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Slow algae, fast fungi: exceptionally high nucleotide substitution rate differences between lichenized fungi *Omphalina* and their symbiotic green algae *Coccomyxa*

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Abstract

Omphalina basidiolichens are obligate mutualistic associations of a fungus of the genus *Omphalina* (the exhabitant) and a unicellular green alga of the genus *Coccomyxa* (the inhabitant). It has been suggested that symbiotic inhabitants have a lower rate of genetic change compared to exhabitants because the latter are more exposed to abiotic environmental variation and competition from other organisms. In order to test this hypothesis we compared substitution rates in the nuclear ribosomal internal transcribed spacer region (ITS1, 5.8S, ITS2) among fungal species with rates among their respective algal symbionts. To ensure valid comparisons, only taxon pairs (12) with a common evolutionary history were used. On average, substitution rates in the ITS1 portion of *Omphalina* pairs were 27.5 times higher than rates in the corresponding pairs of *Coccomyxa* since divergence from their respective ancestor at the base of the *Omphalina*/*Coccomyxa* lineage. Substitution rates in the 5.8S and the ITS2 portions were 2.4 and 18.0 times higher, respectively. The highest rate difference (43.0) was found in the ITS1 region. These are, to our knowledge, the highest differences of substitution rates reported for symbiotic organisms. We conclude that the *Omphalina* model system conforms to the proposed hypothesis of lower substitution rates in the inhabitant, but that the mode of transmission of the inhabitant (vertical versus horizontal) could be a prevailing factor in the regulation of unequal rates of nucleotide substitution between co-evolving symbionts. Our phylogenetic study of *Coccomyxa* revealed three main lineages within this genus, corresponding to free-living *Coccomyxa*, individuals isolated from basidiolichens *Omphalina* and *Coccomyxa* isolated from ascolichens belonging to the Peltigerales.

Keywords: Nucleotide substitution rates; Coevolution; Mutualistic symbiosis; Lichens; Inhabitant/exhabitant; Basidiomycota; *Omphalina*; *Coccomyxa*

1. Introduction

Fungi play an important role in many ecologically significant mutualistic systems, such as in mycorrhizae, endophytes, and lichens. More than one-fifth of all extant fungal species are known to be lichenized, living in a close (obligate) mutualistic association with photoautotrophic green algae, cyanobacteria, or both types of photobionts (Hawksworth, 1991; Hawksworth et al., 1995). More than 99% of this diversity is found within the Ascomycota, where transitions to the lichenized state are assumed to be old (Lutzoni et al., 2001). The remaining lichenized fungal species are part of the Basidiomycota and are likely to have originated more recently (Kranner and Lutzoni, 1999; Moncalvo et al., 2000).

Law and Lewis (1983) proposed that in mutualistic ectosymbiotic systems in which one partner (the inhabitant) lives extracellularly inside the other (the exhabitant), the inhabitant should show lower rates of genetic change. This could be due to variation in abiotic environments and competition from other organisms, which forces the exhabitant to respond in an adaptive manner, leading to genetic changes. The inhabitant is expected to live in a much more stable environment, provided by the

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exhabitant, with less abiotic variation, change, and competition. In such an environment the inhabitant is assumed to experience fewer genetic changes. In addition, the hypothesis suggests that selection against sexual reproduction should act on the inhabitant.

Lichens seem to conform to the proposed trend of reduction in genetic change if the number of genera and species is used as a proxy for genetic change. A comparison of the numbers of fungal and algal genera and species-forming lichens shows a markedly higher diversity within the fungi. About 525 mycobiont genera, including approximately 13,500 lichenized species, are recognized (Hawksworth, 1988; Hawksworth, 1991). In contrast, only about 40 photobiont genera, with about 100 species of green algae and 100 species of cyanobacteria are accepted, some of them of questionable taxonomic value (Friedl and Büdel, 1996; Hawksworth et al., 1995; Tschermak-Woess, 1988). About 20% of all lichens described seems to involve just one genus of algae, *Trebouxia* (Ahmadjian, 1970; Friedl and Büdel, 1996). However, more recently Kroken and Taylor (2000) have shown that there can be a considerable diversity within the algae as well, depending on the characters that are used to circumscribe species (either based on morphology or multiple molecular markers). Lichens seem also to confirm the hypothesis of a reduction of sexual reproduction, as propagation of most known algae inside lichen thalli is largely asexual and by autospores (Friedl and Büdel, 1996; Tschermak-Woess, 1988). Evidence of recombination and hence the possibility of sexual reproduction of the algae was found in only one of the seven *Trebouxia* species Kroken and Taylor (2000) proposed.

In an attempt to test the hypothesis of a low rate of genetic change in the inhabitant, we have chosen to investigate substitution rates in the *Omphalina* model system (Lutzoni and Vilgalys, 1995). These lichens consist of a basidiomycete of the genus *Omphalina* (exhabitant) and an associated unicellular green alga *Coccomyxa* (inhabitant). *Omphalina* includes approximately 40 species, eight of which are lichenized. Recently, the latter eight species have been segregated to form a new genus, *Lichenomphalia* (Redhead et al., 2002). The five known lichen-forming species included in this study resulted from a single transition to the mutualistic state, *Omphalina ericetorum* being part of the first speciation event within this symbiotic lineage (Lutzoni, 1997). Lutzoni and Pagel (1997) detected an accelerated rate of nucleotide substitution in this monophyletic lineage when compared to non-lichenized omphalinoid species. The *Omphalina* thallus is either crustose-globulose or squamulose and grows directly on soil, mosses, plant remains or wood. *Omphalina ericetorum* has the broadest ecological amplitude (Kranner and Lutzoni, 1999). Dispersal of symbionts is either through thallus fragments (presumably important for local and medium range dispersal) or through spores (presumably more important for long distance dispersal). After dispersal through spores, the two symbionts have to re-establish the lichenized state, a slow and complex process. *Omphalina* fruiting bodies can be found regularly, it is however not clear how important the dispersal via spores is compared to fragmentation (Kranner and Lutzoni, 1999).

In this study we compared nucleotide substitution rates in the nuclear ribosomal internal transcribed spacer region (ITS1, 5.8S, ITS2) of lichenized *Omphalina* species and their symbiotic green algae (*Coccomyxa*). To ensure the validity of substitution rate comparisons, only fungal and algal pairs sharing the same evolutionary history were used to calculate rate ratios. In addition to rate ratio estimations of mycobionts and photobionts found in basidiolichens, substitution rates were investigated in free-living *Coccomyxa* species and *Coccomyxa* associated with lichenized ascomycetes. The results are discussed in the light of the hypothesis that rates of nucleotide substitution are lower in the inhabitant than the exhabitant as a result of the mutualistic interaction. If the hypothesis by Law and Lewis (1983) applies to these lichens, substitution rates in algal species pairs (the inhabitant) should be considerably lower compared to rates in the fungal pairs (the exhabitant). If the lower rate of nucleotide substitution recorded for the lichenized *Coccomyxa* is a consequence of a transition to mutualism, their rate should also be lower than for free-living *Coccomyxa*. If however, substitution rates were generally low in *Coccomyxa*, then all species, including the free-living, should have about the same low rates, and suggest that slow evolving *Coccomyxa* was perhaps predisposed to form this mutualistic association with *Omphalina*.

2. Methods

2.1. Species sampling and cultures

Thallus fragments and basidiomata of six lichenized basidiomycetes, *Omphalina hudsoniana*, *O. ericetorum*, *O. grisella*, *O. velutina*, *O. luteovitellina*, and one undescribed *Omphalina* species, discovered during this study, were collected at two localities in Greenland, one in Iceland and one in Eastern Canada (Table 1). All specimens collected at a single location were growing within a 2 m distance from each other. Algal symbionts were isolated from the lichen thalli and axenically cultured on Bold's Basal Medium (Bischoff and Bold, 1963). In addition, four lichenized and one non-lichenized *Coccomyxa* strains were obtained from the Culture Collection of Algae at the University of Texas at Austin, USA (UTEX): *Coccomyxa solorinae var. croceae*, *C. chodatii*, *C. peltigerae var. variolosae*, *C. solorinae var. saccatae*, and *C. solorinae var. bisporae*

Table 1
ITS sequences from fungal species (*Omphalina*) constitute dataset LO. ITS sequences from algal symbionts (*Coccomyxa*) form dataset LC. Lichen species used for the substitution rate ratio comparisons

Lichen species	Collection No.[a]	Locality	Fungal symbionts (dataset LO)[b]		Algal symbionts (dataset LC)[b]	
			Specimen	GenBank Accession No.	Specimen	GenBank Accession No.
O. ericetorum	930810-2	Disko Island, Greenland	OE1	AY293955	C2	AY293940
O. ericetorum	930822-2	Schefferville, Québec, Canada	OE2	AY293956	C9	AY293944
O. ericetorum	930822-8	Schefferville, Québec, Canada	OE3	AY293957	C12	AY293932
O. ericetorum	930724-2	Nuuk, Greenland	OE4	AY293958	C8	AY293938
O. ericetorum	930724-1	Nuuk, Greenland	OE5	AY293959	C10	AY293934
O. ericetorum	930822-4	Schefferville, Québec, Canada	OE6	AY293960	C19	AY293945
O. ericetorum	930805-5	Myvatn, Iceland	OE7	AY293961	C5	AY293942
O. grisella	930822-6	Schefferville, Québec, Canada	OG1	U66443	C13	AY293936
O. grisella	930822-5	Schefferville, Québec, Canada	OG2	AY293949	C20	AY293946
O. hudsoniana	930724-3	Nuuk, Greenland	OH1	AY293950	C6	AY293937
O. hudsoniana	930822-3	Schefferville, Québec, Canada	OH2	AY293951	C18	AY293933
O. hudsoniana	930724-6	Nuuk, Greenland	OH3	AY293952	C4	AY293941
O. hudsoniana	930811-6	Disko Island, Greenland	OH4	AY293953	C16	AY293947
O. hudsoniana	930805-6	Myvatn, Iceland	OH5	AY293954	C14	AY293948
O. luteovitellina	930812-2	Disko Island, Greenland	OL	AY293962	C1	AY293935
O. sp.	930724-5	Nuuk, Greenland	OS	AY293963	C7	AY293943
O. velutina	930812-1	Disko Island, Greenland	OV	U66454	C15	AY293939

[a] Collected by FL and deposited at DUKE.
[b] All sequences except OG1 and OV were generated as part of this study.

Table 2
Additional ITS sequences from lichenized and non-lichenized *Coccomyxa* strains and algal outgroup species part of dataset AC

Species name	Collection No.[a]	Symbiotic state	GenBank Accession No.[b]
Coccomyxa peltigerae var. variolosae	UTEX 271	Lichenized with Ascomycota	AY293964 *
C. solorinae var. croceae	UTEX 276	Lichenized with Ascomycota	AY293965 *
C. solorinae var. bisporae	UTEX 275	Lichenized with Ascomycota	AY293966 *
C. solorinae var. saccatae	UTEX 277	Lichenized with Ascomycota	AY293967 *
C. chodatii	UTEX 266	Non-lichenized	AY293968 *
C. peltigerae	SAG 216-5	Lichenized with Ascomycota	AY328522 *
C. subellipsoidea	SAG 216-13	Lichenized with Basidiomycota	AY328523 *
C. rayssiae	SAG 216-8	Non-lichenized	AY328524 *
Outgroup			
Chlamydomonas callosa	None	Non-lichenized	U66945
Dunaliella tertiolecta	None	Non-lichenized	U66956
Pandorina morum	None	Non-lichenized	AF376740

[a] *Algal culture collections*. UTEX: Culture Collection of Algae at the University of Texas at Austin, USA. SAG: Culture Collection of Algae at the University of Göttingen, Germany. None: Sequences obtained from GenBank.
[b] GenBank accession numbers followed by an asterisk indicate sequences generated as part of this study.

(Table 2). We also included sequences for three *Coccomyxa* species obtained from T. Friedl (SAG, Culture Collection of Algae at the University of Göttingen, Germany): *C. subellipsoidea*, *C. peltigerae*, and *C. rayssiae*. Three green algae belonging to the order Volvocales (*Pandorina morum*, *Chlamydomonas callosa*, and *Dunaliella tertiolecta*) were chosen as outgroup species. These three nucleotide sequences were obtained from GenBank (Table 2).

2.2. Molecular data

Total DNA was isolated from basidiomata and algal cultures using a DTAB-CTAB extraction method (Armaleo and Clerc, 1995). Polymerase chain reaction (PCR) primers ITS5 and ITS4 (White et al., 1990) were used to amplify both strands of the nuclear ribosomal ITS region (ITS1, 5.8S, and ITS2). PCR was performed in a 50-μl reaction volume, containing 33.7 μl H_2O, 5 μl dNTP, 5 μl 10× Taq reaction buffer, 0.3 μl Taq polymerase (Boehringer–Mannheim), 2 μl of each primer (ITS5 and ITS4 at 10 μM), and 2 μl of template DNA. PCR was performed in a thermal cycler (PTC-200, MJ-Research) using the following protocol: initial denaturation at 94 °C for 1 min; 34 cycles of 94 °C for 30 s, 52 °C for 45 s, 72 °C for 1 min; final extension of 5 min. PCR products were cleaned with low binding cellulose filter units (Millipore). Both strands of the PCR products

were cycle sequenced in a 10-μl volume using BigDye (Applied Biosystems) with the primers ITS5, ITS4, 5.8S, and 5.8SR (Vilgalys and Hester, 1990; White et al., 1990). Polyacrylamide gel electrophoresis was performed on an ABI 377 automated DNA sequencer (Applied Biosystems).

The resulting DNA sequences were assembled using Sequencher 3.1 (Gene Codes Corporation) and optimized by eye. Three sequence datasets were created, one for the lichenized *Omphalina* species (LO), one for lichenized *Coccomyxa* isolated from lichen thalli part of dataset LO (LC), and one for all *Coccomyxa* species including the outgroup algae (AC). Secondary structures of the fungal ITS1 and ITS2 sequences were constructed using Mfold 3.0 with energy parameters version 2.3 (Zuker et al., 1999; Mathews et al., 1999) with default parameters, except that the folding temperature was set at 15 °C. The resulting five to eight secondary structures per fungal species were compared by hand to identify the most stable sequence regions, particularly stems and loops, across all species. This procedure enabled us to improve the alignment of fungal sequences and to distinguish eight unambiguously aligned sequence portions in ITS1 and eight in ITS2. The low variability within the ITS region of the algal symbiont did not require the use of secondary structure information to improve the alignment.

2.3. Phylogenetic analyses and testing for co-lineage sorting

Phylogenetic analyses were done using maximum parsimony (MP) and maximum likelihood (ML) criteria as implemented in PAUP* version 4.0b8a (Swofford, 2000). For maximum parsimony analyses, gaps were used as a fifth character state for the unambiguous portions of the alignment. These sites were subjected to step matrices with cost values inversely proportional to the frequency of changes for each type of substitutions (6) and indels (4). In PAUP* the command 'Show character status—full details' was chosen. In the resulting character state table, the column 'States,' showing all the nucleotide states found at each position of the alignment, except the excluded sites, was saved as a separate text file. This file was then used as input for the program STMatrix (written by SZ and available on request), which computed the step matrix values by calculating the minimum frequency of reciprocal changes from one state to another (including gaps) and converting those to cost of changes using the negative natural logarithm of the probability (Felsenstein, 1981; Wheeler, 1990). Heuristic maximum parsimony searches with 1000 random addition sequence replicates, TBR branch swapping, and Multrees option in effect were performed on the three datasets (LO, LC, and AC), each with their specific step matrix and gaps treated as a fifth character state. Bootstrap support values for topological bipartitions were obtained by doing 1000 bootstrap replicates with 10 random addition sequence replicates each and the same search settings as for the heuristic tree searches.

To determine which model of nucleotide substitution with the least number of parameters best fit the data, hierarchical likelihood ratio tests were performed as implemented in the program Modeltest 3.04 (Posada and Crandall, 1998). A general time-reversible model (Lanave et al., 1984) with gamma distributed among site rate variation (GTR + G) was selected for the LO dataset and the AC dataset, and a Tamura–Nei-93 (Tamura and Nei, 1993) model was selected for the LC dataset. Heuristic maximum likelihood searches with 1000 random addition sequence replicates, TBR branch swapping and Multrees option in effect were performed on all three datasets. Bootstrap support values were obtained by doing 300 bootstrap replicates with three random sequence addition replicates each and the same search settings as for the heuristic searches.

Algal and fungal tree topologies (datasets LO and LC) from the maximum likelihood analysis were compared using the Kishino–Hasegawa (KH) and Shimodaira–Hasegawa (SH) tests as implemented in PAUP*, using likelihood optimization (Kishino and Hasegawa, 1989; Shimodaira and Hasegawa, 1999). Both tests assess the same property of the trees and sequences under consideration, the KH test in a parametric and the SH test in a non-parametric manner. The null hypothesis assumes that the average of the differences in likelihood for each nucleotide site is zero and the distribution normal. The null hypothesis is rejected, and consequently the trees are assumed to be significantly different, when the observed difference is significantly greater than zero. The tests were performed using either the algal or the fungal nucleotide dataset as basis, with full optimization and 1000 RELL bootstrap replicates (Kishino et al., 1990).

Because the long outgroup branches in the analysis of dataset AC attached to the longest ingroup internode and, due to this, the lichenized *Coccomyxa* species were not monophyletic, we were concerned about the possibility of long branch attraction (Felsenstein, 1978), which could lead to the recovery of an incorrect topology even under the ML criterion. In order to test if ML would suggest non-monophyly of the lichenized species when in fact monophyly was the topologically correct solution, we performed a computer simulation as follows. An ML search with a constraint for monophyly of the lichenized species was conducted, using the same model settings and search options as for the original search. The resulting tree had a zero length internode leading from the non-lichenized to the lichenized species. However, for simulating the data an internode greater than zero was required. Therefore, we arbitrarily set that

internode length equal to half the error margin given by PAUP* for this branch (PAUP* command: ''describe trees''). This amended tree and the model parameters from the original tree search were then used to simulate 100 nucleotide datasets with Seq-Gen 1.2.5 (Rambaut and Grassly, 1997). On all 100 datasets, ML tree searches were performed using the same model settings and search options as for the original AC dataset, with the exception of implementing only 10 random addition sequence replicates. Topologies and branch lengths were recorded. A significant uncertainty about the accuracy of the non-monophyly of lichenized *Coccomyxa* revealed by the original search would be assumed if searches on simulated datasets chose non-monophyly in more than 5% of the datasets.

Cospeciation, host switching, duplication, and lineage sorting events were estimated with TreeMap 1.0b (Page, 1995), using the ''exact search'' option. The algal tree topology found in the ML analysis was mapped onto the three fungal ML tree topologies. Significance tests for the number of co-lineage sorting (''cospeciation'' in TreeMap) events were conducted using the Markovian model and the proportional model as implemented in TreeMap. All three implemented options of tree randomization were explored. In each case 1000 random trees were generated.

2.4. Substitution rate estimations

To assure valid rate ratio comparisons, only species pairs with matching evolutionary history should be considered. Species pairs that involve for example, horizontal transfers should not be considered (Huelsenbeck et al., 1997). The TreeMap reconstructions

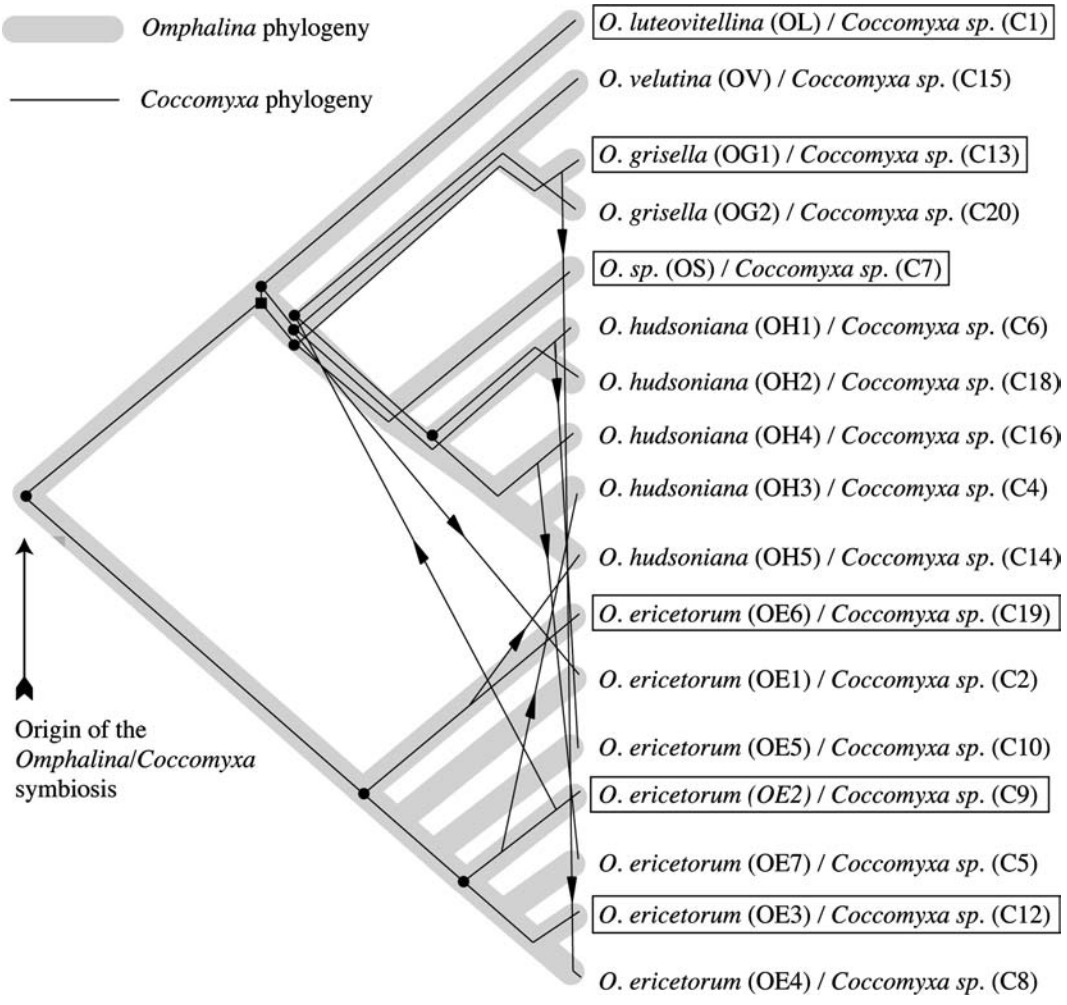

Fig. 1. TreeMap reconstructions of co-lineage sorting (''cospeciation''), host switching, duplication, and lineage sorting events for the *Omphalina–Coccomyxa* model system using one of three most likely *Omphalina* trees. The algal tree (black lines) is mapped onto the fungal tree (wide gray lines). Eight co-lineage sorting events (black dots), one duplication event (black box), and seven host switching events (black lines with arrows) were reconstructed. Paths from one taxon to another that do not involve host switching events are valid for the rate ratio comparisons (e.g., OL to OE2). All six taxa that are available for valid comparisons (for this reconstruction) are highlighted with black boxes. Not all valid pairs in the presented TreeMap reconstruction were also valid in the two other equally plausible reconstructions (using the two other equally likely *Omphalina* trees). Only 12 pairs were valid in all three TreeMap reconstructions.

suggested many disqualifying events, in particular many host switching events. Therefore, for the rate ratio calculations, only taxon pairs that did not involve host switching along the path from one taxon to the other in all three best TreeMap reconstructions were considered (e.g., path from OL to OE2 in Fig. 1).

Nucleotide substitution rates (number of substitutions per 100 sites) were calculated for the ITS1, 5.8S, and ITS2 sequence portions of the lichen datasets LO and LC separately (six subsets in total), and for the additional *Coccomyxa* species of dataset AC (three subsets). For all valid pairs of sequences within a subset, the substitution rates were recorded. Rate estimations were performed in PAUP* using the topologies and likelihood models from the maximum likelihood reconstructions. Substitutions among taxon pairs were estimated by summing up the rates along branches leading from one taxon to the other. Rates for dataset LO are based on the three equally most likely phylogenies and calculated as the average of the estimated rates on the three trees. Substitution rate ratios between corresponding valid fungal and algal taxon pairs were then obtained by dividing the rate of the fungal pair by the rate of the corresponding algal pair, except if one of the values was estimated to be zero. Averages and standard errors were calculated using StatView 5.0.1 (SAS Institute).

3. Results

3.1. Phylogenetic analyses

The final alignment for the fungal sequences (dataset LO) consisted of 907 sites. Eighteen ambiguously aligned regions with 568 sites were excluded, resulting in a total of 339 sites that were included in the phylogenetic analyses. Seventy-nine characters were parsimony-informative. The parsimony (MP) analyses produced 18 equally most parsimonious trees (length = 268.52 steps) found in each of the 1000 replicates. Parsimony bootstrap analysis supported all putative species. The likelihood (ML) analyses of the fungal data yielded three equally most likely trees (ln likelihood = −975.506). These trees were found in each of the 1000 replicates and disagreed only in the placement of *O. ericetorum* specimens within the *O. ericetorum* clade (Fig. 2A). The strict consensus tree is identical to the strict consensus tree resulting from the MP analyses. The monophyly of all species represented by more than one individual were highly supported by the ML bootstrap analysis (Fig. 2A).

The final alignment for the lichenized *Coccomyxa* sequences (dataset LC) consisted of 627 sites of which 602 were constant and 11 were parsimony-informative. None of the sites was found to be ambiguously aligned. MP analyses yielded 38 trees (length = 47.10 steps). ML analyses yielded one most likely tree (ln likelihood = −1047.84216). This tree was found in all of the 1000 random addition sequence replicates. The ML tree (Fig. 2B) is topologically identical to one of the most parsimonious trees and similar to the strict consensus tree of the parsimony analyses. MP and ML bootstrap support values for most partitions of the *Coccomyxa* tree were below 50% (Fig. 2A). Only the clade containing the two samples C5 and C14 from Iceland was highly supported in both MP and ML bootstrap analyses.

The final alignment for all *Coccomyxa* sequences including the outgroup species (dataset AC) consisted of 823 characters. Twelve ambiguously aligned regions with 180 sites were excluded, resulting in a total of 643 sites that were included in the phylogenetic analyses, of which 144 were parsimony-informative characters. MP analyses yielded 36 trees (length = 419.8 steps). ML analyses yielded one most likely tree (Fig. 3A) with ln likelihood = −2801.52724. The same tree was found in all of the 1000 random addition sequence replicates. The likelihood tree is topologically identical to one of the most parsimonious trees and similar to the strict consensus tree of the parsimony analyses. MP and ML bootstrap support values were high except for internodes within the lichenized *Coccomyxa* lineages (Fig. 3A).

Tree searches on the 100 simulated datasets recovered a total of 111 trees representing four different topologies. The topology under which the data were simulated (monophyly of the lichenized *Coccomyxa* species; L/O and L/P) was found only 82 times (74%, Fig. 3B). Thirteen times (12%) the free-living *Coccomyxa* (F) and the *Coccomyxa* associated with *Omphalina* (L/O; Basidiomycota) grouped together, 10 times (9%) the free-living *Coccomyxa* (F) and the *Coccomyxa* associated with members of the Peltigerales (L/P; Ascomycota) grouped together, and 6 times (5%) the relationship of the free-living (F) and the two lichenized groups (L/O and L/P) was unresolved (Fig. 3).

The phylogenies of the lichenized fungi and algae do not show any obvious congruence, and no pattern in regard to geographical origin is detectable, except that two *Coccomyxa* samples from Iceland form a distinct group with high bootstrap support (Fig. 2A). All Kishino–Hasegawa and Shimodaira–Hasegawa tests suggested a significant difference among the fungal and algal tree topologies ($p < 0.01$). The TreeMap analysis of the three ML tree pairings (one algal tree mapped onto three equally likely fungal trees) revealed three best reconstructions suggesting eight co-lineage sorting (“cospeciation”) events each. Duplication events were found to be rare (1, 0, 0, respectively), host switching was more abundant (7, 8, 8), and lineage sorting events were suggested to be frequent (17, 12, 16). None of the randomization tests implemented in TreeMap was able

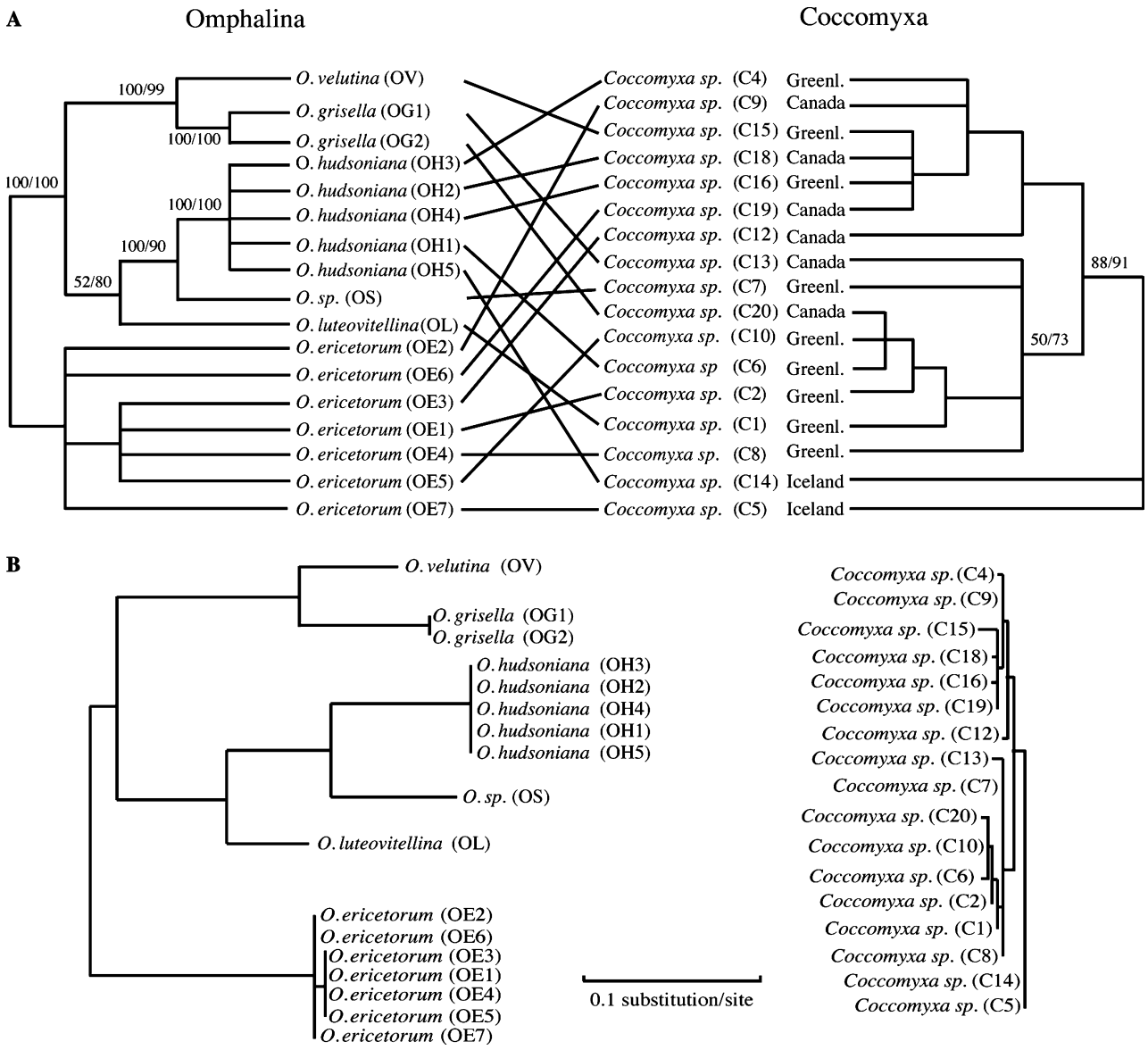

Fig. 2. Phylogenetic relationships of the lichenized fungus *Omphalina* and alga *Coccomyxa*, based on nuclear ribosomal ITS1, 5.8S, and ITS2 data. The cladogram (A) and the phylogram (B) are one of three most likely trees for *Omphalina* (dataset LO, left side) and the single most likely tree for *Coccomyxa* (dataset LC, right side). The phylogram trees are drawn to the same scale. Maximum parsimony and maximum likelihood bootstrap support values greater than 50% are given before and after the backslash, respectively. The geographic origin of the samples is given next to the *Coccomyxa* specimen codes. Symbionts from the same lichen thallus are connected with a line in the cladogram. The fungal tree was rooted on the internode between *O. ericetorum* and the rest of the lichenized taxa as shown in a broader phylogenetic study of omphalinoid mushrooms (Lutzoni, 1997). The *Coccomyxa* tree is arbitrarily rooted with the two specimens from Iceland (C5, C14).

to reject the possibility that eight speciation events could match due to chance alone (lowest p value = 0.07, highest p value = 0.16).

On average, over all three TreeMap reconstructions, and disregarding events where source and destination location were the same, Canadian *Coccomyxa* lineages were 4.66 times the source and 1.33 times the destination in host switching events. Lineages in Greenland were 2.33 times the source and 3.66 times the destination, and lineages in Iceland never served as source but were 2 times the destination. When corrected for the number of specimens sampled in each region, every Canadian lineage, on average, served 0.78 times as source and 0.22 times as destination, and each Greenland lineage served 0.26 times as source and 0.41 times as destination.

3.2. Substitution rates

Average numbers of substitutions per 100 sites in the lichenized *Omphalina* (dataset LO) ITS1 portion were

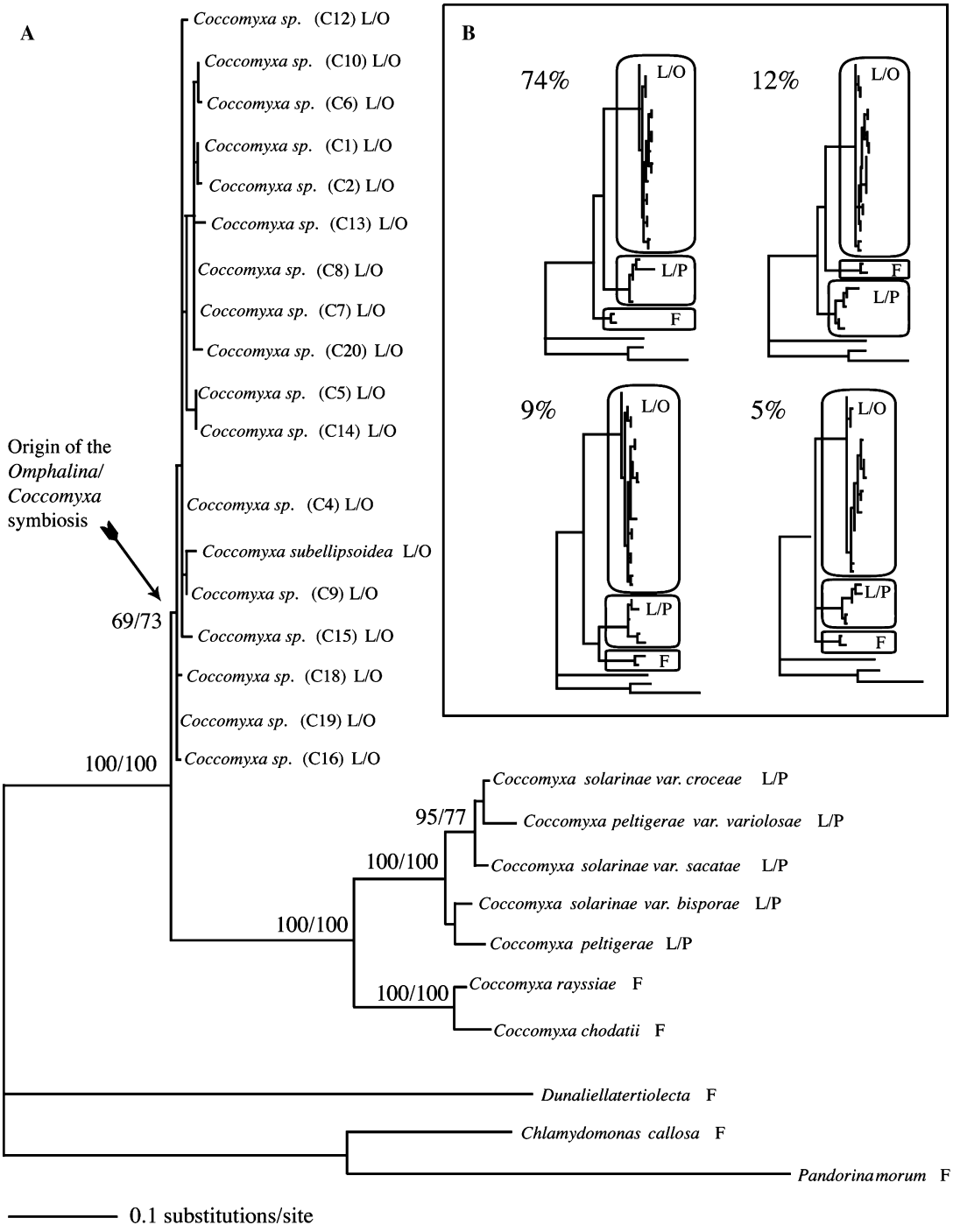

Fig. 3. Phylogenetic relationships among lichenized and non-lichenized *Coccomyxa* algae (dataset AC) as revealed by an ML search using ITS (ITS1-5.8S-ITS2) sequences. *Coccomyxa* isolated from lichenized *Omphalina* species (Basidiomycota) are denoted by a bold L/O after the name. *Coccomyxa* isolated from members of the Peltigerales (Ascomycota) are annotated with a bold L/P. Free-living species are denoted by a bold F. (A) Single most likely tree with maximum parsimony and maximum likelihood bootstrap support values greater than 50% given above internal branches, before and after the backslash, respectively. (B) Frequency of the four topologies recovered by the ML analysis from 100 simulated datasets. The topology under which the data were simulated (monophyly of the lichenized *Coccomyxa* species) was recovered only 74% of the times (top left).

28.31, 3.81 in the 5.8S portion, and 19.71 in the ITS2 portion. The corresponding substitution rates for *Coccomyxa* associated with *Omphalina* (dataset LC) were 1.82 (ITS1), 1.04 (5.8S), and 2.19 (ITS2). Average numbers of substitutions per 100 sites among the *Coccomyxa* associated with Ascomycota and the free-living species were 12.49 for the ITS1 portion, 1.23 for the 5.8S portion, and 7.03 for the ITS2 portion.

The procedure to identify valid rate ratio comparisons, based on the TreeMap reconstructions, revealed a total of 12 suitable taxon pairs, out of 58 possible pairs. The valid comparisons are: OL–OG1, OL–OS, OL–OE2, OL–OE3, OL–OE6, OG1–OS, OG1–OE2, OG1–OE3, OG1–OE6, OS–OE2, OS–OE3, OS–OE6, and their corresponding *Coccomyxa* algae pair (Table 1; Fig. 1). Substitution rates in the ITS1 portion for pairs of *Omphalina* sequences ranged from 6.78 to 43.01 times greater than rates in the corresponding pairs of *Coccomyxa*, with a mean of 27.45 (standard error, ±4.34). The corresponding values for substitutions in the 5.8S portion ranged from 1.81 to 2.73 with a mean of 2.36 (±0.22) and substitutions in the ITS2 ranged from 2.50 to 33.87 with a mean of 18.00 (±3.23). The highest rate difference (43.01) was found in the ITS1 regions of the species pair *O. ericetorum* (OE6)–*O. sp* (OS), and their corresponding *Coccomyxa* C19 and C7, respectively.

4. Discussion

Our phylogenetic analyses of the fungal sequences are in agreement with previously identified relationships within the genus *Omphalina* (Lutzoni, 1997). The very low number of substitutions detected among the lichenized *Coccomyxa* species associated with *Omphalina*, the fact that they group together with *C. subellipsoidea* (specimen SAG 216-13, isolated from *O. ericetorum* by O. Jaag), and the support in both maximum parsimony and maximum likelihood bootstrap analyses for this group, led us to conclude that potentially a single phylogenetic species, *C. subellipsoidea*, is shared by the six lichenized *Omphalina* species. This is even more remarkable as the specimens have been collected at widely separated locations in Canada, Greenland and Iceland (our samples), and Austria (culture specimen SAG 216-13, *C. subellipsoidea*).

The Canadian *Coccomyxa* lineages served 3 times more often as source in host switching events and were almost 2 times fewer the destinations than the Greenland lineages. The two Icelandic lineages never served as source. The Canadian location was the most southern of the three areas sampled. These findings are particularly intriguing in the light of past glaciation periods and linked extinction and recolonization events. Greenland and Iceland were almost entirely covered with ice in the last glaciation period, rendering them unsuitable for *Omphalina* lichens, and had most likely to be recolonized from refugia in either continental Europe or North America, were the species were able to survive in southern, ice-free locations. The separate phylogenies of the fungi and algae themselves are not informative about the location of refugia or the geographic origin of the lineages that recolonized Greenland and Iceland. However, the TreeMap host switching pattern, with an excess of source lineages in Canada, and an excess of destination lineages in Greenland and Iceland, presents preliminary evidence that the *Omphalina* lichens recolonized Greenland and Iceland from a continental location in North America. Additional, thorough sampling of both symbionts particularly in Europe and in North America will be needed to corroborate these findings.

The computer simulation designed to test the possibility of long branch attraction in the AC dataset analysis (Fig. 3) revealed considerable uncertainty about the attachment of the outgroup. Although we evolved data along a topology with a monophyletic grouping of the lichenized *Coccomyxa* and basal placement of the free-living species, the subsequent ML searches recovered other topologies in 29 (26%) of the trees, of which 10 (9%) were identical to the topology found in the original analysis of dataset AC (Fig. 3). Therefore, we cannot reject the possibility that the non-monophyly of the lichenized *Omphalina*, as revealed by the original search (Fig. 3A), was due to long branch attraction (Felsenstein, 1978). The phylogenetic uncertainty associated with this dataset (AC) also impinge a high level of variation when comparing rates of nucleotide substitutions among lineages of *Coccomyxa*, ranging from a drastically lower rate of evolution in the L/O group (Fig. 3A) to a faster rate in the lichenized versus free-living (L/O and L/P) *Coccomyxa* (Fig. 3B). Therefore, this study does not allow us to determine if a shift in rates of nucleotide substitution is associated with transitions from a free-living to a mutualistic state in the algal genus *Coccomyxa*. However, this uncertainty does not compromise our comparison of rates of nucleotide substitution between fungal and algal symbionts. Results presented in Fig. 3 also show that *Coccomyxa* found in ascolichens belonging to the Peltigerales are very likely part of a different lineage than *C. subellipsoidea* found in *Omphalina* basidiolichens. Based on the same phylogenetic evidence, free-living *Coccomyxa* seems to belong to a lineage separate from lichenized *Coccomyxa*, for a minimum of three main lineages within *Coccomyxa*.

Fungal and algal symbionts (datasets LO and LC) show remarkably high differences in nucleotide substitution rates, with up to 43 times higher rates in fungal pairs. The sequence regions that show the highest rate differences are the nuclear ribosomal ITS1 and ITS2. Even the selectively constrained 5.8S region evolved on average almost 2.5 times faster in the *Omphalina* species than in the associated *Coccomyxa*. These high rate ratios are, to our knowledge, the highest differences of substitution rates reported for symbiotic organisms. If the large ambiguously aligned portions of the fungal ITS1 and ITS2 had not been excluded from the analyses, even higher rate differences would have resulted. Higher rate differences have been reported between endosymbiotic mitochondrial (CO1) and nuclear (EF-1α) genes in lice

(Johnson et al., 2003). However, evolutionary processes acting on organelles are very different than for self-sustainable symbiotic organisms. Our estimates of substitution rate ratios of lichenized *Cladonia* (Ascomycota) and their mutualistic green algae, derived from ITS1 and ITS2 maximum likelihood trees published by Piercey-Normore and DePriest (2001), are comparable, but generally lower.

The fact that substitution rates are so low among *Coccomyxa* specimens associated with *Omphalina*, even in the rapidly evolving ITS region, and no significant cosorting is detectable let us conclude that speciation in this symbiotic system is likely to be primarily restricted to the fungal symbionts. Generally, mutualistic symbionts are closely associated and synchronized but it is reasonable to assume that they still have slightly different optima of abiotic environmental parameters, amount and type of nutritional requirements, and differences in life cycles. Strong conflicts in life cycles, nutritional requirements or environmental optima, could destabilize the mutualistic association and, therefore, must be reduced (Herre et al., 1999). Consequently, adaptations that reduce conflicts would be advantageous. Several factors have been identified that could lessen conflicts and align the requirements of closely associated symbionts. Important in the context of this study are genotypic uniformity of the inhabitant or even a slowdown of genetic change (nucleotide substitution) in the inhabitant (Axelrod and Hamilton, 1981; Doebeli and Knowlton, 1998; Trivers, 1971) and the suppression of sexual reproduction in the inhabitant (Herre et al., 1999). Law and Lewis (1983) have proposed that mutualistic ectosymbiotic systems in which one partner lives inside the other, would promote selection against sex and lower rates of genetic change in the inhabitant. We have shown that in the *Omphalina–Coccomyxa* system the inhabitant shows remarkably low nucleotide substitution rates compared to the exhabitant. Average substitution rates among all *Coccomyxa* algae (dataset AC) were generally lower than in the fungi (approximately 3 times lower for the whole ITS region) but they are particularly low in the individuals that are associated with the *Omphalina* species (approximately 15 times lower).

Based on results and findings from other studies (Kranner and Lutzoni, 1999; Lutzoni, 1997; Lutzoni and Pagel, 1997; Lutzoni and Vilgalys, 1995) we conclude that the assumption of a common time frame (i.e., a single origin of lichenization) for the *Omphalina* fungi and the associated *Coccomyxa* algae is the most likely scenario. Several facts support this conclusion. First, the phylogenetic reconstructions clearly show that a single strain/species of *Coccomyxa* is associated with all *Omphalina* fungi investigated and that this group is unequivocally distinct from free-living *Coccomyxa* and from *Coccomyxa* associated with ascolichens (Peltigerales). This holds true even in the rigorous long branch attraction tests. Second, the *Omphalina* lichenization is comparably young, particularly in comparison to ascolichens (Lutzoni et al., 2001; Moncalvo et al., 2000). Third, *Omphalina* samples from geographically widely separated places are associated with the same strain/species of *Coccomyxa*. Additionally, if the present *Coccomyxa* strain/species was acquired only after the speciations of the *Omphalina* fungi took place, it would have had to replace completely any other algal symbionts in all the *Omphalina* species in all geographic localities.

Given the uncertainty about the dispersal biology and life cycle of the *Omphalina–Coccomyxa* association and the low bootstrap support on the algal phylogenetic tree, we cannot rule out the possibility of a common evolutionary history for at least some lineages. It is therefore prudent to apply the TreeMap procedure to estimate substitution rate ratios. Furthermore, in the case that the underlying assumptions of the TreeMap based reconstructions are not met, the approximated substitution rate ratios will be conservative estimates of the true values. In other words, if the TreeMap approach was erroneously applied, the high substitution rate ratios presented in this study, would actually be even higher.

One could argue that the rate ratio comparisons are biased, because the lichenized *Omphalina* fungi comprise several species and the lichenized *Coccomyxa* algae probably only one. In other words, the comparison involves among fungal species versus within algal species variation, which inherently must result in lower substitution rate estimates for the alga. However, under the assumption of a common time frame, the mycobionts and photobionts had the same amount of time to diverge and speciate since the origin of the lichenization in these lineages (Figs. 1 and 3, see also Kranner and Lutzoni, 1999; Lutzoni and Pagel, 1997). The fact that the symbiotic *Omphalina* lineage diverged into several distinct species since then, with very high sequence variation, while the associated algae did not diverge and accumulated only few substitutions, supports the hypothesis of lower substitution rates in the inhabitant, independent of the level of comparison (species/population) and accuracy of the calculations.

Another plausible scenario, involving speciation in the symbiotic algal lineage, could still lead to the same conclusion. This scenario assumes that the symbiotic *Coccomyxa* did speciate since this mutualism originated, but the new algal species always ''escaped'' the symbiosis and reverted to a free-living mode or did at least not form a symbiotic association with *Omphalina*, leaving behind an essentially unchanged species of *Coccomyxa* associated with *Omphalina*. The overriding principle here, would be that only a genetically stable algal lineage, accumulating few substitutions over time, would be able to maintain the association with the

fungal symbiont. The expectation under this scenario would be that further sampling of free-living *Coccomyxa* would reveal that some are nested within the lichenized *Coccomyxa* lineage associated with *Omphalina*.

The question arises, if such disparities in rates among mutualistic symbionts are a general pattern or rather the exception. Only few studies have investigated sequences of symbiotic inhabitants and their results are somewhat conflicting. They deal mostly with bacterial species and are obscured by the fact, that rarely have both symbionts been investigated and if they have, rarely have the same DNA regions or genes been sequenced. Nevertheless, there seems to be a trend of a lower nucleotide substitution rate in inhabitants, supporting the hypothesis of Law and Lewis (1983). Based on ITS1 and ITS2 sequences, such a tendency can be seen for example in *Cladonia* (lichenized Ascomycota) and their green algal symbionts (Piercey-Normore and DePriest, 2001). Cyanobacterial symbionts of the genus *Nostoc* (inhabitants) of four lichen species were investigated by Paulsrud and Lindblad (1998) and found to belong to very closely related strains. These strains showed only a few nucleotide substitutions and deletions/insertions in their $tRNA^{Leu}$ introns, compared to non-symbiotic clades. The fungal exhabitants were not investigated, and therefore, no information is available about their relative substitution rates.

Gast and Caron (1996) analyzed the small subunit ribosomal DNA (SSU rDNA) of two lineages of symbiotic dinoflagellates (inhabitants) associated with three foraminifera host species and six planktonic radiolarian host species, respectively. The dinoflagellates SSU rDNA within the two lineages showed low nucleotide substitution rates compared to non-symbiotic dinoflagellates. However, no information on the host species SSU rDNA was published. Lower substitution rates in the inhabitant have been reported in a system of marine algae and their bacterial symbionts (Ashen and Goff, 2000). However, the authors did not compare the same DNA region, as they were concerned with phylogeny and not substitution rates.

Several other studies clearly oppose the idea that rates of evolution in inhabitants should slow down. Interestingly, these studies involve bacterial inhabitants with strict vertical transmission and small to very small population sizes. These factors favor a fast fixation of nucleotide substitutions in the inhabitant. For example, Moran and colleagues (Clark et al., 2000; Moran and Wernegreen, 2000; Moran et al., 1995) have found that the aphid-associated bacteria *Buchnera* shows much faster rates of molecular evolution than either its host species and free-living relatives. This points to the mode of transmission of the inhabitant (horizontal versus vertical) as being one of the important factors regulating the rate of nucleotide substitution. Vertical transfer of inhabitants to the next generation would involve faster rates of nucleotide substitution than the horizontal transfer of inhabitants, where the inhabitant needs to reassociate with the exhabitant at each generation.

A final assessment of the hypothesis, especially the proposed genetic slowdown, and under what conditions it is operating, would require detailed information on a number of additional mutualistic symbiotic systems. Setting aside the aphid-*Buchnera* and similar highly specialized systems with vertical transmission of the inhabitants, there appears to exist a trend of lower substitution rates among the inhabitant species compared to their exhabitant symbionts, and this despite the diversity and range of the integration levels of the systems. It is not clear at this point if the low substitution rates observed in symbiotic algae are a direct result of the lichenization, or if these species were favored by the fungi because they already had the predisposition needed for a successful and stable transition to a lichenized state (i.e., low mutation rates, mostly asexual reproduction, etc.). Future studies must show if the proposed mechanism is particularly enhanced in lichens or if other constraints acting on lichens might have led to the exceptionally high differences in substitution rates. Finally, a comparison of rates of nucleotide substitutions between inhabitants transmitted vertically versus horizontally is needed to better understand the causes of molecular evolution in symbiotic systems.

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