Do Photobiont Switch and Cephalodia Emancipation Act as Evolutionary Drivers in the Lichen Symbiosis? A Case Study in the Pannariaceae (Peltigerales)

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Abstract
Lichen symbioses in the Pannariaceae associate an ascomycete and either cyanobacteria alone (usually Nostoc; bipartite thalli) or green algae and cyanobacteria (cyanobacteria being located in dedicated structures called cephalodia; tripartite thalli) as photosynthetic partners (photobionts). In bipartite thalli, cyanobacteria can either be restricted to a well-delimited layer within the thallus (‘pannarioid’ thalli) or spread over the thallus that becomes gelatinous when wet (‘collematoid’ thalli). We studied the collematoid genera Kroszia and Physma and an undescribed tripartite species along with representatives of the pannarioid genera FuscoPannaria, Pannaria and Parmeliella. Molecular inferences from 4 loci for the photobiont and statistical analyses within a phylogenetic framework support the following: (a) several switches from pannarioid to collematoid thalli occurred and are correlated with photobiont switches; the collematoid genus Kroszia is nested within the pannarioid genus FuscoPannaria and the collematoid genus Physma is sister to the pannarioid Parmeliella mariana group; (b) Nostoc associated with collematoid thalli in the Pannariaceae are related to that of the Collemataceae (which contains only collematoid thalli), and never associated with pannarioid thalli; Nostoc associated with pannarioid thalli also associate in other families with similar morphology; (c) ancestors of several lineages in the Pannariaceae developed tripartite thalli, bipartite thalli probably resulting from cephalodia emancipation from tripartite thalli which eventually evolved and diverged, as suggested by the same Nostoc present in the collematoid genus Physma and in the cephalodia of a closely related tripartite species; Photobiont switches and cephalodia emancipation followed by divergence are thus suspected to act as evolutionary drivers in the family Pannariaceae.

Introduction
Several spectacular aspects of the lichen symbiosis have come to light recently, the most surprising for the general public and the most promising for evolutionary studies being the multiple variations of the association between the mycobiont and photobiont partners. The lichen as the icon of consensual and stable symbiosis between two very different partners “for better and for worse” is not the model that molecular studies have produced in recent years. Indeed, some mycobionts can incorporate several algal genotypes in their thalli [1–3], or even different algal species [4–5]. Several phylogenetic studies have demonstrated that photobiont switching is rather widespread [6], even in obligatory sterile taxa where both partners are dispersed together, and may occur repeatedly over evolutionary timescales [7]. Studies of the genetic diversity of both partners within a geographical context revealed that mycobionts can recruit several lineages of photobionts, allowing for ecotypic differentiation and thus for colonization of different ecological niches and distribution [6,8]. Those multiple variations in the association between the partners involved in the lichen symbiosis may take part in their evolutionary trajectory and we here address that matter for a lichen family (the Pannariaceae) in which several very different types of thalli occur together with variation in the number of photobionts involved in their construction.

The Peltigerales, a strongly supported lineage within the Lecanoromycetes, contains many well-known lichen genera, such as Lobaria, Peltigera and Sticta, within 10 families [9–12], including the Collemataceae and the Pannariaceae, two families that will be mentioned in this paper.

Within the Peltigerales, symbiosis includes two different lineages of photobionts [10]: (a) cyanobacteria mostly belonging to the genus Nostoc, or to Scytonema, Hyphomorpha and other taxa in the Sctyonemataceae and Rivulariaceae; (b) green algae, mainly assigned to the genera Coccomyxa, Dictyochloropsis, Myrmecia, all belonging to the Trebouxioideae. The number of photobionts associated with the mycobiont provides the ground for the distinction of bi- and tripartite lichens, the latter case being much more diverse in the way of allocating space for the cyanobacteria [13–15]:

(a) association with a single photobiont partner, either a cyanobacteria or a green algae; these thalli are bipartite and are referable to the cyanolichens or the chlorolichens, respectively [16];
Further two different types of cyanobacterial bipartite thallus can be distinguished on the basis of their response to changes in water availability [19]. A first type is characterized by thalli that swell considerably and become very much gelatinous when wet, and return to a rather brittle and crumpled condition when dry, while the second type has thalli that do not radically change when wet and return to a rather brittle and crumpled condition when dry, but can be morphologically rather similar or very much different one from the other – in the latter case the cyanomorph has a Dendricocaulon-like morphology [14].

(b) association with two partners, a cyanobacteria and a green algae and corresponding thalli referred to as tripartite thalli [17]; the topological organization of the partners can vary: (b1) both photobionts can be present in a dedicated layer within the thallus (chloro-cyanolichen; see [16]); (b2) the green photobiont is present in a dedicated layer within the thallus whilst cyanobacteria are confined to dedicated and morphologically recognizable organs, named cephalodia [18]; (b3) production of different thallus types, either living independently from one another or being closely associated, one with the cyanobacteria and the other one with the green algae; these structures are referred to as « photosymbiodemes », « photopairs » or « photomorphs » and can be morphologically rather similar or very much different one from the other – in the latter case the cyanomorph has a Dendricocaulon-like morphology [14].

Materials and Methods

Taxon Sampling

We assembled material belonging to the Pannariaceae from recent field trips in Madagascar (2008), Reunion Island (2008, 2009) and Thailand (2012). The 36 specimens used for molecular analysis are listed in Table 1. Identification of these collections is based on Jørgensen [27,28,30–44], Jørgensen & Schumm [45], Jørgensen & Sipman [46], Upreti et al. [47], Swinscow & Krog [48] and Verdon & Elix [49].

Molecular Data

Well-preserved lichen specimens lacking any visible symptoms of fungal infection were selected for DNA isolation. Extraction of DNA followed the protocol of Cabero et al. [50]. We sequenced the ribosomal nuclear loci ITS, using primers ITS1F [51] and ITS4 [52], and LSU with primers LROR [53] and either LR7 [53] or LIC2044 [54], the mitochondrial ribosomal locus mtSSU, using primers SSU1 and SSU3R [55], and part of the protein-coding gene RPB1 with RB1AF [56] and RB1CR [57]. We sequenced the 16S ribosomal region of the Nostoc symbiont of 25 of this set of Pannariaceae as well as 2 additional Fuscopannaria leucosticta, 2 additional Physma and 4 from two other genera (Leptogidium and Pseudocyphellaria) belonging to the Peltigerales, using the two primer pairs ID1 [58]–revAL [17] and I712 [59]–rD1 [58]. Amplicons were sequenced by Macrogen® or by the GIGA technology platform of the University of Liège.

Sequences Editing and Alignment

Sequence fragments were assembled with Sequencher version 4.9 (Gene Codes Corporation, Ann Arbor, Michigan). Sequences were subjected to megaBLAST searches [60] to detect potential contaminations. Sequences were aligned manually using MacClade version 4.08 [61]. Ambiguous regions were delimited manually and excluded from the analyses. Substitutions and indels in ITS1 and ITS2 were so numerous that no unambiguous alignment could be realized; therefore ITS sequences were reduced to the less variable 5.8S portion.

Concatenation and Partitioning

Congruence of the four fungal loci was assessed by the comparison of single-locus phylogenetic trees produced with RAxML HPC2 version 7.2.8 [62–63] as implemented on the CIPRES portal [64], looking for the best ML tree and bootstrapping with 1000 pseudoreplicates in the same run, using GTRCAT model and the default settings. No significant conflict with bootstrap values (BS) > 70 was detected and we therefore
Table 1. Voucher table of the specimens used in the study, with the species names for the mycobiont, and the species names of the host for the photobiont, when available; the country of origin and the voucher information; GenBank accessions of the sequences.

| Mycobiont species | Reference | Country of origin and voucher information | ITS | mtSSU | LSU | RPB1 | cyanobacterial 16S |
|-------------------|-----------|--------------------------------------------|-----|-------|-----|------|---------------------|
| Degelia durietzi Arv. & D.J. Galloway | 19 | New Zealand | GQ259022 | GQ258992 | GQ259051 |     |
| Degelia plumbea (Lightf.) P.M. Jørg. & P. James | 93 (ITS), 19 | Norway (ITS), Portugal | AF429265 | AY340491 | AY340543 | GQ259052 |     |
| Erioderma verruculosum Vain. | 117 | ? | DQ972990 | DQ973041 | DQ973062 |     |
| Fuscodermia annulata (D.J. Galloway & P. M. Jørg.) P.M. Jørg & D.J. Galloway | 19 | New Zealand | GQ259024 | GQ258994 | GQ259053 |     |
| Fuscodermia ahlneri (P.M. Jørg.) P.M. Jørg. | 118 (ITS), 19 | Norway (ITS), South Korea | GU570097 | GQ259025 | GQ258995 | GQ259054 |     |
| Fuscodermia confusa (P.M. Jørg.) P.M. Jørg | 118 | Norway | GU570108 | GQ259026 | GQ258996 | GQ259056 |     |
| Fuscodermia ignobilis (Anzi) P.M. Jørg. | 119 (ITS), 117 | ? | HQ650673 | DQ917416 | DQ917417 | DQ986839 |     |
| Fuscodermia leucosticta (Tuck.) P.M. Jørg. | NEW | Reunion Island, R1123 (LG) | KF704257 | JX494238 | JX494264 | JX494284 | KF704325 |     |
| Fuscodermia leucosticta (Tuck.) P.M. Jørg. | 93 (ITS), 19 | USA | AF429277 | DQ900630 | DQ900640 | GQ259055 |     |
| Fuscodermia mediterranea (Tav.) P.M. Jørg. | 118 (ITS), 117 | Norway (ITS) | GU570131 | DQ917418 | DQ917419 |     |
| Fuscodermia praetermissa (Nyl.) P.M. Jørg. | 118 (ITS), 19 | Norway (ITS), Sweden | GU570108 | GQ259026 | GQ258996 | GQ259056 |     |
| Fuscodermia praetermissa (Nyl.) P.M. Jørg. | NEW | Reunion Island, R1060 (LG) | KF704258 | JX494239 | JX494285 | KF704346 |     |
| Fuscodermia sampaiana (Tav.) P.M. Jørg. | 118 | Norway | GU570030 |     |
| Joergensenia cephalodina (Zahlbr.) Passo, S. Stenroos & Calvelo | 96 | Argentina | EU885308 | EU885329 |     |
| Kroswia crystallifera P.M. Jørg. | NEW | Madagascar, M788 (LG) | KF704254 | JX494235 | JX494261 | JX494281 | KF704343 |     |
| Kroswia crystallifera P.M. Jørg. | NEW | Reunion Island, R1055 (LG) | KF704255 | JX494236 | JX494262 | JX494282 | KF704345 |     |
| Kroswia crystallifera P.M. Jørg. | NEW | Reunion Island, R1679 (LG) | KF704256 | JX494237 | JX494263 | JX494283 | KF704344 |     |
| Leciophysma furfurascens (Nyl.) Gyeln. | 19 | Sweden | GQ259028 | GQ258998 | GQ259058 |     |
| Leptogidium contortum (Henssen) T. Sprib. & Muggia | 34 | Chile | JF938195 |     |
| Leptogidium dendrixicum (Nyl.) Nyl. | 34 | USA, Alaska | JF938202 | JF938143 |     |
| Leptogium lichenoides (L.) Zahlbr. | 119 (ITS), 114 | ? | HQ650672 | DQ923210 | DQ917412 | DQ917414 |     |
| Pannaria athroophylla (Stirt.) Elvebak & Galloway | 96 | Argentina | EU885303 | EU885325 |     |
| Pannaria calophylla (Müll. Arg.) Passo & Calvelo | 96 | Argentina | EU885296 | EU885318 |     |
| Pannaria conopea (Ach.) Bory | 93 (ITS), 15 | Norway (ITS) | AF429281 | AY424209 |     |
| Pannaria impensa (Stirt.) Passo, Calvelo & Stenroos | 95 | Argentina | EU885311 | EU885333 |     |
| Pannaria lurida (Mont.) Nyl. | NEW | Madagascar, M786 (LG) | KF704248 | JX494240 | JX494265 | KF704307 |     |
| Pannaria lurida (Mont.) Nyl. | NEW | Reunion Island, R1033 (LG) | KF704252 | JX494247 | JX494272 | KF704311 |     |
| Pannaria lurida (Mont.) Nyl. | NEW | Reunion Island, R1012 (LG) | KF704253 | JX494246 | JX494271 | KF704312 |     |
| Pannaria microphyllizans (Nyl.) P.M. Jørg. | 93 (ITS), 96 | Australia (ITS), Argentina | AF429279 | EU885322 |     |
| Pannaria multifida P.M. Jørg. | NEW | Reunion Island, R942 (LG) | KF704249 | JX494241 | JX494266 | KF704308 |     |
| Pannaria multifida P.M. Jørg. | NEW | Reunion Island, R960 (LG) | KF704251 | JX494242 | JX494267 | KF704309 |     |
| Pannaria multifida P.M. Jørg. | NEW | Reunion Island, R961 (LG) | KF704250 | JX494243 | JX494268 | KF704310 |     |
Table 1. Cont.

| Mycobiont species                                      | Reference | Country of origin and voucher information | ITS      | mtSSU    | LSU      | RPB1      | cyanobacterial 16S |
|--------------------------------------------------------|-----------|-------------------------------------------|----------|----------|----------|------------|-------------------|
| *Pannaria pallida* (Ny.) Hue                          | 96 (ITS, mtSSU), 87 (LSU) | Argentina                                   | EU885301 | EU885323 | GQ927270  |                        |                   |
| *Pannaria rubiginella* P.M. Jorg.                      | 19        |                                           | GQ927269 | GQ259037 | GQ259007 | GQ259074   |                   |
| *Pannaria rubiginosa* (Thumb. ex. Ach.) Delise         | 19        | Portugal                                   | GQ927267 | AY340513 | AY340558 | GQ259073   |                   |
| *Pannaria rubiginosa* (Thumb. ex. Ach.) Delise         | NEW       | Reunion Island, R1008 (LG)                | KF704259 | JX494244 | JX494269 | KF704313 | KF704321 |
| *Pannaria rubiginosa* (Thumb. ex. Ach.) Delise         | NEW       | Reunion Island, R1126 (LG)                | KF704260 | JX494249 | JX494274 | KF704315 |                   |
| *Pannaria rubiginosa* (Thumb. ex. Ach.) Delise         | NEW       | Reunion Island, R1011 (LG)                | KF704261 | JX494245 | JX494270 | KF704314 | KF704323 |
| *Pannaria sp.*                                          | NEW       | Thailand                                   | KF704247 | KF704289 | KF704290 | KF704306 | KF704333 |
| *Pannaria sphinctrina* (Mont.) Hue                     | 96 (ITS, mtSSU, 87 (LSU) | Argentina                                   | EU885302 | EU885324 | GQ927271  |                        |                   |
| *Pannaria tavaresii* P.M. Jorg.                        | 96        | Argentina                                   | EU885294 | EU885316 |                   |                        |                   |
| *Pannaria sp.* (tripartite thallus)                    | NEW       | Reunion Island, R969 (LG)                 | KF704268 | KF704286 | KF704299 | KF704341 |                   |
| *Parmeliella appalachensis* P.M. Jorg.                 | 117       | ?                                          | DQ972992 | DQ973064 |                   |                        |                   |
| *Parmeliella borbonica* P.M. Jorg. & Schumm            | NEW       | Reunion Island, R1122 (LG)                | KF704271 | JX494259 |                   |                        | KF704320 |
| *Parmeliella brisbanensis* (C. Knight) P.M. Jorg. & D.J. Galloway | NEW | Thailand, T1 (LG)                      | KF704246 | KF704280 | KF704292 |                   |                   |
| *Parmeliella brisbanensis* (C. Knight) P.M. Jorg. & D.J. Galloway | NEW | Thailand, T3 (LG)                      | KF704277 | KF704281 | KF704294 | KF704351 |                   |
| *Parmeliella brisbanensis* (C. Knight) P.M. Jorg. & D.J. Galloway | NEW | Thailand, T7 (LG)                      | KF704276 | KF704282 | KF704295 | KF704352 |                   |
| *Parmeliella brisbanensis* (C. Knight) P.M. Jorg. & D.J. Galloway | NEW | Reunion Island, R1019 (LG)            | KF704278 | JX494255 | KF704296 | KF704350 |                   |
| *Parmeliella brisbanensis* (C. Knight) P.M. Jorg. & D.J. Galloway | NEW | Reunion Island, R1247 (LG)            | KF704262 | JX494258 | KF704297 | KF704347 |                   |
| *Parmeliella mariana* (Fr.) P.M. Jorg. & D.J. Galloway | NEW       | Reunion Island, R974 (LG)                | KF704275 | JX494256 | KF704301 | KF704330 |                   |
| *Parmeliella miradorensis* Vain.                       | 12        | Spain, La Gomera                           | HQ268592 | HQ268591 |                   |                        |                   |
| *Parmeliella parvula* P.M. Jorg.                       | 118       | Norway                                     | GU570099 | GU570031 |                   |                        |                   |
| *Parmeliella polyphyllina* P.M. Jorg.                  | NEW       | Reunion Island, R1021 (LG)               | KF704265 | JX494251 | JX494276 | KF704317 | KF704327 |
| *Parmeliella polyphyllina* P.M. Jorg.                  | NEW       | Reunion Island, R1058 (LG)               | KF704267 | JX494252 | JX494277 | KF704319 | KF704326 |
| *Parmeliella polyphyllina* P.M. Jorg.                  | NEW       | Reunion Island, R1120 (LG)               | KF704266 | JX494250 | JX494275 | KF704318 |                   |
| *Parmeliella sp.* (mariana gr.)                        | NEW       | Thailand, T2 (LG)                        | KF704283 |                   | KF704293 | KF704348 |                   |
| *Parmeliella sp.* (mariana gr.)                        | NEW       | Thailand, T6 (LG)                        | KF704279 | KF704284 | KF704304 | KF704349 |                   |
| *Parmeliella stylophora* (Vain.) P.M. Jorg.            | NEW       | Reunion Island, R979 (LG)               | KF704274 | JX494257 |                   | KF704300 | KF704331 |
| *Parmeliella triptophylla* (Ach.) Müll. Arg.           | 120(ITS), 19 | Finland, ITS, Sweden                    | HM448807 | AY652623 | GQ259008 | GQ259075 |                   |
| *Parmeliella triptophyllaides* P.M. Jorg.              | NEW       | Reunion Island, R965 (LG)               | KF704264 | JX494253 | JX494278 | KF704316 | KF704324 |
| *Peltigera aphthosa* (L.) Wild.                        | 121, 122  | Sweden                                     | KC437624 | AY340515 | AF286759 | DQ915598 |                   |
| *Physma byrsaeum* (Ach.) Tuck.                         | 19        | Tahiti                                     | GQ259039 | GQ259010 | GQ259077 |                   |                   |
| *Physma byrsaeum* (Ach.) Tuck.                         | NEW       | Reunion Island, R2847 (LG)               | KF704272 | JX494260 |                   | KF704303 | KF704338 |
| *Physma byrsaeum* (Ach.) Tuck.                         | NEW       | Reunion Island, R2 (LG)                 | KF704273 | KF704287 |                   | KF704302 | KF704340 |
| *Physma byrsaeum* (Ach.) Tuck.                         | NEW       | Reunion Island, R1121 (LG)              | KF704269 | JX494254 |                   | KF704298 | KF704337 |
| *Physma pseudoisidiatum* Aptroot & Simpson              | 19        | USA                                        | GQ259041 | GQ259012 |                   |                   |                   |
| *Physma radians* Vain.                                 | 19        | Japan                                       | GQ259040 | GQ259011 | GQ259078 |                   |                   |
| *Physma radians* Vain.                                 | NEW       | Thailand, T5 (LG)                        | KF704270 | KF704285 |                   | KF704305 | KF704336 |
| *Placynthium nigrum* (Huds.) Gray                       | 119 (ITS), 19 | Sweden                                   | HQ650699 | AY340518 | AF356674 | GQ259079 |                   |
| Mycobiont species                  | Reference                  | Country of origin and voucher information | ITS          | mtSSU        | LSU          | RPB 1        | cyanobacterial 16S |
|-----------------------------------|----------------------------|--------------------------------------------|--------------|--------------|--------------|--------------|-------------------|
| **Protopannaria pezizoides** (Weber ex. F.H. Wigg.) P.M. Jørg. & S. Ekman | 93 (ITS), 19 | Sweden                                    | AF429271     | AY340519     | AY340561     | GQ259081     |                    |
| **Poroma hypnorum** (Vahl.) Gray  | 93 (ITS), 19 | Sweden                                    | AF429272     | AY340523     | AY340565     | GQ259085     |                    |
| **Poroma palaecum** (Fr.) Nyl.    | 96 (mtSSU), 87 | Argentina                                 | GQ927304     | EU885327     | GQ927305     |              |                    |
| **Porophorus pholidotus** Elvebakk & S.G. Hong | 96 (mtSSU), 87 | Argentina                                 | EU885314     | EU885336     | GQ27289     |              |                    |
| **Ramalodium succulentum** Nyl.   | 19 | Australia                                 | GQ259043     | GQ259013     | GQ259086    |              |                    |
| **Staurolemma omphalarioides** (Anz) P.M. Jørg. & Henssen | 19 | Norway                                    | GQ259044     | GQ259014     |              |              |                    |
| **Staurolemma sp.** NEW Reunion Island, R982 (LG) | KF704263 KF704288 KF704291 |              |              |              |              | KF704329     |                    |
| **Vahliella californica** (Tuck.) P.M. Jørg. | 12 | Canada, British Columbia                   | HQ268594     | HQ268593     |              |              |                    |
| **Vahliella leucophaea** (Vahl.) P.M. Jørg. | 94 (ITS), 19 | Sweden                                    | AF429266     | AY652621     | GQ259090     |              |                    |
| **Vahliella saubinetii** (Mont.) P.M. Jørg. | 12 | Croatia                                   | HQ268602     | HQ268601     |              |              |                    |
| **Xanthopsoroma contextum** (Stirt.) Elvebakk | 97 | Argentina                                 | EU885313     | EU885335     |              |              |                    |
| **Xanthopsoroma soccatum** (R. Br. ex Cromb.) Elvebakk | 96, 87 (LSU) | Argentina                                 | EU885315     | EU885337     | GQ272823     |              |                    |

### Cyanobacterial species

**Cyanobacterial species**

(or host when applicable)

| Cyanobacterial species | Reference | Country of origin | ITS          | mtSSU        | LSU          | RPB 1        | cyanobacterial 16S |
|------------------------|-----------|-------------------|--------------|--------------|--------------|--------------|-------------------|
| Anabaena flos-aquae Brebisson ex Bornet & Flauhault | Choi & Oh unpublished |                | DQ234825     |              |              |              |                    |
| Anabaena oryzae F.E. Fritsch & Rich | Mishra et al. unpublished | India         | HM573456     |              |              |              |                    |
| Anabaena vaginicola F.E. Fritsch & Rich | Aghashariatmadari et al. unpublished | Iran         | JN873351     |              |              |              |                    |
| Blastis pusilla 1 L. | Laiimer et al. unpublished | Norway        | EU022724     |              |              |              |                    |
| Blastis pusilla 2 L. | Laiimer et al. unpublished | Norway        | EU022708     |              |              |              |                    |
| Blastis pusilla 3 L. | Laiimer et al. unpublished | Norway        | EU022728     |              |              |              |                    |
| Blastis pusilla 4 L. | Laiimer et al. unpublished | Norway        | EU022717     |              |              |              |                    |
| Chroococcus sp. | 123 | Italy            | FR798931     |              |              |              |                    |
| Collema flexa (Ach.) Ach. | 124 | Finland          | DQ265959     |              |              |              |                    |
| Collema nigrescens (Huds.) DC. | 125 | USA California | JN847352     |              |              |              |                    |
| Cycas revoluta Thunb. | 126 | Italy            | AM711533     |              |              |              |                    |
| Fischereilla muscicola (Thuret) | 127 | strain PCC 7414 | AF132788     |              |              |              |                    |
| Fuscinopannaria leucastica (Tuck.) P.M. Jørg. | NEW | Reunion Island | KF704322     |              |              |              |                    |
| Fuscinopannaria leucastica (Tuck.) P.M. Jørg. | NEW | Reunion Island | KF704353     |              |              |              |                    |
| Glacieopsis sp. | 128 | strain PCC 73106 | AB039000     |              |              |              |                    |
| Gunnera praepeps Hook. f. | 126 | New Zealand      | AM711541     |              |              |              |                    |
| Leptogium furfuraceum 1 (Harm.) Sierk | 125 | USA California | JN847353     |              |              |              |                    |
| Leptogium furfuraceum 2 (Harm.) Sierk | 129 | USA, California | JQ07761      |              |              |              |                    |
| Leptogium gelatinosum (With.) J.R. Laundon | 130 | USA             | DQ185232     |              |              |              |                    |
| Leptogium lichenoides 1 (L.) Zalbr. | 129 | Scotland        | JQ07765      |              |              |              |                    |
| Leptogium lichenoides 2 (L.) Zalbr. | 129 | Scotland        | JQ07766      |              |              |              |                    |
| Mycobiont species                     | Reference | Country of origin and voucher information | ITS | mtSSU | LSU | RPB1 | cyanobacterial 16S |
|--------------------------------------|-----------|-------------------------------------------|-----|-------|-----|------|-------------------|
| Leptogium palmatum (Huds.) Mont.     | 125       | USA Oregon                                |     |       |     |      | JN847344          |
| Leptogium pseudofurfuraceum P.M. Jørg. & A.K. Wallace | 125       | USA California                            |     |       |     |      | JN847347          |
| Leptogium satanninum (Dicks.) Nyl.   | 124       | Finland                                   |     |       |     |      | DQ265957          |
| Leptogium sp.                        | NEW       | Reunion Island, R2848 (LG)                |     |       |     |      | KF704328          |
| Leptogium sp.                        | NEW       | Reunion Island, R2849 (LG)                |     |       |     |      | KF704335          |
| Leptogium sp.                        | NEW       | Reunion Island, R2850 (LG)                |     |       |     |      | KF704334          |
| Lobaria pulmonaria 1 (L.) Hoffm.     | 125       | USA Oregon                                |     |       |     |      | JN847345          |
| Lobaria pulmonaria 2 (L.) Hoffm.     | 125       | Norway                                    |     |       |     |      | JN847357          |
| Lobaria scrobiculata (Scop.) P. Gaertn. | 129     | Scotland                                  |     |       |     |      | JQ007744          |
| Massalongia cariosa (Dicks.) Körb.   | 130       | USA                                       |     |       |     |      | DQ185235          |
| Microcoleus chthonoplastes Thur.     | 131       | Finland                                   |     |       |     |      | DQ460700          |
| Nephroma arcticum (L.) Tons.         | 129       | Finland                                   |     |       |     |      | JQ007764          |
| Nephroma bellum 1(Spreng.) Tuck.     | 120       | Finland                                   |     |       |     |      | HQ591510          |
| Nephroma bellum 2 (Spreng.) Tuck.    | 120       | Finland                                   |     |       |     |      | HQ591518          |
| Nephroma laevigatum Ach.             | 125       | Norway                                    |     |       |     |      | JN847359          |
| Nephroma parale (Ach.) Ach.          | 120       | Finland                                   |     |       |     |      | HQ591521          |
| Nephroma resupinatum (L.) Ach.       | 120       | Finland                                   |     |       |     |      | HQ591528          |
| Nephroma washingtoniense Gyeln.      | 125       | USA Oregon                                |     |       |     |      | JN847341          |
| Nodularia spumigena Mittens          | Beer et al., unpublished | USA Utah                                |     |       |     |      | FJS46713          |
| Nostoc commune 1 Vaucher             | 132       |                                           |     |       |     |      | AB088405          |
| Nostoc commute 2 Vaucher             | Gachon et al., unpublished | South Africa                            |     |       |     |      | HE974995          |
| Nostoc entophytum Bornet & Flahault  | Seq & Yokota, unpublished |                                         |     |       |     |      | AB093490          |
| Nostoc linckia (Roth) Bornet & Flahault | Seq & Yokota, unpublished |                                         |     |       |     |      | AB074503          |
| Nostoc linckia var. arvense C.B. Rhao | 132       |                                           |     |       |     |      | AB325907          |
| Nostoc muscorum 1 C. Agardh ex Bornet & Flahault | 133     | Czech Republic                            |     |       |     |      | AJ630451          |
| Nostoc muscorum 2 C. Agardh ex Bornet & Flahault | 126     | Czech Republic                            |     |       |     |      | AM711524          |
| Nostoc muscorum 3 C. Agardh ex Bornet & Flahault | Mishra et al., unpublished | India                                    |     |       |     |      | HM573462          |
| Nostoc muscorum 4 C. Agardh ex Bornet & Flahault | 126     | Czech Republic                            |     |       |     |      | AM711523          |
| Nostoc muscorum 5 C. Agardh ex Bornet & Flahault (soil) | 130     | France                                    |     |       |     |      | DQ185254          |
| Nostoc punctiforme (Kützing) Harlot Gunnera manicata | 130     | Germany                                   |     |       |     |      | DQ185256          |
| Nostoc sp. 1                         | Liaimer et al., unpublished | Norway                                  |     |       |     |      | EU022737          |
| Nostoc sp. 2                         | Suzuki et al., unpublished |                                         |     |       |     |      | GU062468          |
| Nostoc sp. 3                         | Suzuki et al., unpublished |                                         |     |       |     |      | GU062469          |
| Nostoc sp. 4(root of plant)          | 126       | Italy                                     |     |       |     |      | AM711532          |
| Nostoc sp. 5                         | 134       | South Africa                              |     |       |     |      | AJ344583          |
| Nostoc sp. 6                         | Liaimer et al., unpublished | Norway                                  |     |       |     |      | EU022709          |
| Nostoc sp. 7                         | Liaimer et al., unpublished | Norway                                  |     |       |     |      | EU022729          |
| Mycobiont species | Reference | Country of origin and voucher information | ITS | mtSSU | LSU | RPB1 | cyanobacterial 16S |
|-------------------|-----------|------------------------------------------|-----|-------|-----|------|-------------------|
| Nostoc sp. 8      | Mishra et al. unpublished | strain PCC 7120 |               |        |     |      | HM573458           |
| Nostoc sp. 9      | 132       | strain PCC 7906                           |     |       |     |      | AB325908           |
| Nostoc sp. 10     | Liaimer et al. unpublished | Norway |               |        |     |      | EU022713           |
| Nostoc sp. 11     | 126       | Italy                                     |     |       |     |      | AM711549           |
| Nostoc sp. 12     | 135       | Spain                                     |     |       |     |      | HM623782           |
| Pannaria aff. leproloma 1 | 17   | Chile                                     |     |       |     |      | EF174208           |
| Pannaria aff. leproloma 2 | 17 | Chile                                     |     |       |     |      | EF174213           |
| Pannaria andina 1 P.M. Jørg. & Sipman | 17 | Peru                                     |     |       |     |      | EF174233           |
| Pannaria andina 2 P.M. Jørg. & Sipman | 17 | Chile                                     |     |       |     |      | EFS36022           |
| Pannaria araneosa (C. Bab.) Hue | 17 | New Zealand                              |     |       |     |      | EF174222           |
| Pannaria athroophylla (Stirl.) Elvebakk & Galloway | 17 | Chile                                     |     |       |     |      | EF174202           |
| Pannaria cf. allorhiza | 17 | New Zealand                              |     |       |     |      | EF174206           |
| Pannaria conoplea (Ach.) Bory | 17 | Norway                                    |     |       |     |      | EF174221           |
| Pannaria durietzii (P. James & Henssen) Elvebakk & D.J. Galloway | 17 | New Zealand                              |     |       |     |      | EF174227           |
| Pannaria elixii P.M. Jørg. & D.J. Galloway | 17 | New Zealand                              |     |       |     |      | EF174230           |
| Pannaria fulvescens (Mont.) Nyl. | 17 | New Zealand                              |     |       |     |      | EF174231           |
| Pannaria isabellina 1 (Vain.) Elvebakk & Bjerke | 17 | Chile                                     |     |       |     |      | EF174226           |
| Pannaria isabellina 2 (Vain.) Elvebakk & Bjerke | 17 | Chile                                     |     |       |     |      | EF174223           |
| Pannaria obscura Müll. Arg. | 17 | Australia                                 |     |       |     |      | EF174232           |
| Pannaria patagonica (Malme) Elvebakk & D.J. Galloway | 17 | Chile                                     |     |       |     |      | EF174204           |
| Pannaria rubiginella P.M. Jørg. | 17 | Chile                                     |     |       |     |      | EFS36024           |
| Pannaria rubiginosa (Thunb. ex. Ach.) Delise | 17 | Norway                                    |     |       |     |      | EF174220           |
| Pannaria sphinctrina Zahlbr. | 17 | Chile                                     |     |       |     |      | EF174205           |
| Parmeliella triptophylla (Ach.) Müll. Arg. | 125 | Norway                                    |     |       |     |      | JN847361           |
| Peltigera aphthosa (L.) Willd. | 130 | Switzerland                               |     |       |     |      | DQ185253           |
| Peltigera canina 1 (L.) Willd. | 130 | USA                                       |     |       |     |      | DQ185230           |
| Peltigera canina 2 (L.) Willd. | 130 | Norway                                    |     |       |     |      | EU022726           |
| Peltigera didactyla (With.) J.R. Laudon | 130 | Poland                                    |     |       |     |      | DQ185245           |
| Peltigera evansiana Gyeln. | 129 | USA, Oregon                               |     |       |     |      | JQ007784           |
| Peltigera leucophlebia 1 (Nyl.) Gyeln. | 136 | Finland                                   |     |       |     |      | FJ815321           |
| Peltigera leucophlebia 2 (Nyl.) Gyeln. | 129 | Svalbard                                  |     |       |     |      | JQ007783           |
| Peltigera malacea (Ach.) Funch | 137 | Finland                                   |     |       |     |      | EF102280           |
| Peltigera rufofuscens 1(Weiss) Humb. | 130 | Germany                                   |     |       |     |      | DQ185219           |
| Peltigera rufofuscens 2 (Weiss) Humb. | 130 | Germany                                   |     |       |     |      | DQ185215           |
| Peltigera scabrosa Th. Fr. | Liaimer et al. unpublished | Norway |     |       |     |      | EU022727           |
| Peltigera sp. | 129 | Argentina                                |     |       |     |      | JQ007785           |
| Physma byrsæum (Ach.) Tuck. | NEW | Reunion Island, R1 (LG)                   |     |       |     |      | KF704342           |
| Physma byrsæum (Ach.) Tuck. | NEW | Reunion Island, R2846 (LG)                |     |       |     |      | KF704339           |
concatenated the different loci. As several species are represented by sequences obtained from specimens collected in the different parts of the world, mostly with ITS, we further assembled a 3 loci dataset excluding ITS. We thus produced three matrices, two for each part of the world, mostly with ITS, we further assembled a 3 loci concatenated dataset, as well as on a subset of 20 trees (10 from each run of the Bayesian analysis) and with Mesquite version 2.75 [71–72] using the likelihood parameters and the default settings, calculating the average probabilities of the ancestral states based on the same subset of 20 trees.

We also used BayesTraits version 1.0 [73] on a set of 2 trees: the best tree produced by the ML analysis on the Pannariaceae 4 loci concatenated dataset and on the best tree of the concatenated analysis without 5.8S, as they were slightly different, to constrain some branches (ancestors) to be to a certain state. We compared the harmonic mean of the iterations, which is an approximation of the marginal likelihood of the model, calculating the Bayes Factor, which is twice the difference of likelihood between the models, with each state of ancestor, to see which state of the ancestor leads to the best likelihood of the model. A positive Bayes Factor suggests that the first character state tested has a better likelihood than the second one, and a Bayes Factor above 2 is considered significant (Bayestraits Manual, available at http://www.evolution.rdg.ac.uk/BayesTraits.html). We used reversible jump and a gamma hyperprior whose mean and variance vary between 0 and 10. We ran the program for 50×10⁶ iterations for each constrained state. The character reconstructed was the type of thallus, and the character states considered were tripartite, pannarioid bipartite and collematoid bipartite.

### Maximum Likelihood and Bayesian Phylogenetical Analyses

For each matrix, we produced the best likelihood tree and bootstrapped for 1000 pseudoreplicates in the same run using RAxML version 7.4.2 [62–63] with the default settings and the GTRCAT model. We further ran a Bayesian analysis using MrBayes version 3.1.2 [66]. Each analysis consisted of 2 runs of 5 heated chains and 1 cold one. We assessed the convergence using Tracer version 1.5 [60] and stopped the runs after checking with AWYTT [69] that convergence was reached for each run and that tree topologies have been sampled in proportion of their true posterior probability distribution. The analysis for the family Pannariaceae was stopped after 15×10⁶ generations, the analysis on _Nostoc_ 16S after 35×10⁶ generations.

### Ancestral State Reconstruction

We reconstructed ancestral character states using SIMMAP version 1.5.2 [70], with default settings, on the consensus Bayesian tree produced by the MrBayes analysis on the Pannariaceae 4 loci concatenated dataset, as well as on a subset of 20 trees (10 from each run of the Bayesian analysis) and with Mesquite version 2.75 [71–72] using the likelihood parameters and the default settings, calculating the average probabilities of the ancestral states based on the same subset of 20 trees.

We tested different tree topologies on the concatenated dataset of 4 loci for the Pannariaceae. We generated 8 constrained best trees with RAxML, with the same settings as above, and using the following constraints: (1) the 3 accessions of _Kroswia, Physma_ and the undescribed species with a tripartite thallus, including the four loci 5.8S, mtSSU, LSU and RPB1 or including only the latter three, and one with the _Nostoc_ 16S data.

For the concatenated analysis of the four loci, we partitioned the data in different subsets to optimize likelihood. We used PartitionFinder [65] to choose the best partition and determine the best models for the different subsets. We used BIC as the criterion to define the best partition, and compared all models implementable in MrBayes [66]. The partition tested for the analysis on the four loci was composed of 6 subsets: _RPB1_, 1st codon position, _RPB1_, 2nd codon position, _RPB1_ 3rd codon position, mtSSU, LSU, 5.8S. For the 16S analysis on _Nostoc_, we used MrModelTest version 2.3 [67] to determine the best model.

### Topological Tests

We tested different tree topologies on the concatenated dataset of 4 loci for the Pannariaceae. We generated 8 constrained best trees with RAxML, with the same settings as above, and using the following constraints:

1. The 3 accessions of _Kroswia_ forming a monophyletic group;
2. _Kroswia_ as a monophyletic group basal to a group formed by _Fuscopannaria ahnieri, F. confusa, F. leucosticta_ and _F. praetermissa_; (3) _Kroswia_ as a monophyletic group basal to all accessions of _Fuscopannaria_ except _F. sampaiata_; (4) all accessions of _Fuscopannaria_ except _F. sampaiata_ as basal to the _Physma_ clade (which includes _Pannaria_ clade (all _Pannaria_ except the _Nostoc_ 16S gene analysis).
[19] and Spribille & Muggia [10]; (5) the tripartite species annotated as the tripartite R969 as basal to a group formed by all accessions of Parmeliella mariana group and Physma resolved in the same clade; (6) all accessions of Physma as basal to all accessions of Parmeliella mariana group and the tripartite R969 in the same clade; (7) Parmeliella borbonica basal to all accessions of Physma; (8) all accessions of Physma basal to all accessions of Parmeliella mariana group including Parmeliella borbonica in the same clade. We computed the likelihood of 100 trees (the best constrained tree, the best unconstrained tree and a random sample of 98 bootstrap replicate trees from the unconstrained analysis), estimating parameters on a NJ tree, using an HKY model with a gamma rate of heterogeneity and 4 gamma categories (parameters choice and methodology suggested by [74]). We performed the 1sKH test [75–77], the SH test [75] and the ELW test [78] on the constrained tree using TreePuzzle v. 5.2, [79]. Due to its very low power (see for instance [74]), we did not consider the results of the SH test.

Results

Molecular Data

We amplified ITS, mtSSU and RPBI for all 36 selected specimens, except one for RPBI. We amplified LSU for 21 specimens, all 15 negative results being resolved in a single clade comprising all accessions of Physma, the Parmeliella mariana gr. (P. brachanensis, P. mariana and P. stylophora), Parmeliella borbonica and the undescribed tripartite ‘Pannaria’ R969 (here annotated the tripartite R969). Wedin et al. [19] could amplify the LSU loci for three species of Physma, but, for unknown reasons, all our attempts to amplify LSU for this clade failed.

Matrix Assemblage and Concatenation

For the analysis on the Pannariaceae mycobiont, we could include the following newly sequenced specimens: 21 specimens with all 4 loci, 14 with 3 loci (lacking LSU) and 1 specimen with 2 loci (lacking LSU and RPBI). We added 46 taxa retrieved from GenBank to complete our sampling, 39 members of the Pannariaceae, and 7 outgroup taxa all belonging to the Peltigerales (3 Vahliellaceae, 1 Collemataceae, 1 Placynthiaceae, 1 Peltigeraeaceae). Those included either the 4 loci or a subset of them. Detailed information can be found in table 1. For the 16S dataset on Nostoc, we produced 36 new sequences; we added 93 Nostoc sequences retrieved from GenBank, chosen either on the phylogenetic position of their fungal partner or their nucleotide similarity to our sequences, based on megaBLAST searches [60], and 14 outgroup sequences, belonging to other genera, to complete our sampling.

Partitioning and Model Selection

For the analysis on the Pannariaceae mycobiont, PartitionFinder divided the partition in 4 subsets: one composed of RPBI 1st and 2nd codon positions with LSU, one with mtSSU only, one with 5.8S only and one with RPBI 3rd codon position only. For the first subset, the model selected was GTR+I+G, as well as for mtSSU and RPBI 3rd codon position; for 5.8S, the model selected was K80+I+G. For the analysis on the Nostoc 16S dataset, the model selected was GTR+I+G.

Phylogenetic Analyses

The 50% Bayesian consensus tree of the analysis of the Pannariaceae mycobiont dataset comprising 4 loci is presented in Figure 1, with the bootstrap values of the ML analysis and the Bayesian PP values written above the branches. The same consensus tree obtained with the 3 loci dataset is available in the Supplementary Material (Figure S1). The 50% Bayesian consensus tree of the analysis of the Nostoc 16S dataset is presented in Figure 2, with the bootstrap values of the ML analysis and the Bayesian PP values written above the branches.

Phylogeny of the Family Pannariaceae (Fig. 1)

Topology of the family.

The analysis of the 3 and 4 loci datasets yielded the same topology, albeit with less support for some branches for the former; as expected the 5.8S loci provides an interesting resolution power to discriminate branches at the generic and infrageneric level. We retrieved the Pannariaceae as a monophyletic group, divided into two strongly supported clades: the first one includes all Parmeliella accessions, incl. the genus type P.triphyllea, except for the P. mariana group and P. borbonica which are resolved with strong support in the other clade. The so-called Parmeliella s. str. clade further includes Degelia (here resolved as polyphyletic, as already detected by Wedin et al. [19]), Erioderma, Leptogium and the monotypic foerensensia which represents the only tripartite species in this clade. The second clade can be divided into three groups: (1) the first one is not supported in ML optimization but gets a PP = 0.95 in Bayesian analysis; it is composed of Xanthoparmelia, Physma, Pannaria, mariana group, Parmeliella borbonica and the tripartite species R969, and will be referred to as the Physma group; (2) a group not supported in ML optimization but getting a PP = 0.94 in Bayesian analysis, composed of Pannaria, Staurolema, Ramalodium, Fuscodermia, Pororna and Porophorus, that will be referred to as the Pannaria group; and finally (3) a group composed of Fuscopannaria, Krossia, Protoxopannaria, Lecophysma and Parmeliella parvula, that will be referred to as the Fuscopannaria group.

Wedin et al. [19] and Spribille and Muggia [10] retrieved the Parmeliella s. str. group, the Pannaria group and the Fuscopannaria group with similar topology as ours. However, in their studies, their single or multiple accessions of Physma is or are nested within the Pannaria group. With our dataset, which includes a larger sampling of Physma and representatives of the closely related Parmeliella mariana gr., P. borbonica and the tripartite R969, the hypothesis of the whole Physma group nested in the Pannaria group and the Fuscopannaria group as basal is strongly rejected by two topological tests (ELW and 1sKH tests; see table 2).

Monophyly of Several Genera

Our accessions of Kroszia crystallifera (the type species of the genus; [27]) gathered in Madagascar and Reunion are not resolved as a monophyletic group; they are nested within Fuscopannaria, and closely related to its type species F. leucosticta [38]. Even with the exclusion of species now referred to Vahliella [12,30], the genus Fuscopannaria is not resolved as monophyletic, unless F. sampaiana is excluded and Kroszia crystallifera included. Two strongly supported clades can be distinguished if the genus is so recircumscribed: one with F. ignobilis and F. mediterranea and the other with the type species and Kroszia crystallifera.

Pannaria is resolved as a diverse but nevertheless well-supported genus, including several tripartite species formally placed in the genus Pororna and which were transferred to Pannaria following the detailed studies by Elvebakk [81–84], Elvebakk & Bjørke [85], Elvebakk & Galloway [86] and Elvebakk et al. [17]. Interestingly, our single accession of the tripartite Pannaria-like R969 is not resolved amongst other tripartite Pannaria but within the Physma clade with strong support. It therefore appears that the tripartite Pannaria-like species are more diverse than expected and that the tripartite habit is widespread amongst the Pannariaceae, being absent only in the Fuscopannaria group. Two recently described and
tripartite genera *Xanthopsoroma* and *Psorophorus*, segregated from *Psoroma* [87], are retrieved as a part of the *Physma* gr. with support only in the Bayesian analysis for the former, and as sister to *Psoroma s. str.* in the *Pannaria* group for the latter.

*Parmeliella* (type species: *P. triptophylla*) is a well-supported monophyletic group if the *Parmeliella mariana* gr., *Parmeliella borbonica* and *P. parvula* are excluded. The latter is resolved with strong support within the *Fuscopannaria* gr. whilst the others are resolved within the *Physma* group, on a long and strongly supported branch. Further, *P. borbonica* appears nested inside *Physma*, which is therefore paraphyletic.

**Nostoc Phylogeny (Fig. 2)**

We defined phylotypes (A to G) on the *Nostoc* tree based on well-supported monophyletic groups containing sequences from our representatives of the *Pannariaceae* family. All our sequences are part of *Nostoc* clade 2 (*sensu* [59,88]) except phylotype G, which seems related to *Nostoc clade 3 sensu* Svenning et al. [59].

There is no evidence suggesting coevolution or cospeciation events between the mycobiont and the photobiont. The phylogeny of *Nostoc* involved in the lichen symbiosis does not match the phylogeny of the *Pannariaceae*.

**Topological Uncertainties (Table 2)**

The tests do not reject the monophyly of *Kroswia*, either its position outside of the polytomy including *Fuscopannaria leucosticta* and *F. praetermissa*, although the difference of likelihood with the best unconstrained tree is relatively high (13.68). However, the position of *Kroswia outside of Fuscopannaria s. str.* (including *F. mediterranea* and *F. ignobilis*) is significantly rejected by the ELW and 1sKH tests. Therefore *Kroswia crystallifera* should be considered as part of *Fuscopannaria*.
Concerning the position of the tripartite R969, the topological tests do not reject its position at the base of the Physma group as a whole. However, its position at the base of the Parmeliella mariana gr., with Physma basal to both of them, is significantly rejected by the ELW and 1sKH tests.

Concerning the position of Parmeliella borbonica, the topological tests do not reject its position neither as basal to Physma, nor as basal to the Parmeliella mariana gr., with Physma basal to both of them, although the difference of likelihood for the latter case is relatively high (10.29). We consider that the weak resolution of the test regarding the position of Parmeliella borbonica might be due to a large amount of missing data as only 2 loci are available for this accession, reducing its impact on the likelihood of the trees. More material should therefore be studied before the taxonomic status of P. borbonica can be revised.

As commented above, we also tested the topology proposed by Wedin & al. [19] and Spribille & Muggia [10] where their accessions of Physma are resolved within the Parmelia gr. Such a topology is rejected on our dataset by the ELW and 1sKH tests.

Reconstruction of Ancestral States (Fig. 1, Table 3)

Results of the SIMMAP reconstructions on the Bayesian consensus tree are shown in pie charts on Figure 1. Results of the BayesTraits and Mesquite reconstructions, as well as the SIMMAP reconstruction on 20 trees are shown in table 3.

Even though the probability values can vary quite widely from a reconstruction method to the other, the same ancestral character state is recovered for most branches.

For the Fuscopannaria group, a pannarioid ancestor is strongly supported, incl. for the Fuscopannaria s. str. clade (all Fuscopannaria except for F. samponiana). Within the Parmelia group, two deep nodes are recovered with a tripartite ancestor (the unresolved clade with all accessions of Parmelia, Physma, Poronema and Porophorus) as well as the node supporting the whole group. The node supporting both groups (the Fuscopannaria and the Parmelia gr.) also has tripartite thalli as the most likely ancestral type. For the clade comprising Physma, the Parmeliella mariana gr., P. borbonica and the tripartite R969, reconstructions favor a pannarioid ancestor without much support, except the Bayes Factor that slightly favors a tripartite ancestor. However, for the whole group and thus including both accessions of Xanthoparmelia, reconstructions recover a tripartite ancestor with strong support. The node supporting the three groups (Fuscopannaria-, Parmelia-, and Physma-group) has most likely a tripartite thallus, as recovered by all four methods. The Parmeliella s. str. group most probably had a pannarioid ancestor, as well as the family Pannariaceae.

Discussion

Nostoc from Collematoid and Pannarioid Thalli (Fig. 2)

Thalli belonging to the collematoid or pannarioid types never share the same Nostoc phylotype. Phylotypes A, E and F only contain symbionts from collematoid thalli. Moreover phylotype F also contains symbionts associated with the lichen genus Leptogium, a typical representative of the collematoid type, these accessions being resolved in a strongly supported clade together with the Kroswia symbionts. Phylotype E includes the photobiont of several Physma accessions together with that of the cephalodia of the tripartite R969, and these cephalodia have the same homomorphous structure as the thalli of Physma hysaenum (Fig. 3a, c).

Phylotypes B, C, D and G only contain symbionts from pannarioid thalli. Phylotype B which contains the photobiont of our accession of the terricolous Fuscopannaria protermitina is closely related to sequences from terricolous-muscicolous Nephroma arcticum photobionts whereas phylotypes C and D contain Nostoc sequences from epiphytic Lobaria, Nephroma and Pseudocyphellaria, along with our accessions of epiphytic Pannariaceae with pannarioid thalli. This confirms that Nostoc from epiphytic heteroimerous thalli cluster together, although they group in a polyphyletic assemblage of different phylotypes [17,89,90]. These data strongly suggest that many pannarioid thalli share Nostoc strains between them and with other representatives of the Peligerales that also have Nostoc in a well-defined thin layer. Furthermore collematoid thalli can share

Table 2. Topology tests.

| Constraint | logL best tree | diff. with unconstrained | 1sKH test | ELW test |
|------------|----------------|--------------------------|-----------|----------|
| Kroswia monophyletic | −19700.43 | 2.77 | 0.312 | 0.0816 |
| Kroswia out of F. leucosticta group | −19711.34 | 13.68 | 0.145 | 0.0239 |
| Kroswia out of Fuscopannaria s. str. | −19741.75 | 44.09 | 0.002 | 0 |
| Physma group in Parmelia group, Fuscopannaria group | −19730.55 | 32.89 | 0.019 | 0.011 |
| R969 basal out of Physma/Parmeliella mariana group | −19701 | 3.34 | 0.299 | 0.0816 |
| Physma basal to R969/Parmeliella mariana group | −19731.4 | 33.75 | 0.007 | 0 |
| R1122 basal to Physma | −19703.25 | 5.59 | 0.165 | 0.041 |
| R1122 basal to P. mariana group; Physma outside | −19707.95 | 10.29 | 0.094 | 0.018 |

Likelihood values of the best trees and results of the 1sKH test and ELW test on the different constraints on the topology of the tree. Results in bold significantly reject the concerned topologies.

doi:10.1371/journal.pone.0089876.t002
Table 3. Reconstruction of ancestral states.

| Node                                             | SB     | S20    | M      | BF[T>P] | BF[T>C] |
|--------------------------------------------------|--------|--------|--------|---------|---------|
| *F. leucosticta* + *F. praetemissia*              | P = 0.99 | P = 0.99 | P = 0.99 |         |         |
| *Fuscopannaria* s. str. (incl. *F. ignobilis*, w/o *F. sampaiana*) | P = 0.99 | P = 0.99 | P = 0.99 |         |         |
| *Fuscopannaria* gr. (incl. *F. sampaiana*)       | P = 0.99 | P = 0.97 | P = 0.73 |         |         |
| genus *Pannaria*                                 | T = 0.99 | T = 0.98 | T = 0.91 | 9.66    |         |
| genus *Pannaria* w/o *P. implexum*               | T = 0.99 | T = 0.8  | T = 0.84 |         |         |
| *Porophora* + *Psorophorus* + *Fuscoederma*      | T = 0.98 | T = 0.93 | T = 0.83 |         |         |
| *Pannaria* group (incl. *Porophora*, *Staurolemma* etc.) | T = 0.94 | T = 0.86 | T = 0.81 |         |         |
| *Fuscopannaria* + *Pannaria*                     | T = 0.91 | T = 0.84 | T = 0.77 | 1.4     |         |
| *Physma* + *Parmeliella mariana* gr.              | P = 0.58 | P = 0.5  | P = 0.39 | 0.32    | 3.94    |
| *Physma* + *Parmeliella mariana* gr. + *Xanthopsoroma* | T = 0.99 | T = 0.99 | T = 0.91 | 11.7    | 8.7     |
| *Fuscopannaria* + *Pannaria* + *Physma*          | T = 0.92 | T = 0.89 | T = 0.815 | 1.06    |         |
| *Parmeliella* s. str. (incl. *Erioderma* etc.)   | P = 0.98 | P = 0.99 | P = 0.87 |         |         |
| family *Pannariaceae*                            | P = 0.7  | P = 0.71 | P = 0.46 |         |         |

T = tripartite, P = pannarioid, C = collematoid. SB = SIMMAP results on the 50% consensus Bayesian tree; S20 = SIMMAP results on the subset of 20 trees, M = Mesquite results, BF = Bayes Factor of the BayesTraits analysis, T>P = Tripartite rather than pannarioid ancestor, T>C = Tripartite rather than collematoid ancestor.

doi:10.1371/journal.pone.0089876.t003

Figure 3. Selected pictures of studied Pannariaceae. Column, from left to right: a: tripartite R969, b: pannarioid *Parmeliella mariana*, c: collematoid *Physma byrsaeum*, d: pannarioid *Fuscopannaria leucosticta*, e: collematoid *Kroswia crystallifera*. Top row: macroscopic pictures showing the general aspect of the thallus; arrow point to cephalodia. Middle row: microscopic pictures showing the position of the *Nostoc* cells inside the thallus. Bottom row, left: Microscopic picture showing the position of the green algal cells in the thallus; right: macroscopic picture showing the aspect of *Kroswia* when wet.

doi:10.1371/journal.pone.0089876.g003
Nostoc with representatives of the Collemataceae that also have Nostoc chains throughout their thallus.

These results strongly suggest that the thallus type (collematoid versus pannarioid), and the organization of the Nostoc cells inside it, depend on the phylotype of the Nostoc with which the mycobiont associates. Therefore, it seems that in the family Pannariaceae, the Nostoc associated with the mycobiont would have more impact on the morphology of the thallus formed than the phylogenetic origin of the mycobiont. The corollary might be true as well, the Nostoc selection by the mycobiont is more affected by the morphological and ecophysiological characteristics of the association than by the phylogenetic position of the mycobiont. Extracellular polysaccharides substances (EPS) produced by many bacterial lineages, incl. cyanobacteria, are involved in the physiological and ecological characteristics of those organisms [91]; in Nostoc, the biochemistry and structure of the dense sheath of glcyan strongly participate in the desiccation tolerance of Nostoc commune [92]. Although no clear evidence is available, we suspect that variations in the glcan sheath characteristics amongst the various strains of Nostoc involved in the lichenization events within the Pannariaceae drive the differences between the collematoid and the pannarioid thallus types.

Occurrence of Collematoid Thalli All across the Pannariaceae (Fig. 1)

We found collematoid thalli in the four main groups of the family. Kroasia and Lectophysuma appear as part of the Fuscopannaria group, Kroasia being nested within Fuscopannaria s. str., excluding F. sampanana; Staurodemma and Ramalodium are part of the Pannaria group and Pannaria sartessansi was described as a collematoid thallus species; Physma is in the Physma group, along several taxa with pannarioid thallus; and finally Lepidogium is part of the Parmellales s. str. group. These results suggest that thalli switched from pannarioid type to collematoid and possibly vice versa several times along the evolutionary history of the family.

These results also suggest that the thallus type organized by the association between a mycobiont and a photobiont is primarily driven by the identity of the latter, the Nostoc phylotype with which it associates rather than by the phylogenetic identity of the mycobiont. Indeed, unlike the original assumption that all collematoid thalli were part of the Collemataceae and all pannarioid thalli were part of the Pannariaceae, many collematoid thalli are actually members of the Pannariaceae, as already detected by Wedin et al. [19] and Otálora et al. [35]. Moreover, they do not form a monophyletic group inside the Pannariaceae, but are present all across the family, suggesting the absence of phylogenetic pattern of the mycobiont related to the collematoid morphological and anatomical thallus type.

Evidence for Coincidence between Photobiont Switch and Change of Thallus Type

The most spectacular and straightforward example lies with the type species of Kroasia which is nested inside Fuscopannaria s. str.: it exhibits a drastic change of morphology (see figure 3d–e) of the thallus (all representatives of this genus so far have typical pannarioid thalli), and it associates with a Nostoc phylotype (phylotype F) that is totally different from the one associating with the closely related Fuscopannaria leucosticta (phylotype D). Moreover, phylotype F has also been found associated with the typically collematoid Leptogium lichenoides. The duo Kroasia/ Fuscopannaria thus provides the best example of the influence of the Nostoc on the shape of the thallus. Actually, K. crystalliifera is a species of Fuscopannaria with little genetic divergence with its related species such as F. leucosticta and F. praetemissar, this divergence however precludes any assumption that it could be considered as a photomorph of one of them. Its thallus is dramatically different because it switched to a different Nostoc, one that triggers the collematoid format for the thallus. Jorgensen [24], when studying the apothecia characters of the other species assigned to that genus (K. gemasseni), concluded that “the characters of the hymenium and the chemistry of the thallus certainly place it close to Fuscopannaria (...)”. Quite interestingly another photobiont switch can be postulated in that group as the phylogenetic position of Moelleropopsis usbalsa as sister to F. leucosticta has been retrieved by Ekman & Jorgensen [93] and more recently announced as confirmed [94]. This species exhibits granulose thalli with clusters of Nostoc interwoven and covered by short-celled hyphae and very much different from the pannarioid thallus type, and thus most probably associated with a different Nostoc phylotype.

Occurrence of Tripartite Thalli All across the Pannariaceae (Fig. 1)

We could detect tripartite thalli in all main groups within the family, except in the Fuscopannaria group. This absence might be caused by incomplete sampling as the only tripartite species known in Fuscopannaria (F. viridescens, associated with a green algae and producing cehalodia; [95]) as well as both species of Dogelilina (forming tripartite thalli; [42]) could not be included in our dataset. Pannaria, Psorophorus and the tripartite representatives of Pannaria are resolved in the Pannaria group, Xanthoparmelia and the tripartite R969 belong to the Physma group, and the characteristic Jorgensenia is included in the Parmellaceae group. Until the seminal papers by Elvehakk & Galloway [86] and Passo et al. [96], all tripartite Pannariaceae were assigned to a single genus (Pannaria) assumed to form a monophyletic group. Within the three main groups of the Pannariaceae where they are resolved, the species with tripartite thalli are mixed up with species with bipartite thalli, mainly of pannarioid type but also with collematoid type. These results suggest that several times through the history of the family, mycobionts switched from a tripartite to a bipartite thallus or vice versa.

Evidence for Cephalodia Emancipation

Switches from a tripartite to a bipartite thallus may involve the cehalodia and their emancipation from their green algae-containing thall. Although cehalodia are usually associated with rather small, firmly attached, or even included, structures, there are many examples of tripartite Pannaria and Psoroma in which cehalodia are large and easily detached, or proliferating and developing large squamules that can be easily detached from their “host” thalli (examples in [17,81,97,98]). The cehalodia of the tripartite R969 start their development as modest blue gray squamules over the thallus, but eventually grow up to 0.7 cm across and develop a foliose habit with denticulate to deeply lobulate margin (see figure 3a).

More interestingly, the Nostoc photobiont in several accessions of Physma byssaeum (annotated R1, R2, R2846 and R2847; phylotype E) is very closely related to the one found in the cehalodia of the tripartite R969. As the latter is basal to the clade containing all accessions of Physma, it can be postulated that several species belonging to this genus arose from cehalodia emancipation from their common ancestor. Indeed, the common ancestor of the whole Physma clade is recovered as producing tripartite thallus. Furthermore, the disposition of the Nostoc cells inside the cehalodia of R969 is similar to the one inside Physma thalli (see figure 3a–c): they are enclosed in ellipsoid chambers delimited by
medulla hyphae, these structures being responsible for the maculate upper surface of thalli (**Physma**) or cephalodia (**R**69).

Besides the tripartite **R**69, the clade included both accessions of the recently described genus *Xanthophorsoroma* [67], which also develops tripartite thalli, with a green algae as the main photobiont and *Nostoc* included in cephalodia. The three species recognized within the *Parnelia mariana* gr. may have arisen from cephalodia emancipation of their common tripartite ancestor or from a photobiont switch from a **Physma** ancestor. Quite interestingly, the pannarioid *Parnelia bornowica*, nested within **Physma**, is associated with phototype D of *Nostoc*, shared by most accessions of the *Pannaria* and *Parnelia s. str.* groups (as well as other distantly related species of the *Peltigerales*), and not phylotypes C or G, chosen by all our accessions of its closely related species of the *Parnelia mariana* gr. When excluding both accessions of *Xanthophorsoroma*, the **Physma** gr. is a well-supported clade on a long branch and includes a tripartite species, species with pannarioid as well as collematoid thalli. The long branch may indicate that our sampling is too scarce and geographically too restricted. However, as both *Physma* and the *Parnelia mariana* gr. have a pantropical distribution, we can confidently assume it would not collapse in future studies.

In figure 4, we illustrate the different possible scenarios to switch from tripartite to bipartite, and from collematoid to pannarioid thalli and vice versa, and emphasize on the possibility to obtain, with switches and time, the three types of thalli from the same tripartite ancestor.

As a matter of fact, earlier workers came close to the conclusion that cephalodia can emancipate and start their own evolutionary trajectory. Ekman & Jørgensen [93] pointed to the « homology » between the cephalodia of the green algae-containing *Psoroma hymnorum* and the thallus of the cyanobacterial autonomous species *Santessoniella polychioides*; Passo et al. [96] retrieved the latter as sister to *Psoroma aphthosum*, a green algal species with coralloid-subfruticose cephalodia, very much akin the thallus of *Santessoniella polychioides*. We strongly suspect this case represents a further case of cephalodia emancipation, and subsequent divergence. This scenario implies that emancipated cephalodia can reproduce sexually as most species of *Physma* and *Santessoniella polychioides* produce apothecia and well-developed ascospores. There is indeed no reason to believe that thalli newly formed by cephalodia emancipation and containing only *Nostoc* as photobiont would not be able to produce apothecia, as only the mycobiont is involved in such formation. An interesting alternative would be that, when expelled out of the ascus, the ascospore produced by the mycobiont involved in the ancestral tripartite thallus, would collect or recapture the *Nostoc* of the cephalodia.

Several representatives of the Lobariaceae produce photomorphs, mainly within the genera *Lobaria* and *Sticta* [14,99]. These duos involving the same fungus lichenized either with a green algae or with a *Nostoc* comprise thalli morphologically rather similar or not (see Introduction), and living attached (thus forming tripartite thalli) or not. Although molecular studies on these duos have mainly sought to demonstrate the strict identity of the fungus involved in each part, the separation or “living apart” of one from the other has long been recognized in several taxa, such as *Lobaria amplissima* and its cyanomorph *Dendricocaulon umbansense* and *Sticta canaensis* and its cyanomorph *S. dufouri* [100]. There is a priori no reason to exclude that the duos can separate on “a permanent basis” and thus emancipate; each morph would eventually run its own evolutionary trajectory, as recently suggested for divergence patterns in *Sticta* photomorphs [101]. Such a scenario can be interpreted as a variant of cephalodia emancipation as advocated here for the evolution of thallus types within the Pannariaceae.

The alternative scenario for the complex phylogenies including bi- and tri-partite thalli implies that a cyanolichen would capture a green algae from the environment (or from another lichen), adopt it as its main photobiont and confine its *Nostoc* into cephalodia. This hypothesis has been suggested by Miadlikowska & Lutzoni [32] for the sect. *Pelidina* in the genus *Peltigera* but so far has not been confirmed. Our data and reconstruction of ancestral state do not support it in the Pannariaceae, with a possible exception for *Joergensenia cephalodina*, but a better sampling is needed in that group to reconstruct the ancestral states.

**Conclusions and Perspectives**

Field observations of the lichen species belonging to the widespread and well-known order Peltigerales on the tiny and remote island of Reunion in the Indian Ocean instigated our studies on the relationships between photomorphs in the Lobariaceae (14) and the present study on the Pannariaceae. Indeed, we were intrigued by the occurrence, several times at the same locality or even on the same tree, of representatives of that family with collematoid and pannarioid thalli, and more locally of tripartite thalli.

Collematoid and pannarioid thalli are represented throughout the Pannariaceae. Each thallus type mostly appears mingled within complex topologies. Switches between those thallus types are thus frequent throughout the family. We could demonstrate that both collematoid genera in the Pannariaceae we examined from Reunion material (**Kroswia** and **Physma**) are involved in photobiont switches. We suspect that such a scenario could be detected elsewhere in the Pannariaceae and may act as an important evolutionary driver within the whole family, and perhaps elsewhere within the fungi lineages containing lichenized taxa.

The tripartite thallus type is shown to be the ancestral state in the clade we could study (the **Physma** gr.). Although a larger sampling is needed before such an result could be confirmed, we can postulate that cephalodia emancipation and subsequent evolutionary divergence is the most likely scenario within that clade. The data available support the same scenario in other clades of the Pannariaceae, and it can be suspected in the Lobariaceae where it is represented by the separation and subsequent divergence of photomorphs.

The photomorph pattern in the Lobariaceae demonstrates that a single mycobiont can recognize and recruits phylogenetically unrelated photobiont partners and these associations result in morphologically differentiated thalli. We show here that the use of different lineages of *Nostoc* or the association with only one partner instead of two might lead to the same consequences. Recognition of compatible photobiont cells is carried out by specific lectins produced by the mycobiont, characterized by their ligand binding specificity [102]. *Peltigera* species have served as models in the studies of lectins and their involvement in the recognition of symbiotic partners [103-106]. A lectin detects compatible *Nostoc* cells at the initiation of cephalodium formation in *P. aphthosa* and this process is highly specific [107], as further demonstrated by experiment of inoculation of several *Nostoc* strains into the cephalodia of the same species [108]. The biochemical process sustaining the recognition of both partners in two lichen species associated with green algae has been elucidated by Legaz et al. [109] and extended to cyanolichens with collematoid thalli by Vivas et al. [110]. The genes coding for two lectins assumed to be involved in photobiont recognition have recently been identified [111-112]. Evaluation of the variation of those genes is of tremendous interest in the context of photobiont switching and
cephalodia emancipation as lectins have been shown to be under selection pressure by the symbionts in corals [113–114] and a coevolutionary process could thus be highlighted and demonstrated in lichenized fungi. A preliminary study with *Peltigera membranacea* material from Iceland could demonstrate a significant positive selection in LEC-2 but not due to variation in photobiont partner [112].

Further research should thus assemble larger dataset of tripartite taxa within the Pannariaceae and reconstruct their evolutionary history, especially as to the fate of their cephalodia. Numerous methods for detecting genes under positive selection are available [115] and could be applied to the Pannariaceae. Genomics studies of lectins associated with photobiont recognition on tripartite taxa as well as those involved in obvious photobiont switches (pannarioid to collematoid and vice versa) could therefore bring to light a nice model of coevolution [116].

The taxonomical consequences of these results are published in a companion paper, dedicated to new taxa and new combinations.

**Data Accessibility**

All newly produced sequences are deposited in GenBank. All matrices used in the analyses are deposited in Treebase.

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**Supporting Information**

Figure S1 Phylogenetic relationships in the family Pannariaceae, based on the best ML tree of the analysis on 3 loci (LSU, mtSSU, *RPB1*). Values above branches represent ML bootstrap.

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**Acknowledgments**

Field studies in Reunion were made possible with the help and advice from the “Parc National de La Réunion”, especially through the courtesy of Mr B. Lequette. Dr Cl. Ah-Peng and Prof. D. Strasberg of the University of La Réunion in Saint-Denis and Dr. J. Hivert of the Conservatoire Botanique National de Mascarin (St-Leu) were also very helpful. A first field trip to Reunion in 2008 was conducted with our colleagues and friends Maarten Brand and Pieter van den Boom. The field trip to Madagascar was organized with the logistical support of the “Parc Botanique et Zoologique de Tsimbazaza” in Antananarivo, and with collecting and export permits of scientific material issued by the “Ministère des Eaux & Forêts”; it was organized with our colleagues and friends Damien Ertz, Eberhard Fischer Dorothee Killmann and Tahina Razafindrahaja. We thank them all very warmly. We further thank the curators of the following herbaria for the loan of type collections or relevant material: H, US. Field trip in Thailand was organized as an IAL post-symposium excursion by the Ramkhamhaeng University, and we warmly thank the Lichen Research Unit and the organizers of the field trip, Kavinmat Buurang, curator of the herbarium, Wetchasart Poliyiam and Pachara Mongkolsook. We also thank Therapat
Author Contributions
Conceived and designed the experiments: NM ES. Performed the experiments: NM ES. Analyzed the data: NM ES. Contributed reagents/materials/analysis tools: NM ES. Wrote the paper: NM ES.
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