegetative thalli). Pairwise comparisons of the sites also confirms that site FE15 is significantly dryer than both FE06 and FE20 (p<0.001), and that site FE20 is significantly dryer than FE24 (p<0.001).

The visually rather clear observation that the ‘wet’ site FE20 is also much dryer than the ‘dry’ site FE06, is only poorly supported by a marginal significance of p = 0.062. However, after arcsin-transformation of the dataset (which then still meets the ANOVA assumptions), the difference between FE20 and FE06 becomes truly significant (p=0.006), while the significance levels of all other pairwise site comparisons did not change.

Table 25. Thallus hydration measurements and modelled light transmission of the four treatment sites on 7th June 2013, the day when all samples of the stress physiology experiment have been harvested. The site category column gives in brackets the reproductive state (sexual or vegetative) of thalli naturally growing there. This table is intended to provide evidence for the correct classification of the treatment sites.

<table>
<thead>
<tr>
<th>site category</th>
<th>mean thallus hydration (n=10)</th>
<th>% light transmission on 7th June</th>
<th>site</th>
<th>direct</th>
<th>diffuse</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>wet, open (sex.)</td>
<td>18.1 ± 5.6</td>
<td>115.1 ± 25.6</td>
<td>FE06</td>
<td>32.9</td>
<td>84.6</td>
<td>58.7</td>
</tr>
<tr>
<td>dry, open (veg.)</td>
<td>9.5 ± 6.3</td>
<td>47.2 ± 20.8</td>
<td>FE15</td>
<td>1.6</td>
<td>72.6</td>
<td>67.1</td>
</tr>
<tr>
<td>dry, shade (veg.)</td>
<td>14.2 ± 5.3</td>
<td>89.5 ± 17.2</td>
<td>FE20</td>
<td>0.0</td>
<td>34.0</td>
<td>33.0</td>
</tr>
<tr>
<td>wet, shade (sex.)</td>
<td>28.0 ± 8.8</td>
<td>133.9 ± 23.9</td>
<td>FE24</td>
<td>0.0</td>
<td>39.3</td>
<td>27.2</td>
</tr>
</tbody>
</table>
Figure 41. Dot-plot (left) and box-whisker-plot (right) of ‘dummy’ thallus water measurements in all four treatment sites on 7th June 2013, at the time when all samples of the stress physiology reciprocal transplant experiment were harvested from those sites. The site moisture classification (and reproductive mode of the sites) is colour coded, red for wet (and sexual) and blue for dry (and vegetative). Significantly different groups, according to Tukey’s Test, are indicated by different lower case letters above the x-axis in the box-plot (lower case letters in brackets are results for arcsin-transformed data, where the previously marginal significance of p=0.062 for the comparison of FE06 and FE20 becomes p=0.006). A box-plot of arcsin-transformed data is shown below for comparison.

Table 26. Additional moisture measurements of other experiments and annual light model of the four treatment sites, intended as further evidence for the correct classification of the treatment sites.

<table>
<thead>
<tr>
<th>site category</th>
<th>1st thallus hydration experiment (n=5)</th>
<th>hydro-cloth experiment (n=10)</th>
<th>% annual light transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m(water) [mg]</td>
<td>relative % water</td>
<td>m(water) [mg]</td>
</tr>
<tr>
<td>wet, open (sex.)</td>
<td>7.7 ± 7.0</td>
<td>47.5 ± 14.2</td>
<td>29.1 ± 11.1</td>
</tr>
<tr>
<td>dry, open (veg.)</td>
<td>13.8 ± 13.3</td>
<td>52.5 ± 05.0</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>dry, shade (veg.)</td>
<td>15.8 ± 9.2</td>
<td>93.8 ± 10.4</td>
<td>0.1 ± 0.2</td>
</tr>
<tr>
<td>wet, shade (sex.)</td>
<td>24.7 ± 18.6</td>
<td>102.8 ± 14.1</td>
<td>50.6 ± 14.1</td>
</tr>
</tbody>
</table>
4. Discussion

4.1 Micro-habitat

The reciprocal transplant experiment in the Blue Mountains indicates a correlation between the reproductive mode of *I. splachnirima* and the micro-habitat conditions. Apothecial initials of thalli in vegetative sites are dormant or mature at a very slow rate, while they mature quickly when a thallus is transplanted into a sexual site. In several replicates this maturation of apothecial initials was accompanied by a reduction or loss of soralia. Conversely, formation of sorediate sections on sexual thalli was only observed in two transplants placed in vegetative sites.

In one replicate (no. 01), the untouched control in the vegetative site also showed a high level of apothecial maturation, and a little maturation was also observed in the moved control. This does not conform with the hypothesis, but neither does it contradict it; this would be the case, if the corresponding transplant in the sexual site had lacked apothecial development, but in fact its level of development was similar. Another contradictory outcome would have been reduction of soralia in the controls of the vegetative site and formation of soralia in the controls of the sexual site, but neither were observed.

In several cases untouched controls of vegetative sites showed some apothecial development which may be caused by better water penetration of the substrate through holes created by the adjacent transplant and moved control.

The outcome of the reciprocal transplant experiment in the Blue Mountains confirms the results of a comparable pilot study conducted on Swampy Summit, reported by Ludwig (2012). This initial experiment differed in that only 10 replicates were set up, no moved control was used, and the sites were reassessed 22 month after the reciprocal transplant (rather than after 12 month as in the Blue Mountains experiment). A notable difference in the outcome of the two studies was, that in the pilot study a higher proportion of replicates (5 of 10) showed development of soralia on previously esorediate and fertile transplants placed in vegetative sites. As formation of fairly small soralia on sexual transplants was observed in only 2 of 20 replicates in the Blue Mountains experiment after 12 months, this might indicate that a switch from sexual to vegetative reproduction requires a longer exposure to the triggering cues (presumably adverse conditions), than the activation of apothecial maturation in vegetative specimens (presumably by more favourable conditions).

Sexual transplants placed in vegetative sites do still continue apothecial growth, even though development of apothecial initials of ‘native’ vegetative thalli is inhibited. Therefore it can be assumed that the environmental cues which suppresses initial apothecial development of vegetative thalli cannot affect apothecial development in later stages. It seems that the regulatory system involved is more complex than a simple on/off switch responding to a threshold of certain environmental factors.
The enormous abundance of maturing apothecia in almost all replicates indicates a locally balanced mating type ratio in the Blue Mts. population, similar to the population near Dunedin, for which a balanced MAT ratio has been genetically proven (Chapter 4).

It is difficult to find published studies for comparison with the presented results; the present study appears to be unique in the attempt to transplant thalli in order to manipulate their reproduction directly. A recent review by Smith (2014) on lichen translocation techniques shows that these have almost always been used in order to vegetatively propagate rare lichens, or alternatively to conduct physiological measurements. Should future climate change severely reduce or impair viability of populations of *I. splachnirima*, the successful transplantation procedure employed in the present study could be used by conservationists to propagate it.

The transplantation studies by Gauslaa *et al.* (2006) and Gauslaa (2006) have nicely shown how biomass production of *Lobaria pulmonaria* responds to several light and moisture combinations, but they did not examine the effect of environmental variables on soredia production, although they had semi-quantitative records of the amounts of soredia. A drawback of their study is that they had only three different sites with 9 treatments, but a good number of replicates per site and treatment. Therefore it would be interesting if their dataset would be reanalysed with focus on a potential correlation of microclimate and production of soredia. Unfortunately, the study of the above authors is also not comparable to the present study with regard to a comparison of sexual versus vegetative reproduction, because *Lobaria pulmonaria* is rarely fertile (only 3% of thalli in the dataset bore apothecia), although both species are heterothallic (Singh *et al.*. 2012; Chapter 4). However, ‘sexed’ transplants of compatible mating partners could be conducted to promote and examine sexuality in *L. pulmonaria* (or other heterothallic species), though this would likely be a long-term experiment spanning several years.

Ott *et al.* (2004) write that ascocarps of *Peltigera aptosa* and *Nephroma arcticum* develop only under favourable conditions, but this seems to be based on field observations, as they do not provide data or references to support this statement.

### 4.2 Light environment

While no significant differences were found in overall light regimes between sites occupied by vegetative thalli and sites occupied by sexual thalli, the means shown in Fig 4 trend in the predicted direction, namely that sites with vegetative thalli have a higher mean % direct and indirect light. However the wide and essentially identical range of light environments associated with both reproductive modes strongly suggests that other environmental factors rather than just light may be responsible for the observed differences.
Observations on numerous thalli in 2008/09, led to the initial hypothesis, that sexual thalli inhabit more sheltered and vegetative thalli more exposed sites (Ludwig 2011). Later, this hypothesis had to be modified (Ludwig 2012), when a few sites were found, where sexual thalli grew fully exposed, and also a few very shady sites with vegetative thalli. Notably, the rare sexual thalli found in open sites grew also in exceptionally wet situations (e.g. immediately above the water table of open bogs or on seepages), where they did not desiccate even on sunny summer days, and the rare vegetative thalli grew not only in shady sites, but also in unusually dry situations.

Therefore, while it initially appeared from casual observations (between 2008 and 2010) that sexual thalli grow predominantly in shady sites and vegetative thalli occur mostly in exposed sites, it is important to point out that they are not confined to these contrasting light conditions. Instead, hundreds of field observations since 2010 suggest that an apparent correlation of light environment and reproductive mode may be an over-simplification, possibly based on the indirect effect of high light exposure on the water regime (desiccation), which in turn may affect the reproductive mode directly. Only a few exposed sites are wet enough to compensate for the permanent evaporation in direct sunlight, and in these sites I. splachnirima does reproduce sexually in spite of high light conditions.

On the other hand, only special micro-topographical and edaphic conditions create an effective barrier for soil-water-flow in the usually boggy habitat, which allows frequent desiccation of shady sites. Under these special conditions, I. splachnirima reproduces only vegetatively, although high light stress can be ruled out. This made it clear, that it was necessary to document the range of light environments in which thalli of either reproductive mode do occur, rather than for instance counting the number of thalli of either reproductive mode in a given level of light exposure; the latter approach would certainly create a biased result, suggesting a strong light dependency of the reproductive mode.

The major strength of the technique used here is, that hemispherical fish-eye photos allow modelling of the annual light incidence on a given spot based on a single photograph, involving only one day of field work instead of continuous or repeated light measurements over extended periods of time. However, the method has several drawbacks. Because of the height of the camera plus mounted fish-eye lens and adapter, the photo actually represents the light environment of a spot ca. 20 cm above the thallus on the ground, even if the camera has been placed immediately on top of a thallus. Furthermore, the fieldwork is extremely weather dependant, as it must not be rainy or windy and the sky should be either uniformly overcast or totally clear. Meeting all these criteria in an open, subalpine environment is difficult. Transport of the bulky camera, lens and tripod to and among the field sites is challenging and setting the equipment up to take a picture takes at least 15 minutes (usually rather 30 minutes). These preparations include stable mounting and precise orientation of the fish-eye camera, marking of the North reference, recording of GPS coordinates, photographic site
documentation while the fish-eye camera is in place, and often waiting for the wind to ease. Manual editing of the pictures is unavoidable and take usually at least one hour, sometimes up to three hours. These drawbacks are the reasons why not more fish-eye photos were taken and analysed for this study.

4.3 Moisture regime

Finding an appropriate and feasible way to quantify the substrate or thallus moisture was a challenging task. Commercial soil moisture probes, for example, measure the below ground water content, which hardly varies in bogs in depth of more than 2 cm, and do not pick up surface desiccation, which is what affects the ground-dwelling thalli of *I. splachnirima*. Schuster *et al.* (2002) have develop a very sophisticated method to measure thallus hydration in the field with self-made miniature clip electrodes connected to data-loggers, allowing extended periods of parallel measuring on several thalli. This method, however, was not feasible for several reasons. The skills for manufacturing such sensor-clamps were lacking (in spite of detailed instructions by Schuster *et al.* 2002), the distances between thalli to be compared were too large, and for the use in bogs the whole setup would have to be completely water-proof.

Moisture measurements using the ‘dummy’ thalli are certainly the ecologically most meaningful method that was available, but it turned out to have several drawbacks. Sourcing of the thalli is destructive and limited by natural supply, it is virtually impossible to use a large number of thalli with uniform size, shape and weight, which limits the comparability and reliability of the measurements. That means they are too different to be truly comparable. Furthermore, removal of adhering substrate from the lower thallus surface is an extremely time consuming and laborious process. After the second experimental setup of the ‘dummy’, clear signs of decay were visible on several thalli, probably due to the stress and physical damage associated with the cleaning process and dry storage. This means that tediously cleaned thalli can only be used a few times before they become useless.

The hydro-cloth disc approach overcomes several disadvantages of the ‘dummy’ thalli. Cloth discs are extremely robust and durable, of uniform shape, thickness and more or less similar weight, easier and quicker to manufacture and handle, and their availability is virtually unlimited. They are very absorbent and of similar thickness to thalli. However, they weigh much less (relative to surface area) than thalli, and seem to have a comparatively short moisture retention time, which should be taken into account for interpretation of the results. The maximum water holding capacity of cloth-discs is about 350% of the disc’s dry weight, while fully saturated thalli of *I. splachnirima* can hold only about 140% of their dry weight.
Trends in two of the three experiments were in the predicted direction and the difference was significant in the third experiment, which showed that moisture rather than exposure or light per se is a significant environmental determinants of reproductive status.

The results of the first round of ‘dummy’ thallus measurements indicate no correlation of moisture regime and reproductive mode. However, this was essentially a test run to see whether ‘dummy’ thalli can be used for that purpose at all, and the harvest day (20.03.2013), just after a period of a few rainy days, was probably poorly chosen. It was hoped that the vegetative sites had experienced stronger desiccation since the last rain than the sexual sites, but apparently ‘dummy’ thalli were harvested too early for that.

The second round of ‘dummy’ thallus measurements followed a rather dry period, and revealed a significant difference between reproductive modes; however, its sample size was very small, because it was only meant to provide moisture measurements of the treatments sites of the stress physiology experiment.

The hydro-cloth disc measurements, which is considered the most reliable approach, revealed a significant correlation between reproductive mode and moisture content. The only site representing a thallus with apothecia and soralia (FE10) was totally desiccated. Presumably that this site was more moist in the past than it is now, because the thalli sit on very loose soil and rotting plant matter with large pores. Before erosion and decomposition took place, it must have been more dense, which would have given it a better water holding capacity and better capillary water flow.

A correlation of moisture regime and fertility was found for other lichens by Jahns et al. (1978), Ott (1989) and Jahns et al. (2004). In the tandem-studies of Gauslaa et al. (2006) and Gauslaa (2006) on micro-climatic conditions and vegetative growth of Lobaria pulmonaria, the authors assume ‘a delicate balance between light availability and desiccation risk’ and point out a ‘physiological trade-off between growth potential and fatal desiccation damage, both of which increase with increasing light’, which might be what affects the reproduction of L. splachnirima as well. Regrettably, Gauslaa and colleagues have not examined the effect of the microclimate on vegetative reproduction, which would have been interesting but possibly not very meaningful, as they had only three different sites, though with numerous replicate thalli per site. However, their data-set contains semi-quantitative data on the size of soralia, which could still be analysed later with focus on micro-climate. Also their study species L. pulmonaria is unfortunately very rarely fertile, in contrast to L. splachnirima, although both species cannot self-fertilize (Singh et al. 2012; see Chapter 4).

4.4 Stress Physiology

The experimental results indicate that the change from sexual to vegetative reproduction seems likely to be a stress response. A differential response of sexual specimens to the moisture treatment was the only consistent pattern found, with increased levels of the stress-marker in dry treatments only.
Even though statistical support depends in part on data-transformation, this correlation is far from random and also becomes clear by visual inspection of the data. This result is well in accord with the hypothesis that sexual development coincides with less stressful habitats (stress being desiccation) as it allows resource allocation to fruiting body formation instead of stress resistance mechanisms.

The uniformly low lipid hydroperoxide (LHP) values and means of virtually all vegetative specimens are in striking contrast to the clearly moisture-dependant pattern observed among sexual specimens, among which only the wet-treatments exhibit similarly low values. While the low values for oxidative damage in wet-treated sexual specimens must be interpreted as indicative of a low-stress environment, the same conclusion would be misleading and even contradictory in the case of the vegetative specimens.

Rather than being suggestive of low-stress growth conditions experienced by all vegetative specimens (regardless of the treatment), their uniformly low LHP values are better interpreted as indication for increased hardiness or stress tolerance, owing to adaptation to their more adverse dry natural habitats. Regrettably, there was not enough tissue material available to run assays testing for such an adaptation. The nature of this putative adaptation remains speculative in the absence of further experimental work, but it is likely to involve some sort of radical scavenging and/or repair mechanisms for overcoming oxidative damage.

The data suggest that vegetative specimens cope better with abiotic environmental stress (in particular desiccation) than sexual specimens, and this allows for the conclusion that their improved physiological stress tolerance may be acquired at the expense of sexual reproduction, owing to a trade-off in resource allocation. The results of the moisture measurements and light modelling in the treatment sites allow one to conclude that observed differences in LHP levels are indeed a response to different moisture conditions rather than light incidence. Overall, this experiment provides objective evidence for the hypothesised causal relationship of microclimatic conditions and the switch between the two reproductive modes.

It remains unclear whether the mycobiont or the photobiont or both are directly affected by the stress factor. Weissman et al. (2005) have observed that desiccation and rehydration related nitric oxide production occurred only in the mycobiont but not in the green-algal partner. On the other hand, it is also possible that only the photobiont of I. splachnirima experiences direct disruptive stress, and due to its nutritional dependence, the mycobiont is indirectly affected by limitation stress (malnutrition).

4.5 Conclusions

The reciprocal transplants in the Blue Mountains show that a correlation of reproductive mode and micro-habitat conditions exists. The light levels can be ruled out as direct effector of the reproductive mode, based on the similar range of different light environments observed for either reproductive
mode. The moisture regime is likely to be the driving force behind the bimodal reproduction of *I. splachnirima*, but simultaneous quantification of the thallus hydration status using a decent sample-size *in situ* still represents a major challenge, due to the great deal of time required when collecting samples. However, the results of thallus moisture measurements together with the stress physiology experiment provide strong support, though no conclusive proof, for the hypothesis that, oxidative stress associated with desiccation impairs the species ability to develop sexual fruiting bodies. A trade-off regarding resource allocation towards either sexual reproduction or physiological mechanisms to cope with oxidative stress is a plausible explanation for the usually mutually exclusive occurrence of the reproductive modes. A switch from sexual to metabolically cheaper vegetative propagation would also be a logical consequence when resource allocation towards stress adaptation has priority owing to adverse ambient conditions. A trade-off was also reported between vegetative reproduction and growth in *Lobaria pulmonaria* by Gauslaa (2006). This is not quite comparable with the present study, but it shows that reproduction does compete for resources with other physiological functions.

Jahns & Ott (1997) generalise that lichen ‘species adapted to constantly humid conditions’ do not ‘show a high tolerance against unfavourable conditions’, a statement that is in accord with the results of the present study. Findings of Bidussi et al. (2013) support the assumption that increased desiccation could prevent the maturation of apothecial initials in *I. splachnirima*. Their study showed that extending the hydration and active metabolism from light periods into nights substantially enhances lichen growth. This would surely also apply to growth of fruiting bodies of better hydrated thalli. Gauslaa & Solhaug’s (2004) observation that photoinhibition depends on the hydration status of a lichen is also in accord with the conclusion that sufficient moisture prevents cellular stress and thus allows for investment in sexual reproduction.

It could be argued that sexual reproduction should be favoured under stressful conditions, as sexual recombination increases fitness. Regarding this it is important to point out that vegetatively reproducing specimens of *I. splachnirima*, which often experience drought stress, are virtually always covered with apothecial initials. This means that fertilisation has already occurred and only the maturation of sexual propagules is delayed until it becomes metabolically affordable. Meanwhile, vegetative reproduction via soredia still allows to ‘escape’ from the current, unfavourable site. In this respect reproduction via soredia is no disadvantage compared to ascospores, although the latter are smaller and get wider dispersed. This is because the distance between unfavourable and favourable site conditions within a suitable habitat is mostly in the range of less than 1 m to a few hundred meters, i.e. well in the short-distance dispersal range of soredia.

A correlation of reproductive mode and thallus size (as a proxy for age or maturity) does apparently not exist in *I. splachnirima*, as the author observed a lot of sexual and vegetative thalli in all sizes from about 1 cm to 30 cm diameter. In other lichens thallus size/age was reported to correlate with reproductive modes. For example *L. pulmonaria* reproduces vegetatively for several years (up to
decades) before apothecia are formed in old, larger thalli (Martinez et al. 2012, MacDonald & Coxson 2013), if thalli become fertile at all. However, findings of MacDonald et al. (2013) show as well that very small, i.e. young, thalli of less than 1.4 cm² surface area are already able to produce apothecia, even though it is not common. For Peltigera didactyla it is known that juvenile (smaller) thalli reproduce vegetatively, but later the soralia disappear usually and older (larger) thalli reproduce sexually (Jahns 1973, Goffinet et al. 2003). However, according to Ott & Jahns (1997) this transition to sexual reproduction in P. didactyla is also dependant on (not further specified) suitable microclimatic conditions, similar to I. splachnirima and possibly also L. pulmonaria (Martinez et al. 2012). Also a deficiency of suitable mating partners of heterothallic (self-incompatible) species like L. pulmonaria (Singh et al. 2012) could create the false impression that sexual reproduction occurs only in old thalli.

Seasonal variability of the light and moisture regimes should also be considered, as these factors determine the suitability of the micro-habitat. In general, seasonality appears to be of little relevance, since apothecia take more than one year to mature and last for several years, i.e. they grow and persist regardless of the season. However, it is unclear whether spore production in mature apothecia occurs throughout the year or in seasonal intervals. Suboptimal effective photoperiods in both summer and winter conditions could potentially impede investment in apothecial growth and spore production. Although days are long in summer, the effective photoperiod may be only a few hours before thalli desiccate, unless in really damp sites. In winter, thalli may be sufficiently hydrated but the short daily photoperiod could be not sufficient to gain a carbon surplus for sexual reproduction. This would mean that apothecial growth is generally inhibited during winter time in both sexual and vegetative thalli, and in summer only those thalli can benefit from additional daylight hours and develop apothecia, which remain hydrated because of a suitable, damp micro-habitat. This hypothesised seasonal growth of apothecia requires further examination, e.g. by well replicated photo-documentation in monthly intervals over a period of a whole year or longer.

5 Summary

The hypothesis was tested that a switch from sexual to vegetative reproduction occurs in Icmadophila splachnirima as a response to micro-environmental conditions; it found support by four field-experimental approaches. Reciprocal transplants and measurements of lipid hydroperoxide levels as a stress marker support the idea that micro-site conditions are more stressful for sorediate and sterile thalli than for fertile and esorediate thalli. Quantification of light and moisture regimes associated with thalli of both reproductive modes indicates that moisture rather than light conditions affect the reproductive mode; increased desiccation likely causes an inhibition of sexual maturation and triggers the formation of soralia.
CHAPTER 6

Notes on the occurrence and distribution of *Icmadophila ericetorum* in New Zealand and the Southern Hemisphere

1. Introduction

*Icmadophila ericetorum* (L.) Zahlbr. is a distinctive crustose lichen with striking pink apothecia, which is widely distributed in the Northern Hemisphere, e.g. in the tundra regions and heathlands of Eurasia and North America and upland moorlands (see references in results section), where it grows on damp peaty, rotting wood and bryophytes. It has a thick granular-warty crustose thallus, which is differentiated into a thick, white medulla and an upper cortex of pale greenish-grey colour; ascospores are (1-)3-septate, ellipsoid-fusiform and 13-27 × 4-6 μm in size (Wirth 1995a+b, Hitch et al. 2009).

Because this species has been reported from New Zealand (NZ) (Galloway 1985, 2007, James & Brightman 1992) it was hoped to include a NZ specimen of *I. ericetorum* (L.) Zahlbr. in a molecular phylogenetic revision of the genus *Icmadophila* Trevis. (Chapter 2). However, it soon turned out that several herbarium specimens labelled as *I. ericetorum* were misidentified collections of *Dibaeis absoluta* (Tuck.) Kalb & Gierl. This raised doubts about the presence of this species in New Zealand. Therefore a detailed literature search and re-examination of herbarium specimens was conducted. Both *I. ericetorum* and *D. absoluta* have similar sessile to shortly stalked pink discoid apothecia, but they can be easily distinguished macroscopically by their thallus colour and structure, and microscopically by spore septation and size (see e.g. Thomson 1997, Gierl & Kalb 1993).

In contrast, *Dibaeis absoluta* has a smooth, very thin, varnish-like thallus of dark green colour (when fresh), which is not differentiated into medulla and cortex. Within a few years the colour of herbarium specimens fades to a beige-ochre, making its thallus almost invisible when grown over substrates of similar colour like clay soils, while the thallus colour of *I. ericetorum* does not fade even in very old herbarium specimens (author’s personal observation). Sometimes the thallus of *D. absoluta* can appear more granular or structured at first glance, but that is merely the structure of the underlying substrate. The ascospores are simple, and ca. (7-)12-16 × 4-5 μm in size (Tuckerman 1859, Gierl & Kalb 1993, Galloway 2007).

The aim of this study is to clarify the presence and distribution of this species for NZ and also for the Southern Hemisphere.
2. Material and Methods

The following specimens collected in New Zealand and labelled as *I. ericetorum* (L.) Zahlbr. were re-examined, but all represent *Dibaeis absoluta* (Tuck.) Kalb & Gierl:


**AK 205088**, New Zealand, Sounds-Wellington Ecological Region, Sounds Ecological District, Queen Charlotte Sound, Mount Stokes, track from Kenepuru Saddle to summit, Alt. 620 m, 41 05 S 174 08 E, Coll. A E Wright 11864, 05 January 1992, on clay at trackside through mixed broadleaf forest.

**AK 204903**, New Zealand, Sound-Wellington Ecological Region, Sounds Ecological District, Queen Charlotte Sound, track from Resolution Bay to Ship Cove, Alt. 130 m, 41 06 S 174 14 E, Coll. A E Wright 11718, 01 January 1992, on clay bank at trackside through open red beech forest.

**AK 205320**, New Zealand, Sound-Wellington Ecological Region, Sounds Ecological District, track between Resolution Bay and Ship Cove, near saddle, Alt. 200-240 m, 41 06 S 174 14 E, Coll. B W Hayward, 01 January 1992, on soil.


**UPS:BOT:L-018626**, Leg. Leif Tibell, 10/01/1990, Field number: 19001, locality: Tararua State Forest, 1.4 km WNW of Mt. Holdsworth Lodge, along Mt. Holdsworth Track. In Nothofagus fusca dominated forest. On the track. Wellington (province), New Zealand, 40.9151°S, 175.4627°E.

**UPS:BOT:L-019907**, Leg. Mats Wedin, 10/01/1990, Field number: 2446, locality: Tararua State Forest, along Mt Holdsworth Track at Rocky Lookout. Wellington (province), New Zealand, 40.8984°S, 175.4461°E.

The following specimens labelled as *Dibaeis absoluta* (Tuck.) Kalb & Gierl were examined, and the author agrees with this identification (or material collected and identified by the author):


**OTA 052919** Secretary Id., 1000 ft, Murray Collection, 2. 1959.


**CHR 464741**, N.Z., Nelson, Mt Arthur area; Flora to Salisbury Lodge Track c. 3 km WNW of Flora Hut, 41°10.6’S 172°43.1’E, 830m, coll. A.J. Fife 8501 & E. Brown, 3 Feb 1988, Nothofagus menziesii forest on shale bedrock; shale fragments and adjacent soil on track side bank; partial shade.


**CHR 533494**, New Zealand, Otago Land District, Blue Pools Track, State Highway 6, NZMS 260: G38 122649, 340m, coll. D.J. Galloway 5119, 18 October 2001, on damp rocks at side of track in moderate shade.


**CHR 373618**, Chelsea [no further details on this packet]

**CHR 373619**, Smith’s Creek [difficult to read], [coll. could be HHA for H.H. Allan, illegible!], 27/11/43.

**CHR 373620**, Whangarei North., coll. W.A. Given [difficult to read], 12-2-69 [is det. date!].

**CHR 373708**, Wainui-o-mata Hill, [coll. could be H.H. Allan, illegible!], 29/8/35 [difficult to read!], clay bank in beech-forest.

**CHR 587928**, Parua Bay, 2A469 [no further details on this packet].

**CHR 373709**, Tarama Range, c. 1000m, coll. E.M. Heiner, on damp rocks. [all difficult to read]

**CHR 373711**, George Park (Keith George Memorial Park), Haywards, coll. unknown, 4/3/[19]45, clay bank in Nothofagus forest.

**OTA 064306** (duplic. CHR), *Dibaeis absoluta*, Mt. Pirongia, near Hamilton, NZ, 37°59’46.2”S 175°04’53.1”E, ~840 m a.s.l., coll. Lars Ludwig, 27.11.2014, on rock along track between Pahautea Hut and The Cone.

**OTA 064308** (duplic. CHR), *Dibaeis absoluta*, Mt. Pirongia, near Hamilton, NZ, 37°59’36”S 175°05’42”E, ~900 m a.s.l., coll. Lars Ludwig, 27.11.2014, on rock and bryophytes over rock, between Mt. Pirongia peak and Pahautea Hut (further patches at 37°59’32”S 175°06’06”E and further to Wharauroa lookout).
The following specimens were collected outside New Zealand and labelled as *Icmadophila ericetorum* (L.) Zahlbr. After re-examination the author agrees with this identification:


**OTA 053149** (Dupl. of CHR 372586), Scotland: Perthshire-Beinn Deangh, 500 m, leg. D.J. Galloway, 3 xiii 1976, common in peat on Western flanks.

**OTA 063991**, *Icmadophila ericetorum*, Kampenwand bei Grassau, Bayern, Deutschland, 47°45'25”N 12°22'01”E, ca. 1550 m, leg. Lars Ludwig 28.08.2009, verrottender Kiefernstubben, direkt an Wanderweg vor Beginn der Wand, Nord exponiert.

**OTA 061856**, Breitlahnalm, ca. 0.2 km SE of Breitlahn hut, along road to Schwarzensee, Schladminger Tauern, Steiermark, Austria, 47°18'56”N 13°53’16”E, 1075 m, leg. P. Bilovitz & H. Mayrhofer 19222, 11.10.2012, rotting tree stump (willow) on road side.

According to its herbarium label the following specimen of *Icmadophila ericetorum* was collected in New Zealand, and the author agrees with this identification:

3 Results

3.1 New Zealand

The first literature record of *I. ericetorum* for New Zealand is from Galloway (1985: 202):


This collection has been located in the herbarium of the National History Museum, London (BM001085330), and critical re-examination confirmed its identification as *Icmadophila ericetorum* (see Figure 43 and Figure 44). However, during a visit to Mt. Pirongia in November 2014, the author found only the superficially similar *Dibaeis absoluta* (OTA 064306, OTA 064308) in five sites along the tramping tracks. Although the substrate and habitat conditions there are suitable for this species (lots of damp rotting wood and bryophytes), *Icmadophila ericetorum* has not been found on Mt. Pirongia by the author.

Galloway (2007: 650) lists numerous additional NZ localities of *I. ericetorum*:

“N[North Island]: Northland (Mt Tutamoe – where it is common and well developed on clay tracks in forest), South Auckland (Pirongia). S[South Island]: Nelson (Pelorus Bridge), Marlborough (Mt Stokes, Resolution Bay), Otago (Mt Cargill, Graham’s Bush, Tuapeka West, Black Gully, Blue Mts), Southland (Hump Ra., Lake Hauroko). On forest soil, clay banks, rotting tree stumps, and peat in upland, subalpine areas. Probably more widespread than records show. Widespread in the Northern Hemisphere (Rambold et al. 1993: 224; Obermayer 2004), but known in the Southern Hemisphere only from South Africa and New Zealand.”

No herbarium specimens have been cited directly in Galloway (2007), but specimens from these additional localities (mostly) labelled as *I. ericetorum* were found in AK, CHR and OTA (see list of examined specimens above). However, all of these specimens were misidentified and represent *Dibaeis absoluta* (Tuck) Kalb & Gierl. No herbarium specimen was found for the locality Tuapeka West, but the author collected *D. absoluta* there (on the property of Dr Allison Knight) as well as in nearby Black Gully in the Blue Mountains. Only a specimen from the locality Mt. Tutamoe in Northland is currently missing, but the stated substrate and habitat (“on clay tracks in forest”) is much more typical for *D. absoluta* than for *I. ericetorum*, which commonly grows on organic substrates like damp peaty soil or rotting wood.

Another three specimens from New Zealand, identified as *I. ericetorum*, were found via the GBIF website ([http://data.gbif.org/occurrences/](http://data.gbif.org/occurrences/)) held in UPS (UPS:BOT:L-027393, UPS:BOT:L-018626,
UPS:BOT:L-019907). Upon request, these three specimens were also re-examined by Anders Nordin and likewise identified as *D. absoluta* (pers. comm. A. Nordin, 28.04.2014).

A colour image labelled *I. ericetorum* in Malcolm & Galloway (1997) p. 99 most certainly shows *D. absoluta*, judging by thallus colour and structure. This is also supported by comparison with the colour illustration of *D. absoluta* in Knight (2014), of which the author had the opportunity to examine the voucher specimen (OTA 063774). However, a colour image showing a moist *I. ericetorum* in Wirth (1995b: 433), is indeed highly reminiscent of *D. absoluta*.

Further indication that *I. ericetorum* from NZ has been misidentified is that clay banks are given as substrate in Galloway (2007). Clay banks in native forests are by far the most common places in NZ where the author has collected or seen *D. absoluta*, while *I. ericetorum* is confined to organic substrates like peaty soil, rotting wood and bryophytes according to virtually all Northern Hemisphere treatments or lichen floras, e.g. Ozenda & Clauzade (1970) from France, Wirth (1995a) and Wirth (1995b) from Germany, Thomson (1997) from Canada, James & Brightman (1992) and Hitch *et al.* (2009) from Great Britain, Brodo *et al.* (2001) from USA, Santesson (1993) and Santesson *et al.* (2004) from Scandinavia, and the author’s personal experience in Germany.
Figure 42. *Dibaeis absoluta* from New Zealand, fresh collection but not hydrated (scale bar is 1 mm). Note that the apparently rugged thallus structure does in fact show the exact shape of the substrate underneath (loamy clay soil).
Figure 43. Overview of *Icmadophila ericetorum* collection BM001085330, which is labelled as being collected from Mt. Pirongia, New Zealand. The author confirms this identification but considers its official provenance doubtful.
Figure 44. Detail of *Icmadophila ericetorum* collection BM001085330, which is labelled as being collected from Mt. Pirongia, New Zealand. The author confirms this identification but considers its official provenance doubtful. Note that the granular-warty thallus structure is not caused by the substrate. White medullary tissue is visible along the edges of the fragments (also see previous figure), which does not occur in the undifferentiated thallus of *Dibaeis absoluta*.

3.2 South Africa

Galloway (2007) also writes that *I. ericetorum* is known from South Africa. Although a source for this statement is not cited, it is likely to be Rambold et al. (1993) who writes on p. 224 about its distribution “(...) and South Africa (Drège 1843, Doidge 1950) and New Zealand (Galloway 1985, 1992).”. Doidge (1950) only briefly cited Drège (1843) as follows:

“*Icmadophila ericetorum* Zahlbr.


*Lecidea Icmadophila* Ach., Drège (1843) 80, 85, 88.

on soil and old wood, du Toits Kloof, Drège; Paarlberg, Drège; in krantzes, Table Mt., Drège.”

Drège (1843) merely mentions the name *Lecidea icmadophila* (an old synonym of *I. ericetorum*) among long species lists from certain South African localities, written in German. The first list titled “9. Dutoitskloof, 1000-2000 Fuss, October bis in Januar” starts on p. 78 and contains *Lecidea icmadophila* on p. 80. The second list titled “24. Paarlberg, 1000-2000 Fuss, März, April.” starts on p. 84 with *L. icmadophila* on p. 85. And the third list mentioning *L. icmadophila* on p. 88 is titled “30. Tafelberg, in den Kränzen, 2000-3000 Fuss, zu verschiedenen Zeiten gesammelt.”. The title of Drège’s publication implies that he did collect specimens, but it remains unclear what happened to these collections, i.e.
it was not possible to locate and re-examine them. Notably, the substrate specification “on soil and old wood” given in Doidge (1950) was not provided by Drège (1843).

The three localities for which Drège reported *Lecidea icmadophila*, all in the Cape Town region, could be located thanks to the assistance of Mr Glenn Moncrieff (Cape Town). Coordinates of the general areas were inferred using Google Earth: (1) Tafelberg (German for Table Mount) in Cape Town, 33°58'S 18°25'E; (2) Paarlberg (German for Paarl Mountain) just West of the town Paarl, 33°43'S 18°56'E; (3) Dutoitskloof (Afrikaans for Du Toit’s Valley), a valley few km East of the town Paarl, 33°44'S 19°09'E.

### 3.3 South America

Pereira *et al.* (2006) have reported *I. ericetorum* from Chile, which seems to be the first and only record of this species from South America. All their lichen samples were deposited at the herbarium of Universidad de Talca, Talca, Chile. Iris Pereira has been contacted via e-mail and asked to re-examine the Chilean *I. ericetorum* specimen (collection number L468), considering the possibility of a mix-up with *Dibaeis absoluta*, which is already known from South America (Marcelli 1998). She provided habit images of the specimen (Figure 45), which grew on rotting wood and rock in the precordillera Andes at 500-600 m altitude, and she assured the author that the spores match the description of *I. ericetorum*. Based on this and the habit photographs, the author agrees with this identification, although its growth directly on rock is rather unusual. Regrettably, it was not possible to obtain the specimen as loan for closer examination, because due to recent earthquake damage the Talca University herbarium material has been packed to be transferred into a new herbarium (pers. comm. I. Pereira May 2014).

Galloway & Quilhot (1998) also list *I. ericetorum* in their Chilean lichen checklist. However, their reason for inclusion of the species remains unclear; their only reference (Rambold *et al.* 1993) does not mention Chile or South America in the context of *I. ericetorum*.

Also Litterski (1992) notes that *I. ericetorum* is known from South America (she lists it as “SAM”) and cites 19 reference used for her compilation of its distribution in Europe. It is unclear to which source she refers for the South American record, because several East European references cited by her were inaccessible to the author and she did not respond to an attempt to contact her via e-mail.
Figure 45. *I. ericetorum* from Chile. This specimen is unusual because it grows on rock, except for some parts grown over bryophytes at the top of the picture. Photo and identification by Dr Iris Pereira, Talca, Chile.

4. Discussion

Because almost all specimens from NZ previously identified as *I. ericetorum* have turned out to be *Dibaeis absoluta*, with the only exception being J.K. Bartlett’s collection from Mt. Pirongia from 1982 (Galloway 1985: 202, BM001085330), it appears doubtful whether this specimen really originated from New Zealand. According to Bartlett’s obituary by Edgar (2004), Bartlett visited Canada and subsequently various European countries in 1983, where he might have opportunities to collect *I. ericetorum* as well. Although entirely speculative, it is therefore possible, that a Northern Hemisphere *I. ericetorum* collection somehow got mixed up with other NZ collections, and eventually got erroneously labelled as being from NZ. To back up this assumption, it is necessary to point out two of Bartlett’s habits. First, he was renowned for collecting while rushing through the landscape (“Hurricane” Bartlett), without necessarily recording the exact origin of his collections on the spot (“he could never give me precise localities (grid references) of some of his finds”, Tony Druce in Galloway & Edgar 1987). This was done subsequently but sometimes several months later according to De Lange & Blanchon (2014), who also “have found that details on some Bartlett collections are suspect”. Second, Bartlett apparently also had a habit of driving a car “laden with plants” or “crammed to the roof with lichen specimens” (Galloway & Edgar 1987). These two habits certainly bear the potential to
have caused a mix up of specimens. It seems that only a targeted search for *I. ericetorum* on the “SW slopes of Mt Pirongia” (38°01’S 175°02’ E, near Hamilton) at around 360 m a.s.l. will reveal whether *I. ericetorum* really occurs there. However, the scenario of a potential mix-up of specimens is strengthened by the fact that the author collected *Dibaeis absoluta* on Mt. Pirongia, but failed to find *I. ericetorum* in spite of generally suitable habitat conditions.

It remains unclear why so many NZ specimens of *D. absoluta* were misidentified as *I. ericetorum*, but a noteworthy collection was found in the Auckland Herbarium (AK), which might have contributed to the misunderstanding. The collection AK 309805 from Pelorus Bridge, Marlborough is also a misidentified *D. absoluta*, which according to its label had been identified in 1985 as *I. ericetorum* by H.T. Lumbsch. He is now a famous lichenologist but at that time was still a student in Germany (Kärnefelt *et al.* 2012: 79). Most likely this specimen has been misidentified because Lumbsch was familiar with *I. ericetorum* but not with *D. absoluta*, as it does not occur in Europe (Gierl & Kalb 1993). It is conceivable, although entirely speculative, that this misidentified collection, later served as an authentic reference specimen for the identification of other NZ collections, because it had been identified by a Northern Hemisphere lichenologist.

It should also be mentioned, that in Great Britain *Dibaeis baeomyces* has apparently often been mistaken for *I. ericetorum*, as James & Brightman (1992) write about *I. ericetorum*: “Often confused with *Baeomyces roseus* [= *Dibaeis baeomyces*]; all lowland records need careful checking against this species (...).” A similar statement is made in the updated British Lichen Flora by Hitch *et al.* (2009): “Can be confused with *Dibaeis baeomyces* which has distinctly stalked apothecia. All lowland records need careful checking against that species (...).”

Reports on the presence of *I. ericetorum* in South Africa seem to be solely based on J.F. Drège’s collections and paper from 1843, which was merely cited in subsequent works, e.g. by Doidge (1950) and Rambold *et al.* (1993). It is well possible that Drège had a much wider species concept of *L. icmadophila*, possibly including present-day’s *Dibaeis holstii* (Müll. Arg.) Kalb & Gierl or *Dibaeis absoluta*, as both were first described many years later. *D. holstii* has also been collected in South Africa, while *D. absoluta* is at present unknown from Africa, but has a very wide distribution in both Hemispheres (Gierl & Kalb 1993) and might be present in Southern Africa as well. Unfortunately, the whereabouts of Drège’s South African *L. icmadophila* collections remain unclear, and the author is not aware of any more recent collections of this species there. Therefore, this species’ presence in South Africa should be considered doubtful, until specimens become available to provide unequivocal proof. A targeted search for *I. ericetorum* or potential lookalikes in the localities mentioned by Drège (see Results section for details) would be desirable.
The report of *I. ericetorum* from Chile by Pereira et al. (2006) appears to be correct, but the basis of previous South American records by Galloway & Quilhot (1998) and Litterski (1992) remains obscure. However, since the species’ presence in South America has been confirmed by Pereira et al. (2006), tracing the basis of the two previous records is considered irrelevant. It is still somewhat unusual that Pereira et al. (2006) report it not only growing on bark but also directly on rock. However, the author knows from personal experience in New Zealand that corticolous species and even whole lichen communities occasionally grow on rocks in the forest. Apparently runoff water from trees changes the chemical environment of the rock surface to make it suitable for corticolous lichens.

In conclusion, all New Zealand records examined appear to be a case of mistaken identity, apart from one of somewhat unreliable provenance. No specimen or report could be tracked down to verify Drège’s (1843) report on the species’ occurrence in South Africa. More field work is needed to determine whether *I. ericetorum* really exists in these countries, and in the meantime their occurrence should be re-classified as doubtful. One report from South America was traced and stands as the only reliable record of *I. ericetorum* from the Southern Hemisphere. It would be interesting to include this specimen in future phylogenetic studies of Icmadophilaceae.

5. Summary

Numerous New Zealand collections previously identified as *Icmadophila ericetorum* were critically re-examined. Nearly all collections were misidentified specimens of *Dibaeis absoluta*. Only the very first collection of *I. ericetorum* reported for NZ has been confirmed as this species, but for several reasons it remains doubtful whether this collection really originated from NZ. Very old reports of the species from South Africa are considered doubtful as well, and a recent report from Chile seems to be correct, although it was not possible to examine the specimen.
CHAPTER 7

Discovery of ascomata in the *Siphula decumbens* group, and its placement in the resurrected genus *Nylanderiella* Hue

1. Introduction

*Siphula* Fr. (Icmadophilaceae) is a widespread ground-dwelling lichen genus of currently 18 accepted species (Grube & Kantvilas 2006). It is renowned among lichenologists for its absence of apothecia (Kantvilas 1998, 2000), a trait shared with *Thamnolia* (Sw.) Ach. ex Schaer., placed in the same family. The type species *S. ceratites* has a Holarctic distribution in open tundra-like habitats of northern Eurasia and northern North America (Kantvilas 2002). The *Siphula decumbens* group occurs mainly in the Southern Hemisphere, where its members are widespread over Australia, New Zealand, southern South America and southern Africa. For this study the four species known from New Zealand are of interest (cf. Galloway 2007). These four species were also treated in detail in Kantvilas’ (1998) account on Tasmanian species, namely *S. decumbens* Nyl., *S. fastigiata* (Nyl.) Nyl., *S. dissoluta* Nyl. and *S. gracilis* Kantvilas. Because apothecia were unknown, the current species level taxonomy within the *S. decumbens* group is largely based on secondary metabolite chemistry, which is in part supported by morphological tendencies (Kantvilas 1998).

*Siphula decumbens* and *S. fastigiata* are virtually indistinguishable by morphology, but *S. decumbens* contains thamnolic acid (UV-) while *S. fastigiata* contains baeomycesic and squamatic acids (UV+), which can be easily distinguished using UV light and standard chemical spot tests. In the past both were considered to be chemo-types of the same species (e.g. Galloway 1985), but Kantvilas (1998) opted for recognition of the two chemo-species. *S. gracilis* contains thamnolic acid as well, but is additionally distinguished by extraordinarily narrow and long thalline lobes. *S. dissoluta* is unequivocally characterised by the presence of hypothamnolic acid, which is detectable by UV light and standard spot tests, but it has generally shorter and wider lobes than *S. decumbens* and *S. fastigiata*, and grows in more alpine habitats. However, a considerable range of morphological variability exists within these chemically defined species, and there is a good deal of overlap among them.

Hue (1914) introduced the new genus name *Nylanderiella* Hue for *S. medioxima* Nyl. (now a synonym of *S. decumbens*), based on what he thought was a fertile specimen of the lichen from New Zealand. However, this genus name was never widely used and eventually synonymized with *Siphula* by Kantvilas (1998), who has identified the alleged ascomata of Hue’s *Nylanderiella* type specimen as a parasitic fungus of the genus *Cercidiospora* (this subject is discussed in more detail below).

The seven species previously comprising the strictly austral *S. complanata* and *S. fragilis* groups were recently segregated into the genus *Parasiphula* Kantvilas & Grube, which turned out to be part of Coccotremataceae (Grube & Kantvilas 2006).
Fertile specimens of *S. decumbens* (UV-) were first collected by the author on the summit of Pryse Peak, Stewart Island, NZ, during the John Child Bryophyte and Lichen Workshop in November 2012. The distinctive pink colour of the apothecia immediately led to the assumption that these structures might be the previously unknown teleomorph of the lichen, as the genus was known to be a member of the pink-fruited family Icmadophilaceae (Stenroos *et al.* 2002). Initial microscopic examination also showed that spore characters were in line with other members of Icmadophilaceae.

When the author's collections were shown to Dr Allison Knight, she recalled similar collections from Mt. Webb (Fiordland, NZ) made in 2009. These specimens were *S. fastigiata* (UV+), but their fruits were dismissed as fungal galls, because those specimens were infected by a black lichenicolous fungus, and the occurrence of pink galls is mentioned in Galloway (2007: 1631). During subsequent field trips, we kept watching out for additional fertile specimens.

This previously unreported observation had to be followed up by the author, whose PhD thesis originally dealt only with the reproductive biology of *Icmadophila splachnirima*. However, they are members of the same family and are commonly associated species, which is a sound justification for examining this important aspect of *Siphula*’s reproductive biology as well. Furthermore, previous molecular studies (Platt & Spatafora 2000, Stenroos *et al.* 2002, Grube & Kantvilas 2006) have indicated that the *Siphula decumbens* group could be non-monophyletic with the type species *S. ceratites*, but this has not led to the according taxonomic changes.

It is the purpose of this chapter to test whether the pink apothecia are the true teleomorphic state of *S. decumbens* and *S. fastigiata*, and to settle their systematic and taxonomic placement.

*Siphula decumbens* and *S. fastigiata* are also terricolous members of the family Icmadophilaceae, and grow often together with *Icmadophila splachnirima*, or not far from it in the same general habitat. More importantly, it appears to the author, that the occurrence of apothecia in the *S. decumbens* group could also be correlated to certain micro-habitat conditions, a noteworthy parallel to environmental dependence of the reproductive mode in *I. splachnirima* reported in Chapter 5. Therefore, the inclusion of this chapter in a dissertation mainly focused on the reproductive biology of *I. splachnirima* seems appropriate.
2. Material & Methods

2.1 Collections of fertile specimens of *S. decumbens* and *S. fastigiata*

The following collections of fertile material have been examined:

**OTA 062495**, *Siphula decumbens*, Pryse Peak, Stewart Island, NZ, 46° 56'23.1"S 168°00'20.9"E, 350 m, leg. Lars Ludwig, 25.11.2012, TLC: thamnolic acid, fertile!, duplicate in HO

**OTA 062496**, *Siphula decumbens*, details as in OTA 062495.

**OTA 062497**, *Siphula decumbens*, East slope of Doughboy Hill, Stewart Island, NZ, 47°01'08.0"S 167°45'24.8"E, 320 m, leg. Lars Ludwig, 30.03.2013, TLC: thamnolic acid, fertile!, duplicate in HO. **OTA 062498**, *Siphula decumbens*, 47°01'06.4"S 167°45'23.2"E, all other details as in OTA 062497.


**OTA 062500**, *Siphula fastigiata*, near start of Scott’s Track, Arthur’s Pass, Canterbury, NZ, 45°56'03"S 171°33'32"E, ~800 m, leg. Allison Knight, 02/01/2013, TLC: baemycesic + squamatic acids, fertile!, duplicates in HO and private herbarium A. Knight.

**OTA 064254**, *Siphula fastigiata*, Kaipo River catchment, ca. 4 km N of Mt Thunder Pk., Fiordland, NZ, 44°28'55.7"S 168°00'38.8"E, 1090 m a.s.l., leg. Lars Ludwig, 14.04.2014, W-facing slope of ridge, in open subalpine vegetation with Hebe, bog pine, tussock.


**NV131405467**, DoC TIER1 collection, plot BH97, *Siphula decumbens* (UV-), Heaphy River catchment, 41°00'52"S 172°15'49"E [original NZTM coordinates E1538087 N5459357], leg. Nicky Armstrong, 31 May 2014.
2.2 Analysis of secondary metabolites

Thin Layer Chromatographic analysis of secondary compounds was carried out by Dr Gintaras Kantvilas, Hobart, Tasmania, Australia. The chemical analysis was required for species-level identifications (or its confirmation, respectively), following the chemo-species concept of Kantvilas (1998, 2000).

2.3 Molecular-genetic comparison of thalline and apothecial tissues

DNA extracts of thalline and apothecial tissues from several fertile specimens (see list above) were prepared using a CTAB extraction protocol and subsequent spin column purification with EconoSpin® All-In-One Mini Spin Columns (Epoch Life Sciences Inc.) and home-made buffers as described in Chapter 2.

The ITS region and the 5’ portion of the 28S rRNA gene (nuLSU) were amplified using the primer pairs ITS1F/ITS4A and LROR/LR5 or LROR/LR16. All procedures were performed as described in Chapter 2 on the phylogeny of Icmadophila.

2.4 Molecular phylogenetic placement of the Siphula decumbens group

The phylogenetic placement of the Siphula decumbens group relative to the type S. ceratites has been examined using the ITS region and the 5’ portion of the 28S rRNA gene (nuLSU). This work has been conducted as part of the molecular-genetic revision of the genus Icmadophila in Chapter 2. Therefore, the reader is referred to Chapter 2 of this dissertation for methodological details.

2.5 Literature review on reproduction in Siphula ceratites and Thamnolia vermicularis

Historic and recent literature has been reviewed, in order to find forgotten, overlooked or misinterpreted reports of reproductive structures in Siphula ceratites Fr. and Thamnolia vermicularis (Sw.) Ach. ex Schaer. Both species have been considered strictly sterile and were placed in the family Icmadophilaceae based on previous genetic studies.
3. Results

3.1 Morphology and microscopic examination of apothecia

The apothecia of *S. decumbens* and *S. fastigiata* are globose, ca. 0.27-0.59 mm diameter, with a very narrow disc, mostly growing in raspberry-like clusters of several dozen apothecia. Spores are hyaline, consistently 3-septate, measuring c. (20-)22-26 (-30) × 4-5 μm, 8 spores per ascus.

![Figure 46. Habit photo of fertile *Siphula decumbens* from Pryse Peak, Stewart Island, New Zealand.](image)

![Figure 47. Magnified views of mature apothecial clusters of fertile *Siphula decumbens* from Pryse Peak.](image)
3.2 Chemical analysis

Results of the TLC analyses performed by Dr Gintaras Kantvilas are included in the voucher specimen details above. Specimens containing thamnolic acid were classified as *S. decumbens*, and specimens containing baemycesic and squamatic acids were classified as *S. fastigiata*.

3.3 Molecular-genetic comparison of thalline and apothecial tissues

ITS and nuLSU sequence pairs obtained from apothecial and thalline tissues were identical in all examined fertile specimens of *S. decumbens* and *S. fastigiata*. GenBank accessions are given in Table 10 of Chapter 2 for the examined voucher specimens OTA 062495, OTA 062496, OTA 062499, OTA 062500, OTA 064254; they are also given in Figures 48 and 49 next to the names highlighted in bold.

3.4 Phylogenetic placement of the *Siphula decumbens* group

ITS and nuLSU sequences of the members of the *S. decumbens* group were consistently non-monophyletic with the generitype *S. ceratites* (Figures 48 and 49, modified from Figures 5 and 6). Species of the *S. decumbens* group were not resolved as separate entities; no grouping by secondary chemistry nor by geographic origin was observed. The sequences of *S. decumbens* and *S. fastigiata* are very similar with pairwise inter-species identities of over 99% percent in the ITS region. These two species are about 95% identical with the ITS of *S. dissoluta*. All these three species share a ca. 380 bp intron in the 3' end of the nuSSU gene, which is also highly conserved.

![Figure 48](image.png)

*Figure 48.* Phylogram of Bayesian Inference analysis of the ITS region (modified from Figure 5 in Chapter 2) showing the non-monophyletic position of the *S. decumbens* group relative to *S. ceratites*. The type *S. ceratites* as well as sequences obtained from fertile specimens of *S. decumbens* and *S. fastigiata* are highlighted in bold, followed by GenBank accessions. Branch support values are explained in the caption of Figure 5.
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Figure 49. Phylogram of Bayesian Inference analysis of the nuLSU region (modified from Figure 6 in Chapter 2) showing the non-monophyletic position of the S. decumbens group relative to S. ceratites. The type S. ceratites as well as sequences obtained from fertile specimens of S. decumbens and S. fastigiata are highlighted in bold, followed by GenBank accessions. Branch support values are explained in the caption of Figure 6.

3.5 Taxonomy

Based on the molecular phylogenetic results, the following taxonomic changes are proposed. It should be clarified that this dissertation is not meant to be the place of publication of any nomenclatorial changes. Here merely the necessity and intention to publish such changes is indicated in a manner resembling an article of a scientific journal. Proposed nomenclatorial changes will be validly published in a peer-reviewed international journal soon after this dissertation has been accepted.

Nylanderiella Hue emend. L. Ludw., A. Knight & Kantvilas

Description: Thallus chalky-white, fruticose with flattened strap-like lobes and frequently branched and entangled rhizines penetrating deep into the substrate; true cortex absent. The description of ascomata given by Hue (1914) is hereby rejected and replaced by the following description of apothecia. Apothecia uncommon, biaatorine, laminal to subterminal, not stalked, of pale pink colour, globose, ca. 0.27-0.59 mm diameter with a very narrow disc, often aggregated in raspberry-like clusters of 50 and more individual apothecia, persistent and well-developed proper exciple. Spores are hyaline, consistently 3-septate, measuring ca. (20-) 22-26 (-30) × 4-5 µm, 8 per ascus.
Typus generis: *Nylanderiella decumbens* (Nyl.) Hue emend L. Ludw., A. Knight & Kantvilas.

Etymology: Hue (1914) named this genus in honour of the Finnish lichenologist William Nylander (1822-1899).

The following new combinations are proposed for members of the *Siphula decumbens* group:

**Nylanderiella decumbens** (Nyl.) L. Ludw., A. Knight & Kantvilas, comb. nov.
For additional synonymy see Kantvilas (2000).
Emended description: apothecia as in generic description above.

**Nylanderiella fastigiata** (Nyl.) L. Ludw., A. Knight & Kantvilas, comb. nov.
For additional synonymy see Kantvilas (2000).
Emended description: apothecial characters as for *N. decumbens*.

**Nylanderiella dissoluta** (Nyl.) L. Ludw., A. Knight & Kantvilas, comb. nov.
For additional synonymy see Kantvilas (2000).

**Nylanderiella coriacea** (Taylor ex Nyl.) L. Ludw., A. Knight & Kantvilas, comb. nov.
For additional synonymy see Kantvilas (2000).

**Nylanderiella abbatiana** (Mathey) Kantvilas, A. Knight & L. Ludw., comb. nov.
For additional synonymy see Kantvilas (2000).

**Nylanderiella gracilis** (Kantvilas) L. Ludw., A. Knight & Kantvilas, comb. nov.

**Nylanderiella mascarena** (Mathey) Kantvilas, A. Knight & L. Ludw., comb. nov.
Nylanderiella ramalinoides (Nyl.) Kantvilas, A.Knight & L.Ludw., comb. nov.
For additional synonymy see Kantvilas (2000).

Nylanderiella subulata (Krempelh.) Kantvilas, A.Knight & L.Ludw., comb. nov.

Nylanderiella torulsa (Thunb. ex Ach.) Kantvilas, A.Knight & L.Ludw., comb. nov.
For additional synonymy see Kantvilas (2000).

Nylanderiella verrucigera (J.F. Gmelin) Kantv ilas, A.Knight & L.Ludw., comb. nov.
For additional synonymy see Kantvilas (2000).

3.6 Reproductive structures in *Siphula ceratites* and *Thamnolia vermicularis*

Just after naming the type species *S. ceratites*, Dodge (1973: 115) gives a description of *Siphula* apothecia, which are very similar to those reported in the present study:

“Apothecia very rare, bia torine, stipitate, urceolate, disc pale; asci 4-6-spored; ascospores hyaline, fusiform, 4-locular. Spermogonia very rare, innate in the tips of branches; spermatia short cylindric, slightly curved.”

Apparently his description of apothecia refers to *S. ceratites* (although this was not explicitly stated), and it remains unclear whether he described his own observations or cited older literature. However, he cites only three references in his *Siphula* treatment, apparently for purely taxonomic purposes: “*Siphula* Fries, *Syst. Orb. Vega* 238. 1825”, its synonym “*Dufourea* Ach., *Lichenogr. Univ.* 103.1810” and “*Siphula ramalinoides* Nyl. in Crombie, *Jour. Linn. Soc.* [London] Bot. 15:224. 1876.”. Of these three only Fries (1825) and (Acharius 1810) mention and describe apothecia, but without the details given by Dodge (1973).

Acharius’ (1810) and Fries’ (1825) descriptions most certainly refer to today’s *S. ceratites*, because the first species of the *S. decumbens* group were only described decades later by Nylander (1858-1860). Nylander, who is quoted in Crombie (1876: 226 [not p. 224 as cited by Dodge 1973]), also does not mention apothecia in the original description of *Siphula ramalinoides* Nyl., leaving the source of Dodge’s apothecia description obscure.

Räsänen (1937) reports apothecia for *Siphula patagonica* Vain. However, this is now considered a synonym of the unrelated *Parasiphula complanata* placed in Cocco treme taceae
(Kantvilas 2000, Grube & Kantvilas 2006). Therefore, this report shall not receive any further attention here, although it should be followed up sometime, because apothecia are currently also unknown for *Parasiphula*.

Furthermore, mostly historic reports on the presence of pycnidia in *Siphula ceratites* and *Thamnolia vermicularis* were found, and have already been published as the author’s contribution to the paper by Lord et al. (2013). The author’s claim to the respective parts of this paper is confirmed in its prelude by Lord et al. (2012), although the section on pycnidia in *S. ceratites* was not included in this prelude, because at this time the author’s research on pycnidia in *Siphula* was not yet at a publishable stage. The section of Lord et al. (2013: 407) summarising the author’s findings on old literature reports about pycnidia in *S. ceratites* is reproduced here in full, since that section was written by the author of this thesis:

“(...) pycnidia have been observed in *Siphula ceratites* (Wahlenb.) Fr. by 19th century lichenologists and were later confirmed or cited far into the 20th century (Sommerfelt 1826; Fries 1860; Krempelhuber 1869; Minks 1874; Vainio 1921; Räsänen 1937; Keissler 1960; Dodge 1973), but are now absent from almost all modern *Siphula* treatments (e.g. Kantvilas 2002). Only Kantvilas (1998) briefly mentions that “pycnidia are known in the Northern Hemisphere species, *S. ceratites* (R. Santesson, in litt.)”. However, it remains unclear if this refers to observations made by Santesson, or if Santesson described the older literature mentioned above. The anatomical details given for the putative pycnidia of *Siphula ceratites* are at least superficially reminiscent of the pycnidia in *Thamnolia* and other Icmadophilaceae, as they are described as immersed in the thallus, containing articulated conidiophores with bacilliform conidia (cf. Vainio 1921). Whether or not these putative pycnidia of *Siphula* do really exist or merely represent a lichenicolous fungus is the subject of an ongoing investigation.”

No confirmed reports on apothecia were found for *Thamnolia vermicularis*, but pycnidia were recently rediscovered by Lord et al. (2013). The previously generally accepted interpretation of these pycnidia as being of parasitic origin, was shown to be unjustified by the author’s comprehensive literature review, indicating the absence of any publications demonstrating a parasitic origin for the pycnidia reported for *Thamnolia* in the past. The full publication Lord et al. (2013) is appended to this thesis, with the author’s contributions highlighted in yellow.
4. Discussion

4.1 Molecular-genetic comparison of thalline and apothecial tissues

Sequence comparison of apothecial and thalline tissues confirms that the ascomata reported here for *S. decumbens* and *S. fastigiata* belong to the lichen, rather than to a lichenicolous fungus. This is in accord with anatomical apothecial characters and colour, which fit well with Icmadophilaceae (e.g. Rambold *et al.* 1993). Fertilisation occurs probably via pycnosporial ascomata reported for *S. decumbens* by Mathey (1974), and they appear to fit in the family as well (although they were not seen by the author of this thesis).

It seems likely that apothecia (or immature apothecia) were previously observed in *S. fastigiata*, but were misinterpreted as fungal galls (Galloway 2007: 1631). A few old collections with apothecia as described here were also found in the Allan Herbarium (CHR), although labelled as *S. decumbens*, probably because Galloway (1985) considered both as chemo-types of the same species. Numerous other specimens of *S. decumbens*, *S. fastigiata* and *S. dissoluta* seen by the author in CHR and OTA were also infected by at least two different species of lichenicolous fungi bearing black ascomata. Without the recent molecular-genetic evidence that *Siphula* is a member of *Icmadophilaceae* (Platt & Spatafora 2000, Stenroos *et al.* 2002, Grube & Kantvilas 2006), which means that potential *Siphula* fruits should be pink as in other family members, it is certainly difficult (if not impossible) to tell which fruiting bodies, if any, might be genuine rather than parasitic.

Genuine apothecia are certainly uncommon, and even in areas where they were found by the author, fertile thalli were vastly outnumbered by sterile ones. The sterile thalli usually form extensive swards in very open sites and grow directly on dry, exposed peaty soil or rotting wood. In contrast, the fertile thalli are more scattered individual thalli, which do not form swards and grow in more sheltered and moist sites, usually among moss carpets (e.g. *Racomitrium*). This might indicate a correlation of sexual reproduction with micro-environmental factors, similar to the author’s observation in *Icmadophila splachnirima* (cf. Chapter 5).

The fact that sterile thalli often form extensive, dense swards shows that they still grow in conditions suitable for plentiful vegetative growth. Based on the rarely cited observation of Mathey (1974), who found rhizine-born ‘adventitious thalli’ (French ‘thalles adventifs’) in *S. decumbens*, it seems likely that this growth pattern is a result of vegetative lateral expansion via rhizines, similar to the rhizomes of ferns and horsetails or the caulonema of mosses. Such a mechanism of colonisation of the immediately surrounding area by lateral expansion of rhizomorphs has also been reported for the squamulose or areolate-crustose lichens *Acarospora* cf. *scotia*, *Aspicilia crespiana*, *Candellariella vitellina*, *Endocarpon pusillum*, *Lecanora rhizinata*, *Squamarina cartilaginea* and *Toninia opuntioides* (Sanders & Rico 1992, Letrouit-Galinou & Asta 1994, Sanders 1994, Rico 1999, Sanders 1999). In contrast, no such function of the rhizine-strands of *Cladonia sulphurina* has been observed by Ott *et
al. (1993), but instead Hammer (1997) describes that an underground prothallus serves the same purpose in *Cladonia subtenuis*. To what extent rhizine-mediated short range expansion occurs in the *Siphula decumbens* group (now *Nylanderiella*) could be easily examined by careful dissection and excavation of rhizines of whole swards. A more elegant alternative would be the use of micro-computed tomography to create a 3D model, as recently used to analyse the complex spatial arrangement of *Cladonia portentosa* cushions by Stratford *et al.* (2014; also see cover illustration of British Lichen Society Bulletin no. 110).

Results of the literature search on reproductive structures in *S. ceratites* and *Thamnolia vermicularis* together with the findings of Lord *et al.* (2013) show that it is necessary to critically reconsider the reproduction of all supposedly strictly sterile lichen species. Also, the wording of species descriptions and their interpretation should be carefully considered. A statement like ‘apothecia and pycnidia not seen’ (e.g. repeatedly used by Galloway 2007) must not be interpreted or recited as ‘ascomata or pycnidia do not exist’ or ‘without ascomata or pycnidia’. Logic dictates that it is not possible to provide empirical proof for the non-existence of anything (see e.g. Popper 1963, Lakatos 1970), which means that in case of obvious data-deficiency the only correct statement is that something was ‘not seen’ or ‘not observed’ or is ‘currently unknown’. Brodo & Lendemer’s (2012) report on *Ochrolechia arborea* is another recent example for the occasional occurrence of ascomata in a usually sterile lichen.

### 4.2 Phylogenetic placement of the *Siphula decumbens* group

The *Siphula decumbens* group consistently shows a non-monophyletic relationship to the generitype *S. ceratites*. This result confirms previous findings of Platt & Spatafora (2000), Stenroos *et al.* (2002) and Grube & Kantvilas (2006). Apart from the presence of apothecia, several other morphological and chemical characters distinguish the *S. decumbens* group from *S. ceratites*: the presence of well-developed root-like rhizines penetrating the substrate up to 2 cm deep, the shape of the lobes/branches and the cortical structure. Therefore, the new genus *Nylanderiella* is proposed, together with the transfer of all species currently placed in the *S. decumbens* group.

Hue (1914) introduced the name *Nylanderiella* Hue for *S. decumbens* (Hue called it *S. medioxima* Nyl., which is now a synonym), based on what he thought was a fertile specimen of the lichen from New Zealand. However, this was a misinterpretation of a thallus infected with a lichenicolous parasite of the genus *Cercidospora* (Kantvilas 1998), which bears no resemblance with the fruits reported in the present study. Nylander’s (1888) description of *S. medioxima* was already based on the infected material, and Kantvilas (1998) decided that “the lectotypification of *Siphula medioxima* is here based on the the lichen, not the parasite, and thus *Nylanderiella* becomes a synonym of *Siphula* (R. Santesson in litt.).” Therefore the name *Nylanderiella* has priority over any newer name when segregating *S. decumbens* from *Siphula* s. str. However, it can be argued that Hue’s
new name was obviously intended to accommodate the (parasitic) sexual reproductive structures associated with the lichen. In retrospect, considering that fungal systematics are primarily built on characteristics of sexual fructifications, the name *Nylanderiella* might have better been synonymised with *Cercidispora* instead of *Siphula*.

The flattened branches/lobes of the *S. decumbens* group and the terete thalli of *S. ceratites* are the most obvious macroscopic difference between both, and should not require further discussion. Furthermore, as pointed out by Grube & Kantvilas (2006: 247), thalli of *S. ceratites* have a well-developed pseudoparenchymatous cortex, which is absent in the members of the *S. decumbens* group, and “instead the outermost part of the lobes is a rather poorly defined layer of interwoven hyphae and crystalline inclusions”. Root-like rhizines are a typical character of *Siphula* s. lat., and authors like Keissler (1960) and Kantvilas (2002) mention it without differentiation between the *S. ceratites* and *S. decumbens* groups. However, rhizines appear to be much more pronounced in the *S. decumbens* group (up to 2 cm long) than *S. ceratites*, albeit this is a vague difference. In fact, some descriptions of *S. ceratites* do not even mention rhizines (Nylander 1858-1860, Brodo et al. 2001), although a drawing of a specimen from Finland by Mathey (1974: Fig. 60) shows well-developed rhizines. A branched root (“radix ramosissima”) is also mentioned for that species by Fries (1831: 406-407), which is the valid original genus description according to Brusse (1987). An initial examination of the type collection of *Siphula ceratites* (Wahlenb.) Fr., which is housed in the UPS herbarium (Uppsala, Sweden), was kindly conducted by Anders Nordin, who found two short rhizine-like structures but the major part of the type lacks rhizines. Examination of further *S. ceratites* specimens is necessary to assess to what extent the development of rhizines can be used as additional distinguishing character of *Siphula s. str.* versus the *S. decumbens* group (*Nylanderiella*).

The currently accepted chemo-species *S. decumbens* and *S. fastigiata* are not supported as distinct entities by molecular data, but appear to be conspecific. However, population genetic studies comprising many more specimens of both chemo-species from a wide geographic range should be analysed before both can be declared conspecific with confidence. Should the initial data from the present study receive further support, the correct name (following current taxonomy) of the taxon would be *S. decumbens* Nyl., a species with two chemotypes as in *Thamnolia vermicularis* (1. thamnolic acid; 2. baeomycesic plus squamatic acids; see e.g. Lord et al. 2013). The name *S. fastigiata* Nyl. would have to be reduced to synonymy, a reversal to the taxonomic concept of Galloway (1985). *S. dissoluta* groups among *S. decumbens* and *S. fastigiata* in the presented analysis, therefore it cannot be ruled out that it might be a third chemo-type if all three chemo-species were conspecific. Again the analysis of more specimens is required.

Examination of the mating type locus (*MAT*) of the current *S. decumbens* group would be very desirable for two reasons. First, it would allow to apply the generally accepted Biological Species Concept (BSC) in order to assess whether *S. decumbens* and *S. fastigiata* are conspecific or not. The
BSC states that members of the same species should be able to interbreed and produce fertile offspring (de Queiroz 2005). For ascomycetes this implies that members of the same species would be expected to have (almost) identical MAT genes, even though these genes can vary a lot among closely related taxa. Thus the MAT genes appear to be potentially suitable and meaningful genetic markers for low-level taxonomy and species delimitation in lichenized ascomycetes, which was already demonstrated for phytopathogenic ascomycetes (Silva et al. 2012). Second, mating type screenings in several populations would allow to examine if the rare occurrence of apothecia in *S. decumbens* and *S. fastigiata* is caused by a deficiency of compatible mating partners, or whether apothecium formation is inhibited by other factors, such as suboptimal environmental conditions.

Apothecia are currently only known for *S. decumbens* and *S. fastigiata* (if considered separate species), and molecular data supporting the inclusion in the *S. decumbens* group exists only for *S. decumbens*, *S. fastigiata*, *S. dissoluta* and *S. coriacea*. Nevertheless, the remainder of the *S. decumbens* group *sensu* Kantvilas (1998, 2002) was transferred into *Nylanderiella* as well, in order to maintain nomenclatorial stability. Only *S. pickeringii* remains in *Siphula* s.str, and is thus the only true *Siphula* species present in New Zealand (see Galloway 2007: 1635-1636). This is because in contrast to the chemical classification of Kantvilas (2002), the nuclear LSU and SSU sequences of *S. pickeringii* (as *S. polyschides*) generated by Platt & Spatafora (2000) and reused by Stenroos et al. (2002) suggest that this species belongs in the *S. ceratites* group, together with *S. carassana*. Thus, it seems that the chemical circumscriptions of the *S. decumbens* group is not necessarily congruent with the molecular-genetic relationships. Consequently, the transfer of the following species into *Nylanderiella* is in need for molecular-genetic confirmation: *S. abbatiana*, *S. gracilis*, *S. mascarena*, *S. ramalinoides*, *S. subulata*, *S. torulsa* and *S. verrucigera*. Meanwhile, their new generic status is provisional.

However, the mismatch of chemical versus molecular-genetic placements of *S. pickeringii* might as well be caused by misidentifications, because the specimens extracted for genetic analyses by Platt & Spatafora (2000) were not the same as those used by Kantvilas (2002) for chemical analysis. This is most likely what was implied by Grube & Kantvilas (2006) when they noted that further collections of *S. pickeringii* need to be examined. Therefore, it is still possible that the *Siphula ceratites* group *sensu* Kantvilas (2002) will turn out to be indeed chemically uniform and distinct from the *S. decumbens* group *sensu* Kantvilas (2002; now *Nylanderiella*), provided that re-examination of the *S. pickeringii* vouchers of Platt & Spatafora (2000) confirms a misidentification, and/or examination of additional *S. pickeringii* specimens shows both chemical and genetic affinity to *S. decumbens*. In that case the secondary chemistry would be another distinguishing character of the *S. decumbens* group (*Nylanderiella*) versus *Siphula* s.str., as suggested by Kantvilas (2002).

The nuclear SSU rDNA phylogeny of Stenroos et al. (2002) has already shown that the *S. decumbens* group - represented by *S. decumbens*, *S. cf. fastigiata* and *S. coriacea* - was not monophyletic with the *S. ceratites* group, which was represented by *S. ceratites*, *S. carassana* and *S.
pickeringii (as *S. polyschides*). Notably, in the same study the likewise consistently sterile species *Endocena informis* Cromb. forms a poorly supported clade with the *S. decumbens* group, which might indicate that the *S. decumbens* group should be included in *Endocena* Cromb. rather than a separate genus. Regrettably, a fresh specimen of *E. informis* was not available for the present study, and a separate genus is favoured based on the poor clade support in Stenroos *et al.* (2002) and morphological differences of *E. informis*. These include a lack of root-like structures and a terete rather than flattened thallus (Crombie 1876, Dodge 1973). Bendz *et al.* (1965) report thamnolic acid as a secondary chemical compound in *E. informis*, which is also the case in *S. decumbens*; however, this is still no indication of close relatedness, as thamnolic acid also occurs in several other Icmadophilaceae, and in even more distant genera like *Pertusaria* (cf. Galloway 2007: 1129-1168) or *Ophioparma* (Bjelland 2002).

**Summary**

Fertile specimens of *Siphula decumbens* and *Siphula fastigiata* are reported for the first time from several New Zealand localities. Apothecial characters fit well with the species’ earlier placement in the family Icmadophilaceae. Molecular-genetic comparison of DNA extracts from thalline and apothecial tissues rules out the possibility that the ascomata belong to a lichenicolous fungus. Apothecial characters of *S. decumbens* and *S. fastigiata* are indistinguishable, and initial genetic comparison of samples of both taxa from New Zealand and Tasmania supports the hypothesis that both belong to the same species, with two chemotypes as in *Thamnolia vermicularis*. The name *Nylanderiella* is resurrected for the members of the *Siphula decumbens* group, based on its remote position in molecular phylogenetic analyses relative to *Siphula ceratites*, the type species. Apart from the presence of ascocarps, *Nylanderiella* differs from *Siphula* s. str. in its flattened lobes, the lack of a true cortex and generally better developed rhizines. Additionally a literature search for reports on reproductive structures in *Siphula ceratites* and *Thamnolia vermicularis* has been conducted. Several 19th century authors have reported the presence of pycnidia in both *S. ceratites* and *T. vermicularis*, but this knowledge has vanished from modern treatments of these species, seemingly based on the mere assumption that these were of parasitic origin.
CHAPTER 8
Conclusions

This research has thrown light on the reproductive biology of the rare Australasian lichen *Icmadophila splachnirima*, and also on the mode of dispersal in other globally widespread members of the Icmadophilaceae, whose reproductive biology was previously an enigma. Examined aspects of the biology are the phylogenetic placement of both symbionts, global distribution and population genetic structure, mating system, and reproductive adaptation to changing micro-climatic conditions. The main findings are summarized as follows:

Strong evidence has been found that *Icmadophila splachnirima* is only distantly related to the type species of the genus, *Icmadophila ericetorum*, as well as to any other species currently placed in the family Icmadophilaceae for which reference sequences or specimens were available. The evidence comes from a multi-gene phylogenetic analysis as described in Chapter 2. These findings are in accord with the previous nuclear Small Subunit rDNA phylogeny described by Stenroos *et al.* (2002). Therefore, it is proposed to resurrect the current generic synonym *Knightiella splachnirima*. The view that *I. splachnirima* and *I. ericetorum* were congeneric is much more plausible when assuming that both were sympatric. However, the findings presented in Chapter 6 show that *I. ericetorum* is in all likelihood not present in New Zealand, because almost all herbarium records were misidentified specimens of *Dibaeis absoluta*; the only correctly identified specimen supposedly originating from New Zealand is of doubtful provenance. The photobiont of *I. splachnirima* was confirmed to belong in the green-algal genus *Coccomyxa* s. lat. (Chapter 3), but a more precise identification is currently not possible, due to the absence of suitable and authentic reference material and the urgent need of a taxonomic revision of *Coccomyxa* s. lat. using molecular-genetic techniques.

Further strong evidence has been found that *I. splachnirima* is likely heterothallic, i.e. not capable of self-fertilisation. This evidence comes from mating type analysis, reported in Chapter 4. This is a crucial piece of information for understanding the reproductive ecology and the population genetic structure of the species (Werth 2010). It indicates that sexual reproduction is theoretically limited by two factors: the availability of suitable mating partners and the availability of compatible photobionts for re-lichenization of ascospores. However, since the species is commonly fertile or bears apothecial initials, it appears that the mating type ratio is usually balanced, which was genetically confirmed for one local population. Also the ascospores are not confined to a single strain or species of *Coccomyxa* s. lat. for re-lichenization, since *I. splachnirima* was found to associate with at least two distinct species (89% pairwise ITS nucleotide similarities, OTUs 1 and 2 in Chapter 3). Furthermore, compatible photobionts seem to be fairly abundant, since they were also found in *Lichenomphalia* cf.
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I. splachnirima (reported in Chapter 3). This is a common and wide-spread basidiolichen (see Galloway 2007, and author’s personal observations) rated ‘not threatened’ by de Lange et al. (2012), which grows abundantly in similar habitats as I. splachnirima, often immediately next to it. This may also indicate that I. splachnirima is much more wide-spread in New Zealand than current records show (Chapter 1).

Low genetic variability in mycobiont ITS sequences across the species’ range (Chapter 3) makes it very likely that recent gene flow has occurred among the disjunct populations, since reproductive isolation should quickly lead to differentiation of regional haplotypes, which was found to be the exception rather than the norm in the studied species. High genetic diversity of photobionts among populations allows to conclude that long-distance dispersal between populations is accomplished primarily by ascospores.

Field-ecological experiments reported in Chapter 5 also point towards sexual propagation as the ultimate mode of reproduction in most thalli, even though a transient period of vegetative propagation may precede maturation of dormant apothecial initials. Strong evidence, though no conclusive proof, was found that vegetative propagation via soredia is an alternative/secondary reproductive mode, reserved for periods of unfavourable conditions; it seems likely that insufficient hydration rather than light-stress acts as the stimulus for vegetative propagation (although high-light conditions are often accompanied by increased desiccation). However, reciprocal transplants show that as soon as the growing conditions improve, vegetative thalli with dormant apothecial initials can proceed to sexual maturation. Although this has been concluded from field-manipulations, similar changes may well happen naturally, e.g. in course of plant succession and erosion. Vice versa, favourable micro-climatic conditions of sexual specimens may deteriorate over the long life span of a thallus, and under more stressful conditions the lichen can resort to vegetative reproduction, which is metabolically less expensive and allows for resource allocation towards cellular stress adaptation and repair.

Noteworthy is the apparently very similar ecology of the unrelated Trapeliopsis glaucolepidea (Palice & Printzen 2004). Characteristics of this species shared with I. splachnirima are a similar morphology, substrate and habitat preference, frequent reproduction via apothecia or marginal soralia, and an apparent influence of the micro-environment on the reproductive mode (see discussion in Chapter 3 for details). Therefore, a comparative study on the reproductive ecology of T. glaucolepidea, similar to the present one, is very desirable. An apparent environmental dependence of the sexual reproduction was also observed for Siphula decumbens and S. fastigiata. In the course of the research undertaken for this thesis, the author has observed and proven that sexual reproduction occurs in these wide-spread taxa, which were previously believed to be strictly sterile (Chapter 7). Since these species are also members of the family Icmadophilaceae, and frequently co-
occur in the same habitats as *I. splachnirima*, further investigation of their reproductive ecology would also be a promising endeavour.

The reproductive strategy of *I. splachnirima* can be summarised as follows: The species can reproduce by sexual and vegetative reproduction. Sexual reproduction is the primary reproductive mode and predominant mechanism for long-distance dispersal; however, it is limited by the inability to self-fertilise. Cross-fertilisation is mediated by passively dispersed pycnospores produced in pycnidia. Sexual ascospores are able to re-lichenize with at least two distinct species of the green-algal genus *Coccomyxa* s. lat. Vegetative propagation via marginal soralia is the secondary reproductive mode, which allows to propagate in the absence of suitable mating partners and under conditions that are unsuitable for apothecial development. Both reproductive modes are more or less mutually exclusive, since the lichen can switch back and forth between them, depending on the microclimatic conditions. Sexual reproduction prevails in favourable micro-habitats (rather moist), while vegetative reproduction occurs usually in suboptimal or stressful conditions (rather dry). A trade-off between the relative metabolic costs of either reproductive mode and mechanisms to cope with suboptimal growth conditions is likely to regulate the transition between sexual and vegetative reproduction.

Based on the reproductive strategy outlined above, the author assumes that populations of *I. splachnirima* will be able to maintain themselves in their natural environment, in spite of potentially deteriorating habitat conditions in the course of climatic change. However, especially small and isolated populations may not be able to recover from genetic bottlenecks and habitat loss due to anthropogenic activity. Therefore, monitoring of small and isolated populations is advised, and if necessary, transplants of compatible mating partners should be considered to ensure their long-term survival.


Maximum and the onset of deglaciation on the maritime-Antarctic and sub-Antarctic islands. *Quaternary Science Reviews* 100: 137-158.


