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RANGE DISJUNCTIONS, SPECIATION, AND MORPHOLOGICAL TRANSFORMATION RATES IN THE LIVERWORT GENUS *LEPTOSCYPHUS*

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Bryophytes and angiosperms exhibit similar intercontinental disjunct distributions that have traditionally been explained by continental drift. Such disjunct distributions are, however, typically observed at the species level in bryophytes, whereas they occur at much higher taxonomic level in angiosperms. The corollary of this observation is that morphological evolution in bryophytes is exceedingly slow. These hypotheses can now be explicitly tested with the advent of molecular dating. In this article, we show that the trans-Atlantic disjunctions observed in the mostly tropical liverwort genus *Leptoscyphus* date back to 5.5 Myr, thus largely postdating the opening of the South Atlantic. The temporal calibration of the phylogeny allows us to estimate for the first time the absolute timing of morphological evolution in bryophytes. The time frame necessary for shifts to occur between character states was estimated on average at ca. 4.05 ± 1.86 Myr. As opposed to the traditional view that bryophyte evolution has been triggered by episodic shifts in habitat conditions, our analyses furthermore suggest that morphological and molecular divergence gradually accumulated in the genus, which contrasts with the rapid diversification documented in some tropical trees.

KEY WORDS: **Ancestral character state reconstruction, island calibration, long-distance dispersal, vicariance.**

Historical biogeography has been the subject of major debates among biogeographers. Even today, the controversy around the two central ideas of historical biogeography—vicariance and dispersal—has remained unsettled (McDowall 2004; de Queiroz 2005; McGlone 2005; Waters and Craw 2006; Upchurch 2008). For many decades after Wegener's plate-tectonic theory, the disjunct distributions of the Southern Hemisphere biota were attributed to the sequential break up of the southern supercontinent Gondwana, causing vicariant division of its ancestral biota (Wiley 1988; Morrone and Crisci 1995; Humphries 2000; Nelson and Ladiges 2001; Ebach et al. 2003). This vicariance hypothesis provided a simple explanation for the disjunct distributions shared by many taxa. Because most Gondwana groups were presumed to be poor dispersers, long-distance dispersal across oceanic barriers was discarded as a valid explanation of the disjunct patterns observed. Moreover, the dispersal hypothesis was regarded as unscientific because it is unfalsifiable (Nelson and Platnick 1981; Morrone and Crisci 1995; Craw et al. 1999; Humphries 2000) and biogeographers argued that the congruent or concordant patterns found in so many different groups could not have arisen by the supposedly random dispersal process (Croizat et al. 1974; Craw 1982). If dispersal was conceded for oceanic islands that had never been connected to other landmasses, vicariance was assumed as the more probable explanation for all the other cases of disjunct distribution observed and came to dominate the field of historical biogeography (Wiley 1988; Morrone and Crisci 1995).

Although, in some instances, disjunct distributions can be unambiguously interpreted in terms of ancient vicariance and massive extinctions based on fossil evidence (Tiffney 1985a; Milne and Abbott 2002; Xiang et al. 2005), patterns that are spatially congruent with such hypothesis may, in fact, conceal a complex mixture of relictual distributions and more recent speciation and

dispersal events (de Queiroz 2005; Vanderpoorten et al. 2007). Over the past few years, the infamous oceanic dispersal hypothesis has been the subject of renewed interest among biogeographers and a dramatic change in opinion has come about, the principal cause of which has been molecular dating (Renner 2004; de Queiroz 2005; Milne 2006). Although molecular dating techniques have long been criticized due to methodological shortcomings, including uncertainty associated with the calibration itself, phylogenetic uncertainty, and uncertainty associated with the estimation of rate parameters, the most recent developments incorporated these uncertainties into the final estimation (see Renner 2004; Kumar 2005; and Welch and Bromham 2005 for review). These techniques have increasingly allowed for the falsification of the vicariance hypothesis as an explanation for the disjunct distribution observed in many taxa, bringing back to life its competing hypothesis, that is oceanic dispersal (Renner 2004; Waters and Roy 2004; de Queiroz 2005; McGlone 2005; Yoder and Nowak 2006). In the Southern Hemisphere in particular, wherein the continental drift hypothesis gained much support owing to the very ancient history of Gondwana breakup (McGlone 2005), molecular dating of phylogenetic trees supports oceanic dispersal over tectonic vicariance in various organisms such as carnivores (Yoder et al. 2003), frogs (Vences et al. 2003), and angiosperms such as *Adansonia* (Baum et al. 1998), *Nothofagus* (Knapp et al. 2005), the Malpighiaceae (Davis et al. 2002), Rapateaceae (Givnish et al. 2000), and Atherospermataceae (Renner et al. 2000).

As a matter of fact, however, biogeographic studies have been confronted with an important sampling bias because certain groups of organisms have been much more extensively studied than others (Sanmartin and Ronquist 2004). The possibility remains that the distribution patterns exhibited by entire lineages, which have not been scrutinized yet, is consistent with an ancient vicariance hypothesis. In particular, many bryophyte species share spectacular transcontinental range disjunctions, among which the eastern American-eastern Asian and western American-Mediterranean disjunctions have been especially well documented (Schuster 1983). Because dispersal has long been thought to result in stochastic distribution patterns, the coincidence of so many species that show essentially the same disjunction has traditionally been interpreted in terms of ancient vicariance (Schofield 1988). These disjunctions further coincide with those observed at a much higher taxonomic level in angiosperms. For example, 43% of the moss species found in North America are also found in Europe, whereas 70% of the species found in Europe also occur in North America (Frahm and Vitt 1993). By contrast, 48% of the genera, but only 6.5% of the species, are shared between the North American and European vascular flora (Qian 1999). The similarity in range disjunctions observed in bryophyte species and genera or families of angiosperms has often been interpreted in terms of a common explanation, that is, ancient vicariance (Herzog 1926; Crum 1972; Schofield and Crum 1972; Sharp 1972; Schuster 1983; Schofield 1988; Frey and Beever 1995; Schaumann et al. 2003; Pfeiffer et al. 2004; Schaumann et al. 2004; Stech and Frey 2004; Blöcher et al. 2006). If, as suggested by the ancient vicariance hypothesis, the breakup of continents has in fact shaped the present moss flora, species shared by former Gondwanan and Laurasian supercontinents must have retained their morphological identity for about 105 Myr (McLoughlin 2001), leading to the consideration of bryophytes as "unmoving, unchanging sphinxes of the past" (Crum 1972).

This hypothesis is, at first sight, supported by the available fossil evidence. *Pallaviciniites devonicus*, one of the oldest bryophyte fossils, dates back to the Devonian, and yet resembles extant liverwort genera such as *Pallavicinia* or *Symphyogyna* (Frahm 2000, 2004). The close morphological similarity between fossils dating back from the Cretaceous, Eocene, and Pliocene periods confirms that some groups of mosses have persisted with very little morphological changes for at least 80 Myr (Gradstein 1993; Konopka et al. 1997, 1998; Frahm 2004; Frahm and Newton 2005). Because it is often assumed that rates of morphological and molecular evolution are highly correlated (Barraclough and Savolainen 2001), the corollary is that bryophytes are very slow-evolving organisms that have been considered as a quite conservative group of plants (Frey et al. 1999; Frahm 2000, 2004). In fact, sequence identity among populations of bryophytes that are now scattered on disjunct Gondwanan splinters has sometimes been interpreted as evidence for "stenoevolution," that is, extremely low rates of molecular and morphological evolution (Meissner et al. 1998; Frey et al. 1999; Pfeiffer 2000).

Although the hypothesis of continental drift and its consequences for morphological evolution in bryophytes have once been confirmed by molecular dating (McDaniel and Shaw 2003), the ancient vicariance hypothesis has been questioned by an increasing body of literature (Shaw et al. 2003; McDaniel and Shaw 2005; Hartmann et al. 2006; Henrichs et al. 2006; Feldberg et al. 2007; Vanderpoorten et al. 2008). In bryophytes, however, molecular dating remains extremely limited (McDaniel and Shaw 2003; Wall 2005; Hartmann et al. 2006; Newton et al. 2006; Heinrichs et al. 2007; Wilson et al. 2007; Huttunen et al. 2008), mainly because of the lack of calibration information. Bryophytes are, in fact, poorly represented in the fossil flora of almost all geological periods (Frahm and Newton 2005). The best source of fossil information comes from tissues preserved in Baltic, Saxon, and Dominican amber (Frahm 2000, 2004). The percentage of plant fossils in amber is, however, very low. Ninety nine percent of all fossils from Baltic amber consist of arthropods and, in the remaining percent of plant fossils, bryophytes are extremely rare. To date, only about 100 fossil species are known from the European bryophyte flora (Frahm and Newton 2005). The fossil record for bryophytes is, therefore, extremely incomplete,

either because of low probability of fossilization due to habitat preferences (Krassilov and Schuster 1984) or poor preservation due to absence of woody tissue (Frahm and Newton 2005). In many instances, the question of whether many fossils are actually bryophytes remains open, partly because gametangia or sporophytes are not available (Schofield 1988).

Even when fossils exist, the poor preservation of bryophyte tissues resulted in a very fragmentary fossil material, which obscures the affinities of these fossils to extant lineages. The use of such fossils to calibrate phylogenetic trees is limited because their morphology often does not allow for their definitive placement in the phylogeny, hence increasing the error associated with the estimate of the divergence dates (Heinrichs et al. 2007). Furthermore, the likelihood of fossilization of a particular species depends on its abundance as well as on the presence of decay-resistant compounds in its tissues and the suitability of environmental conditions for plant tissue preservation. Hence, the presence of a species is likely to be recorded in the sediment only well after its actual phylogenetic origin. Consequently, fossils provide a minimum age estimate for the time of origin of particular lineages.

Although bryophyte fossils may usually be assigned to an extant family or genus (e.g., Konopka et al. 1997, 1998; Frahm and Newton 2005), and hence, are suitable for assessing the age of nodes deep in the tree, the uncertainty of their relationships with modern species (Schofield 1988; Ignatov 1992), and the difference between their actual origin and their fossilization preclude their use for dating more recent events. As a consequence, the time scale of molecular and morphological evolution in bryophytes is still extremely poorly documented. In this context, major geological events assumed to have been responsible for lineage divergence (McDaniel and Shaw 2003), and the use of island neo-endemic speciation events in particular (e.g., Richardson et al. 2001a; Emerson and Oromi 2005; Wall 2005; Kim et al. 2008) appear as interesting alternatives for calibrating phylogenetic trees. In this article, we take advantage of the neo-endemic speciation of the Azorean liverwort *Leptoscyphus azoricus* (Vanderpoorten and Long 2006) to calibrate the phylogeny of the genus and revisit competing hypotheses on the origin of the trans-Atlantic disjunction observed in the genus. We explore the limitations of this strategy, discuss the use of alternative calibration points, and determine the extent to which the different assumptions underlying the use of alternative calibration point affects our conclusions. The temporal calibration of the phylogeny will, in turn, allow us to estimate for the first time the average amount of time necessary for a morphological character to switch from one state to the other, which, in the absence of an appropriate time frame, have been until now only examined in a relative way (Vanderpoorten et al. 2002; Vanderpoorten and Jacquemart 2004; Vanderpoorten and Goffinet 2006).

Material and Methods

TAXON AND MOLECULAR SAMPLING

Fourteen of the ca. 18–20 *Leptoscyphus* species (Gradstein et al. 2001) were sampled depending on the availability of suitable material for DNA studies. The sampling almost completely covers the entire distribution range of the genus, which mostly encompasses South America with a few other occurrences in Macaronesia, western Europe, sub-Saharan Africa, Australia, and New Zealand. Sufficiently recent material from New Zealand could, however, not be found despite recent extensive field surveys (D. Glenny, pers. comm.). This sampling corresponds to that of Vanderpoorten and Long (2006), to which a further nine accessions were added so that most species included in this study were represented by multiple accessions to cover as much as possible their entire geographic distribution. *Leptoscyphus australis* and *L. antarticus* were used as outgroups based on the results of a broad molecular phylogeny of *Leptoscyphus* and allied genera (Vanderpoorten and Long 2006).

The chloroplast *atp*B-*rbc*L and *trn*L-F regions were previously sequenced for most of these accessions by Vanderpoorten and Long (2006). To increase resolution and support, these previously published sequences of the chloroplast *atp*B-*rbc*L and *trn*L-F regions were added to sequences from the chloroplast *rps*4 region produced for this study from the same DNA extracts (Table 1).

Although the ITS have often been used in molecular phylogenies of liverworts (e.g., Feldberg et al. 2004, 2007; Heinrichs et al. 2005; Wilson et al. 2007), their use in the present study, together with that of another nuclear gene, namely *rpb*2, proved difficult. The multiloci nature of the ITS region is well known for creating difficulties in phylogenetic reconstructions (see Alvarez and Wendel 2003 for review). In *rpb*2, direct sequencing resulted in conflicting nucleotide calls at many positions, which is consistent with recent reports, in different plant groups, of paralogs in the gene (Oxelman et al. 2004; Luo et al. 2007). Therefore, we decided not to include these nuclear sequences in the dataset.

Chloroplast loci were amplified and sequenced as described in Vanderpoorten and Long (2006) and Buck et al. (2000). Forward and reverse sequences newly obtained for this study were assembled and edited using Sequencher 4.01 (Gene Codes Corporation 1998). Contigs were aligned manually using MacClade 4.1 (Maddison and Maddison 1992), with gaps inserted where necessary to preserve positional homology. Positions that were ambiguously aligned were excluded from the datasets. Potential conflict in the signals displayed by the different genes was searched for by comparing both topologies and posterior probabilities obtained from each one of the three cpDNA datasets. Moreover, an ILD test did not suggest any incongruence among

Table 1. Voucher information and GenBank accession numbers for specimens used in this study. Voucher specimens are deposited in the following herbaria: E, Royal Botanic Garden, Edinburgh, United kingdom; LG, University of Liege, Belgium; GOET, University of Göttingen, Germany; SV, Personal Herbarium of Schäfer–Verwimp; SC, Personal Herbarium of R. Schumacker; B, Personal Herbarium of T.L. Blockeel; EGR, Eger Cryptogamic Herbarium, Hungaria; H, Personal Herbarium of N. Hodgetts.

Taxon sampled; voucher information (country of origin, herbarium, and collection number); *atp*B-*rbc*L, *trn*L and *rps*4 GenBank accession numbers
L. amphibolius #1; Ecuador, SV, *Kottke & Preussing sn*; DQ176676, DQ176723, EU661826
L. amphibolius #2; Mexico, GOET*, Burghardt 4469*; DQ176682, DQ176725, EU661827
L. amphibolius #3; Mexico, E, *Long 29617a;* DQ176688, DQ176717, EU661828
L. amphibolius #4; Mexico, E, *Long 29617b;* DQ176687, DQ176726, EU661829
L. amphibolius #5; Brasil, SV, *Schäfer 14748*; EU350465, EU350474, EU661830
L. australis; Australia, LG, E, *Glenny 9431*; DQ176695, DQ176713, EU661825
L. antarticus; Argentina, E, *Long 31710*; DQ176692, DQ176711, EU661824
L. azoricus #1; Azores (Pico), SC, *Schumacker 20020812*; DQ176679, DQ176728, EU661831
L. azoricus #2; Azores (Terceira), SC, *Schumacker 20030611*; DQ176677, DQ176704, EU661832
L. azoricus #3; Azores (Flores), SC, *Schumacker 20000818/5*; DQ176683, DQ176729, EU661833
L. azoricus #4; Azores (Flores), SC, *Schumacker 20000824*; DQ176685, DQ176730, EU661834
L. cleefii; Venezuela, E, *Long33049*; DQ176689, DQ176708, EU661835
L. cuneifolius #1; Ecuador, SV, *Schäfer 23299/b*; DQ176669, DQ176701, EU661836
L. cuneifolius #2; Scotland, E, *Long 29812*; DQ176698, DQ176732, EU661838
L. cuneifolius #3; Madeira, SV, *Schäfer 26050*; EU350466, EU350475, EU661854
L. cuneifolius #4; United Kingdom, SC, *SBCO 768*; EU350467, EU350476, EU661857
L. cuneifolius #5; United Kingdom, LG, *Vanderpoorten 3101*; EU350468, EU350477, EU661839
L. cuneifolius #6; Ireland, B, TB 31/1325; EU350469, EU350478, EU661837
L. cuneifolius #7; Venezuela, SV, *Schäfer* 12311; EU350470, n/a, n/a
L. cuneifolius #8; Ecuador, SV, *Schäfer 23312/c*; EU350471, EU350479, EU661853
*L. gibbosus #*1; Dominica, SV, *Schäfer 17647*; DQ176670, DQ176702, EU661840
L. gibbosus #2; Costa Rica, SV, *Schäfer SV/H-0364*; EU350472, EU350480, EU661855
L. hexagonus; Venezuela, E, *Long 33049-b*; DQ176696, DQ176714, EU661841
L. infuscatus #1; Malawi, E, *Kungu M3135a*; DQ176693, DQ176712, EU661842
L. infuscatus #2; Malawi, H, *Wiggington M1696b*; DQ176694, DQ176727, EU661843
L. intermedius; Guadeloupe, SV, *Schäfer 22530*; DQ176674, DQ176703, EU661844
L. jackii #1; Ecuador, SV, *Schäfer et al. 24429*; DQ176684, DQ176706, EU661845
L. jackii #2; Ecuador, SV, *Schäfer et al. 24296*; DQ176699, DQ176731, EU661846
L. physocalyx; Venezuela, EGR, *Pocs 05034/H*; DQ176697, DQ176715, EU661847
L. porphyrius #1; Ecuador, SV, *Schäfer 23229/a*; DQ176686, DQ176707, EU661848
L. porphyrius #2; Ecuador, SV, *Schäfer 23448*; DQ176672, DQ176720, EU661849
L. porphyrius #3; Ecuador, SV, *Schäfer 24214/a*; EU350473, EU350481, EU661856
L. porphyrius# 4; Ecuador, GOET, *Gradstein 10125*; DQ176680, DQ176724, n/a
L. sp. nov.1; Costa Rica, SV, *Schäfer SV/H-0342*; DQ176671, DQ176718, EU661851
L. sp. nov.2; Costa Rica, GOET, *Gradstein 9694*; DQ176678, DQ176719, EU661852
L. sp. nov.3; Ecuador, SV, *Schäfer et al. 24282*; DQ176673, DQ176721, EU661850

the three cpDNA datasets ($P = 1.0$), which were therefore combined prior to phylogenetic inference. The combined *atp*B-*rbc*L, *trn*L-F, and *rps*4 dataset is available from TreeBASE at http://www.treebase.org under study number S2176.

MOLECULAR DATING

The phylogeny and divergence times were co-estimated using a Bayesian Markov Chain Monte Carlo method (Drummond et al. 2002) under a relaxed-clock model employing an uncorrelated lognormal model of rate variation among branches in the tree (Drummond et al. 2006). This MCMC, implemented by BEAST 1.4.7 (Drummond and Rambaut 2007), was run under a general time reversible model of nucleotide substitution, with rate variation among sites modeled using a gamma-distribution with four rate categories. One of the trees sampled during a preliminary MCMC analysis implemented in BayesPhylogenies (Pagel and Meade 2004a) was used as a starting tree. The starting tree, however, did not constrain the topology of the trees subsequently sampled by the chain. The divergence dates estimated were thus integrated over the various tree topologies sampled throughout

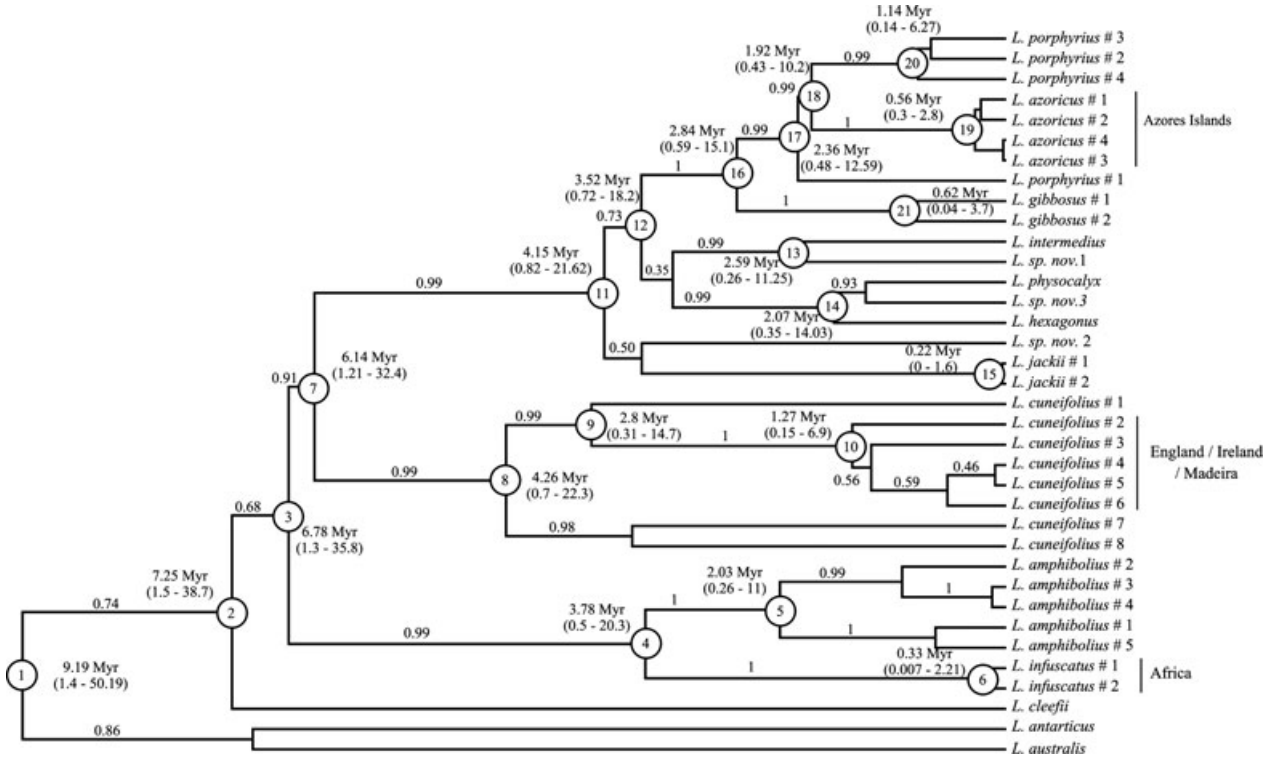

Figure 1. **Divergence times for *Leptoscyphus* as estimated by BEAST version 1.4.7 (Drummond and Rambaut 2007) using a Bayesian Markov Chain Monte Carlo method (Drummond et al. 2002) under an uncorrelated relaxed-clock model (Drummond et al. 2006). The divergence times are indicated only for nodes supported by a posterior probability of > 0.5. The divergence time corresponds to the median of the posterior probability distribution of the node's age in millions years. The number in parentheses represent the 95% highest posterior density interval for the divergence time estimates. Numbers above branches correspond to their posterior probabilities. Node 4 represents the South Atlantic disjunction whereas node 9 represents an intraspecific American/European disjunction. A probabilistic calibration prior, with a maximum and minimum age of 5.1 and 0 Myr (present), respectively, was used on node 18 for calibration. See Table 1 for geographic origin of the taxa sampled.**

the MCMC analysis, and weighted in proportion to their posterior probabilities.

In a first analysis, calibration information was provided by placing a date on the stem node of *L. azoricus* and *L. porphyrius*, that is when *L. azoricus* diverged from its sister South American species (node 18, Fig. 1). This calibration is valid only if the hypothesis of a neo-endemic origin of *L. azoricus* holds true. Alternatively, the same calibration information was placed on the MRCA of *L. azoricus* (node 19, Fig. 1), that is, when *L. azoricus* started to diversify in the Azores. The latter calibration does not imply that *L. azoricus* is a neo-endemic (see Discussion). For both analyses, calibration uncertainties were factored using a probabilistic calibration prior. Accordingly, a normal distribution, with a mean of 5.1 Myr and a standard deviation of 1.5 Myr, was placed on the node being used as a calibration point (either node 18 or 19, Fig. 1). This calibration is based on the formation of the oldest island of the Azorean archipelago, that is, Sta. Maria (5.1 Myr, Schäfer 2003). Under this normal distribution, the likelihood of the divergence of *L. azoricus* increases back in time, which is arguably an appropriate representation of the increasing probability of speciation with time. The normal distribution alone, however, does not model correctly the age of the most recent ancestor of *L. azoricus*, because it encompasses a time period that is older than 5.1 Myr. Consequently, a uniform distribution with a lower and upper bound of 5.1 and 0 (present), respectively, was superimposed to that normal distribution to remove the portion of the normal distribution beyond 5.1 Myr.

A Yule tree prior that assumes a constant lineage birth rate for each branch in the tree was employed (Yule 1924) and four independent MCMC analyses were each run for 10,000,000 steps. Parameter values were sampled every 1000 cycles over the 10,000,000 MCMC steps. Convergence and acceptable mixing of the sampled parameters was checked using the program Tracer version 1.4 (Rambaut and Drummond 2007). After discarding the burnin steps, the four runs were combined to obtain an estimation of the posterior probability distribution of both trees and divergence dates of the ancestral nodes.

The method of molecular dating described above assumes that evolutionary changes occur uniformly along a branch. Evolution is, however, not necessarily a gradual process (Pagel et al.

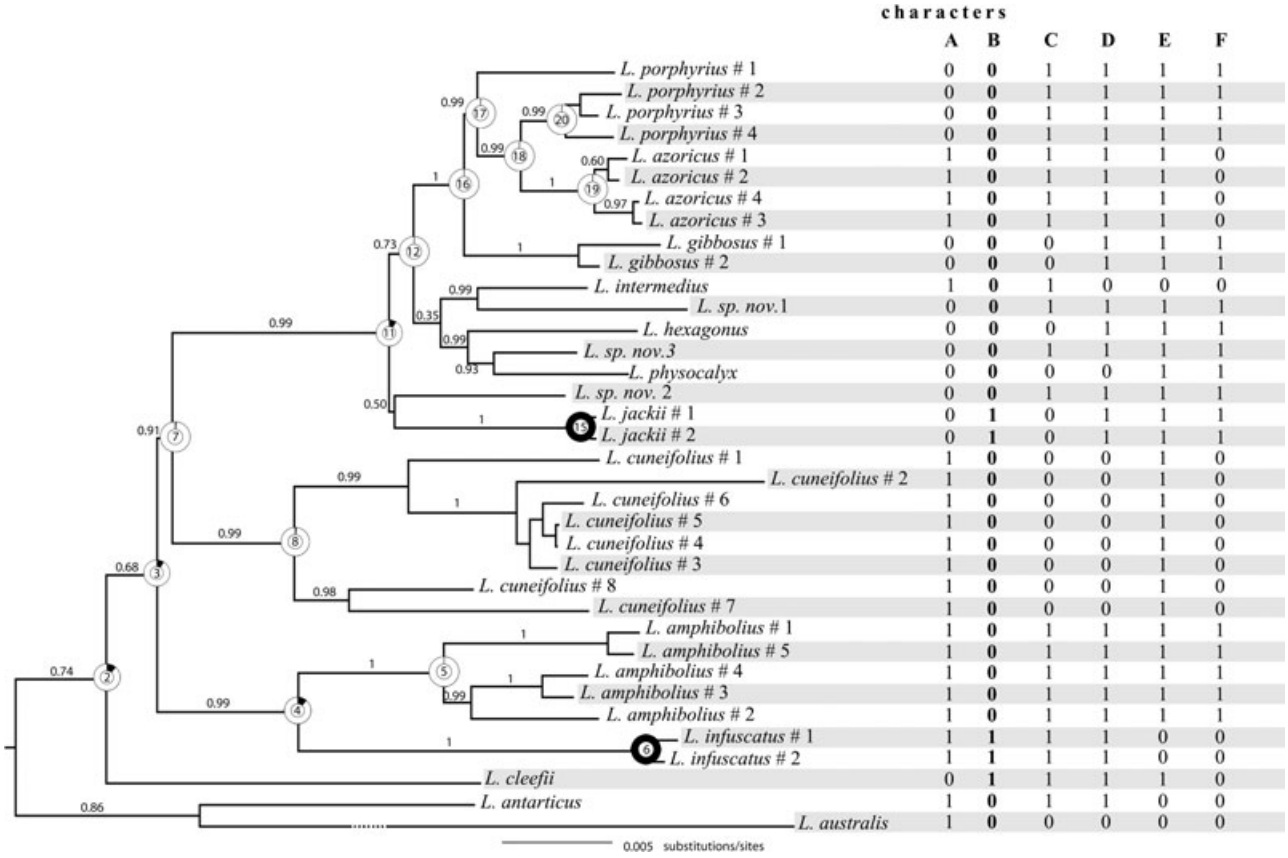

Figure 2. **Majority-rule consensus of the trees (burnin removed) sampled by a Bayesian analysis of *Leptoscyphus trn*L, *atp*B-*rbc*L and *rps*4 sequences implemented in BEAST version 1.4.7 (Drummond and Rambaut 2007). Branch lengths were averaged over the whole sample of trees. Numbers above branches correspond to their posterior probabilities. Pie diagrams indicate the probabilities of ancestral character state for the type of leaf margin (character B; black: denticulate-dentate; white: entire). Those probabilities are derived by averaging the posterior probability distributions of ancestral states sampled by a Markov chain visiting a MCMC sample of trees. The ancestral character states reconstructions are illustrated for selected nodes only. Nodes of interest are numbered as in Table 2 and Figure 1, and are discussed in the text. Character states at terminal taxa for the six variable morphological characters studied (see Table 2 for coding) are indicated on the right.**

2006). Evolutionary divergence among species can, in fact, also be attributed to long periods of stasis followed by short punctuational bursts of evolution associated with speciation. Evidence for punctuational evolution can be found in phylogenetic trees derived from gene-sequence data (Webster et al. 2003). Punctuational evolution in fact involves a positive relationship between path length and the number of nodes along that path, whereas gradual evolution does not (Pagel et al. 2006). In this case, molecular dating techniques relying on a continuous model of nucleotide substitution tend to overestimate actual divergence times. To test for the presence of punctuated molecular evolution in *Leptoscyphus*, that is, to determine if evolution is significantly associated with speciation events independently from time, a dataset including a single accession for each species, as recommended by Pagel et al. (2006), was subjected to a Bayesian analysis as described above. The sample of trees from the posterior probability distribution was then submitted to the Punctuated Evolution program hosted at www.evolution.reading.ac.uk/pe (Pagel et al. 2006). This program estimates, for each tree, the punctuational contribution of speciation to evolution from the relationship between path length (i.e., the sum of the branch lengths along a path from the root of the tree to one of the tips) and the number of nodes along that path (Pagel et al. 2006).

CHARACTER TRANSFORMATIONS AND ANCESTRAL STATE RECONSTRUCTIONS

Morphological evolution within *Leptoscyphus* was examined in a phylogenetic context by reconstructing ancestral shifts in character states for a selection of characters that have been used for species-level taxonomy in the genus (Grolle 1962; Fulford 1976; Hässel de Menendez 2001). Six discrete morphological characters were scored for each species included in our phylogenetic tree. Those characters were scored as follows: (A) perianth mouth entire (0) or dentate (1), (B) leaf margin entire (0) or

denticulate-dentate (1), (C) underleaves divided for $<1/3$ (0) or $>1/2$ (1) of their length, (D) underleaves with entire (0) or denticulate (1) margins, (E) female bracts of similar size (0) or larger (1) than leaves, and (F) underleaves free or attached to leaves on one side (0) or connate with leaves on both sides (1).

Ancestral character state reconstructions were performed using a reverse-jump Markov Chain Monte Carlo (hereafter RJ-MCMC) (Pagel and Lutzoni 2002; Pagel and Meade 2006), as implemented by BayesTraits (Pagel and Meade 2004b). At each iteration, the chain samples a tree from the posterior probability distribution of trees produced by the BEAST analysis, one of four transition models (including one or two rate models, and models wherein either the forward or the backward rate was set to 0), and values of rate parameters. The resulting combination of those parameters is then accepted or rejected depending on the Metropolis–Hastings term. When a combination of tree and rate parameters is accepted, the rate values are used to derive the set of ancestral states at each internal node simultaneously.

To circumvent the issue associated with the fact that not all of the sampled trees necessarily contain the internal nodes of interest, reconstructions were performed using a most recent common ancestor (MRCA) method. This method identifies, for each tree in the Bayesian sample, the MRCA to a group of species and reconstructs the state at the node, then combines this information across trees. Reconstructions of ancestral states were restricted to the nodes, which gained a posterior probability of $> 50\%$ in our Bayesian sample of trees. The rate at which model parameters get changed during the Markov chain (ratedev) was set at the beginning of each run so that the acceptance rate of the proposed changes globally ranged between 20% and 40% (Pagel et al. 2004). Rate coefficients and ancestral character states were sampled every 10,000 generations to ensure independence from successive sampling. Each chain (one per character reconstruction) was run for 10,000,000 generations. An overall estimate of the probability of ancestral state at a particular node was then derived by averaging its posterior state probabilities.

MORPHOLOGICAL EVOLUTION'S TIME FRAME

The temporal calibration of the phylogeny was used to estimate the rate at which morphological transformations operate. Such estimations are, however, only justified if trait transformations, once triggered, gradually occur along the branches of the phylogeny according to a "genetic distance" model (Oakley et al. 2005), in which branch lengths (or a transformation thereof) are used to determine the probability of change on the phylogeny. Traits could, however, evolve according to a "punctuational" (Pagel 1994) or "speciational change" model (Mooers et al. 1999), in which trait evolution is independent of branch lengths. These competing hypotheses can be tested by simulating the impact of branch length transformations on the likelihood of character state reconstructions. The scaling parameter Kappa (hereafter κ) differentially stretches or compresses individual branches. $\kappa > 1$ stretches long branches more than shorter ones, indicating that longer branches contribute more to trait evolution (as if the rate of evolution accelerates within a long branch). $\kappa < 1$ compresses longer branches more than shorter ones. At the extreme, $\kappa = 0$ is consistent with a mode of evolution in which trait evolution is independent of the length of the branch (Pagel 1994). As a consequence, traits have identical probabilities of changing along any branch in the phylogeny, as if transitions were associated with speciation events followed by periods of stasis. To test whether a model allowing for continuous changes along the branches (i.e., a "genetic distance" model) describes the evolution of traits better than a model employing branches of equal lengths by restricting κ to 0 (i.e., a "speciational" model), model performance was compared for each trait by means of Bayes factors. Bayes factors are often used as a goodness-of-fit criterion to determine when a model is to be favored over another. Bayes factors are calculated as the ratio of the marginal likelihoods of the two models being compared (Kass and Raftery 1995). The marginal likelihoods are well approximated by the harmonic mean of the likelihoods when the Markov chain is allowed to run for a very large number (millions) of iterations (Kass and Raftery 1995; Raftery 1996). Strong evidence for a model over another emerges when twice the difference in log-harmonic mean returned by alternative models is > 6 (Kass and Raftery 1995; Raftery 1996).

For characters that evolve in a gradual fashion, absolute rates of character transformations cannot be directly derived from the relative rates sampled by the RJ-MCMC. Indeed, the model employed assumes stationary frequencies of 0.5. Hence, estimates of transitions rates are biased because high transition rates toward the most common state at the tips are often inferred (Stireman 2005). This methodological shortcoming does, however, not affect the likelihood of ancestral states themselves (Nosil and Mooers 2005), so that the time necessary for each morphological transformation can be recorded, providing a dated molecular phylogeny, and averaged for each individual character over all the transformations that occurred along the tree. The time necessary for each morphological character to switch from one state to the other was only estimated for those transformations that occurred between nodes at which reconstruction of ancestral states was supported by posterior probabilities arbitrarily set at > 0.6.

Results

PHYLOGENETIC RECONSTRUCTION AND DIVERGENCE DATE ESTIMATION

The *rps*4 chloroplast gene and the *atp*B-*rbc*L and *trn*L-F intergenic spacers yielded 14.5%, 24%, and 17.9% of variable sites within *Leptoscyphus*, respectively. The 50% majority rule

consensus of the trees sampled by an MCMC implemented by BEAST is presented in Figure 1. With a few exceptions, all the nodes of the trees recovered by the Bayesian analysis received high posterior probabilities. In particular, the south Atlantic disjunction is represented by a fully supported sister relationship between the South American species *L. amphibolius* and the African species *L. infuscatus* (node 4, Fig. 1). Nodes 18 and 19 (Fig. 1), both used for molecular clock calibration, received a posterior probability of 0.99 and 1, respectively. The eight accessions of *L. cuneifolius* form a fully supported monophyletic group. The five European accessions are included within a clade with a posterior probability of 1, which renders the South American accessions paraphyletic (Fig. 1).

Using node 18 as a calibration point, the divergence dates for the node corresponding to the South American/African disjunction in *Leptoscyphus* were estimated at 5.5 Myr (95% highest posterior density: 0.36–11.4, hereafter 95% HPD) before present, whereas the disjunction observed between the three South American and the five European accessions of *L. cuneifolius* was estimated at 4.1 Myr (95% HPD: 0.27–8.4) before present (Fig. 1). The inferred ages for the remaining nodes of the tree are given in Figure 1. Using the alternative calibration point (node 19, Fig. 1), the South American/African disjunction was estimated at 3.78 Myr (95% HPD: 0.5–20.3), whereas the South American/European disjunction was estimated at 2.8 Myr (95% HPD: 0.31–14.7) before present.

Evolutionary divergence in *Leptoscyphus* cannot be attributed to long period of stasis followed by short punctuational bursts of evolution associated with speciation. Indeed, our posterior sample of trees did not reveal any significant punctuated molecular evolution in *Leptoscyphus*, suggesting that a gradual model appropriately describes sequence evolution.

RECONSTRUCTION OF ANCESTRAL CHARACTER STATES

Within *Leptoscyphus*, ancestral character state reconstructions under RJ-MCMC reveal that the six morphological characters scored have a tendency to shift more than once independently along the phylogeny. Figure 2 illustrates, for example, the reconstruction of ancestral states for the type of leaf margin (entire [0] or denticulate-dentate [1]). This character has changed at least three times during the evolution of *Leptoscyphus* (Fig. 2). Probabilities of ancestral state for the other characters investigated are listed in Table 2 for all the major nodes of the phylogeny.

For all the characters investigated, models employing κ-transformed branch lengths to determine the probability of change exhibited a better fit on the data as compared to models using branches of equal length (Table 2). In fact, κ's posterior probability distribution was centred, on average, around 1.23 ± 0.05 and never encompassed 0. Furthermore, constraining the chain to sample models, wherein κ was set to 0, resulted in significantly worse log likelihoods as compared to unconstrained analyses (Table 2). Character state reconstructions combined with information about the divergence time for each node in the phylogenetic tree allowed us to infer the absolute amount of time that is necessary for a shift to occur. According to the divergence date estimated using node 18 as a calibration point, a period of 10.6 Myr (95% HPD: 0.95–20.2) has been necessary for the leaf margin to change from entire to denticulate-dentate along the branch leading to the *L. cleefii* (branch from node 2, Fig. 2). Likewise, 5.08 Myr (95% HPD: 0.35–9.78) were necessary for the same character to shift from one state to the other along the branch leading to *L. infuscatus* (node 4 to 6, Fig. 2). On average, a period of 7.23 Myr, with a minimum and maximum amount of time of 0.59 and 13.47 Myr respectively, is necessary for the leaf margin to change from entire to denticulate-dentate. The average absolute periods of time that are required for the other morphological characters to shift from one state to the other are listed in Table 3.

Discussion

ORIGIN AND TIMING OF THE SOUTH ATLANTIC DISJUNCTION IN LEPTOSCYPHUS

Major geological events assumed to have been responsible for lineage divergence (McDaniel and Shaw 2003), and neo-endemic species found on volcanic islands in particular (e.g., Richardson et al. 2001a; Emerson and Oromi 2005; Wall 2005; Kim et al. 2008), appear as interesting alternative possibilities for calibration of phylogenetic trees when fossil evidence is lacking or incomplete. An insular lineage might be as old as the oldest islands of an archipelago (including islands that are now submerged). However, this type of calibration can only be used with confidence if it can be shown that the insular lineage is not a relict of a once more widespread taxon (i.e., paleo-endemism). Vanderpoorten and Long (2006) argued for a neo-endemic origin of *L. azoricus* by interpreting the paraphyly of its putative parental species, *L. porphyrius*, as the result of incomplete allele sorting owing to the recent origin of the divergence event. Species paraphyly may, however, also result from other processes such as hybridization or cryptic speciation (Funk and Omland 2003). In this case, the hypothesis of a neo-endemic origin of *L. azoricus* does not necessarily hold true. If *L. azoricus* had actually evolved from a continental and now extinct (or unsampled) source population, the use of the age of the Azorean archipelago to calibrate the tree cannot be applied to the MRCA of *L. azoricus* and *L. porphyrius* (Fig. 3A). In Fig. 3A, an unsampled or extinct population is added to the current phylogeny. In this scenario, *L. azoricus* is no longer a neo-endemic species and the calibration that is currently applied to node 18 should in fact be applied to the unknown node "a" (Fig. 3A). However, it is still possible to apply, as an

Table 2. Probability of ancestral character states and parameters of the model of morphological evolution in *Leptoscyphus* for the six morphological characters investigated: (A) perianth mouth entire (0) or dentate (1), (B) leaf margin entire (0) or denticulate-dentate (1), (C) underleaves divided <1/3 (0) or >1/2 (1), (D) underleaves with entire (0) or denticulate (1) margins, (E) female bracts of similar size (0) or larger (1) than leaves and (F) underleaves free or attached to leaves on one side (0) or connate with leaves on both sides (1). From top to bottom: log of the harmonic mean of a model of morphological evolution in which kappa (κ) is sampled by a RJ-MCMC visiting the space of a Bayesian sample of trees, log of the harmonic mean of a model of morphological evolution in which κ is restricted to 0, mean±SD of κ when sampled by the RJ-MCMC (2 × [log of the harmonic mean of the model in which κ is sampled by the RJ-MCMC−log of the harmonic mean of the model in which κ is set to 0] ≥ 6 represents strong evidence against the model in which κ=0), P(0) is the average probability±SD of state 0 at all interior nodes. Nodes are numbered as in Figures 1 and and 2.

		Character					
		A	**B**	**C**	**D**	**E**	**F**
Harmonic mean (when κ estimated)		−14.019852	−11.571114	−15.007446	−11.364217	−10.231243	−14.714929
Harmonic mean (when κ=0)		−22.053387	−15.086397	−23.930081	−23.05181	−15.938281	−22.717335
κ sampled by RJ-MCMC		1.22±0.16	1.29±0.21	1.16±0.14	1.28±0.16	1.30±0.20	1.18±0.15
P(0) at	node 1	0.46±0.17	0.78±0.21	0.12±0.16	0.09±0.11	0.23±0.10	0.52±0.15
	node 2	0.51±0.22	0.79±0.24	0.04±0.08	0.01±0.03	0.02±0.07	0.48±0.21
	node 3	0.29±0.21	0.89±0.19	0.08±0.14	0.026±0.05	0.03±0.08	0.28±0.17
	node 4	0.02±0.03	0.77±0.19	0.011±0.03	0.005±0.1	0.26±0.15	0.23±0.11
	node 5	0.006±0.01	0.99±0.04	0.002±0.01	0.001±0.004	0.009±0.04	0.006±0.01
	node 6	0.001±0.003	0.001±0.01	0.0006±0.002	0.0002±0.0007	0.99±0.014	0.99±0.004
	node 7	0.61±0.16	0.91±0.16	0.23±0.3	0.15±0.19	0.03±0.07	0.38±0.14
	node 8	0.034±0.04	0.96±0.08	0.95±0.04	0.98±0.023	0.03±0.06	0.95±0.05
	node 11	0.99±0.007	0.80±0.22	0.09±0.15	0.0006±0.003	0.008±0.04	0.004±0.01
	node 12	0.99±0.019	0.99±0.04	0.02±0.04	0.004±0.02	0.017±0.064	0.013±0.02
	node 13	0.56±0.15	0.97±0.06	0.012±0.03	0.21±0.24	0.52±0.16	0.45±0.14
	node 14	0.99±0.01	0.98±0.05	0.22±0.28	0.028±0.06	0.013±0.05	0.01±0.025
	node 15	0.99±0.0008	0.0005±0.01	0.99±0.001	0±0.0002	0.002±0.02	0.0004±0.001
	node 16	0.98± 0.032	0.98± 0.05	0.05±0.09	0.002±0.006	0.012±0.05	0.027±0.043
	node 17	0.94±0.06	0.98±0.04	0.003±0.012	0.001±0.004	0.01±0.04	0.07±0.07
	node 18	0.67±0.15	0.98±0.04	0.004±0.017	0.001±0.005	0.012±0.04	0.33±0.15
	node 19	0.0003±0.001	0.99±0.01	0.0002±0.001	0±0.0002	0.002±0.02	0.99±0.001

alternative calibration point, the age of the Azorean archipelago to node 19, which represents the divergence and dispersal of *L. azoricus* within the Azorean archipelago (Fig. 3A). It is worth noting that multiple dispersals from a now-extinct source population cannot be rejected (Figure 3B). However, even if, as illustrated in Figure 3B, multiple dispersals from an extinct population that was present on the mainland occurred, the phylogenetic split at node 19 still tracks the first dispersal event of *L. azoricus*, rendering the use of the age of Azorean archipelago as a calibration point possible.

All our analyses that rely on different sets of assumptions and calibration points converge toward a unique scenario: the trans-Atlantic disjunction observed between *L. amphibolius* and *L. infuscatus* cannot be explained by continental drift following the breakup of Gondwana. Africa and South America started to drift apart ca. 105 Myr ago (McLoughlin 2001). Using molecular dating and under the assumption that *L. azoricus* is a neo-endemic species, the age of the MRCA of *L. infuscatus* and *L. amphibolius* was estimated at 5.5 Myr before present, which clearly indicates that the divergence of the two species largely postdates the Gondwanan fragmentation. Similarly, the node leading to the five European specimens of *L. cuneifolius* was dated at 4.1 Myr, which is not consistent with a divergence caused by ancient vicariance. In fact, the opening of the North Atlantic was completed approximately 50 to 40 Myr ago (Tiffney 1985b) and the last bridges between eastern North America and western Europe sundered 15 Myr ago (Milne and Abbott 2002; Xiang et al. 2005). If *L. azoricus* were not a neo-endemic species, placing a calibration on the MRCA of all four accessions of *L. azoricus* to date their actual in situ diversification results in even younger time estimates.

These divergence times do not need to be corrected for punctuated evolution. In fact, although 43.5% of the phylogenetic trees derived from 40 different plant sequence datasets have been found to contain the signature of past punctuational evolution (Pagel et al. 2006), thereby biasing age estimates from molecular dating techniques assuming continuous rates of evolution, no evidence

Table 3. Minimum, maximum, and average amount of time necessary for a particular morphological trait to switch from one state to the other in *Leptoscyphus* in millions of years. The mean, minimum, and maximum amount of time necessary for each character (see Table 2 for coding) to switch form one state to the other is calculated by averaging over all the transformations that occurred along the tree. Estimations were made for transitions along branches flanked by nodes, for which ancestral state probabilities were > 60%.

Character	Total number of character transformations	Minimum (Myr)	Mean (Myr)	Maximum (Myr)
A	3	0.21	1.99	3.18
B	3	0.59	7.23	13.47
C	5	0.33	3.78	6.85
D	3	0.22	2.84	5.8
E	1	0.35	5.08	9.78
F	3	0.3	3.38	5.91

for punctuated evolution was found in the present data. Thus, in contrast with previous interpretations that largely attributed the many range disjunctions typical of bryophytes to ancient vicariance (e.g., Schuster 1983; Buck and Griffin 1984; Frey et al. 1999; Schaumann et al. 2003, 2004; Pfeiffer et al. 2004; Stech and Frey 2004; Blöcher et al. 2006; Tangney 2007), long-distance dispersal appears as the only likely explanation for the trans-Atlantic disjunction observed in *Leptoscyphus*.

It has, in fact, long been acknowledged that the spores of many bryophytes remain viable under the extremely harsh conditions of cold and drought that prevail in high elevation air currents (van Zanten 1978; van Zanten and Gradstein 1988). Most recently, Munoz et al. (2004) demonstrated that floristic similarities among sub-Antarctic islands are more correlated with wind connectivity than geographic proximity, strongly pointing to long-distance dispersal by wind as the major factor shaping bryophyte distributions. The prevalence of long-distance dispersal in mosses was recently corroborated by analyses of transoceanic gene flow (McDaniel and Shaw 2005; Vanderpoorten et al. 2008). Although it has sometimes been argued that bryophytes can remain unchanged at both the molecular and morphological levels for periods of hundreds of million years (Frey et al. 1999; Pfeiffer 2000), the absence or extremely low sequence divergence among disjunct populations has most often been interpreted as evidence for recent long-distance dispersal (Shaw et al. 2003; Heinrichs et al. 2005). However, our study is among the first ones to clearly reject the ancient vicariance hypothesis based upon an absolute timing of molecular evolution that takes into account calibration and phylogenetic uncertainties. Cases of established ancient vicariance, as in the circumsubantarctic moss, *Pyrrhobryum mnioides* (McDaniel and Shaw 2003), actually become an exception in the context of the recent dispersalist "counter-revolution" (de Queiroz 2005).

CONSEQUENCES FOR MORPHOLOGICAL EVOLUTION

Our results do not support the interpretation that the disjunctions observed are ancient and therefore preclude any need to hypothesize morphological stasis over vast amounts of time. Three of the six investigated characters (leaf margin, female bract size, and the attachment type of the underleaves) have undergone a transformation since the disjunction of the South American/African populations of the ancestor of *L. amphibolius* and *L. infuscatus*. Leaf margin, for instance, was reconstructed as entire in the ancestor of *L. amphibolius* and *L. infuscatus* and evolved into a dentate

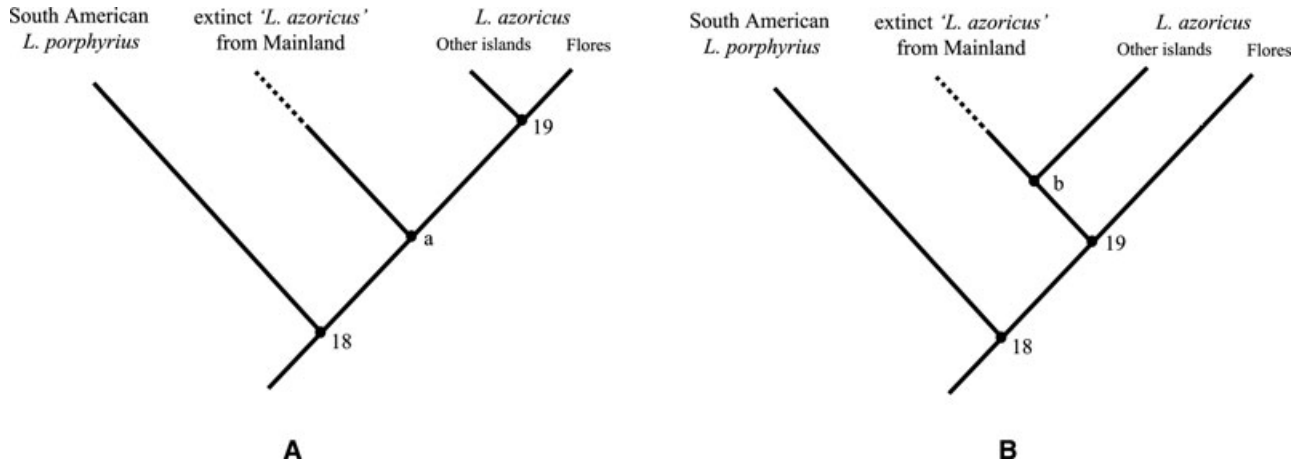

Figure 3. The paleoendemic origin of *Leptoscyphus azoricus*, its impact on the tree topology, and choice of calibration points. (A). *Leptoscyphus azoricus* evolved from a continental and now extinct (or unsampled) source population (node a) and subsequently diversified in the Azores (node 19). (B). Multiple dispersals of *L. azoricus* from a continental and now extinct source population. The phylogenetic split at node 19 tracks the first dispersal event of *L. azoricus* to the Azores. Nodes 18 and 19 correspond to the same nodes in Figure 1 and 2.

margin along the branch leading to the extant *L. infuscatus*. This morphological transformation occurred over a period of 5.08 millions of years (0.35–9.78). Within *Leptoscyphus*, most of the morphological character transitions occurred within a time frame of 4.05 Myr on average.

The complete lack of morphological differentiation between the South American and the European accessions of *L. cuneifolius* is remarkable, however, considering the age of the disjunction and the great molecular divergence observed between the South American and European accession of the species (3.06% of absolute sequence divergence between *L. cuneifolius* #8 and *L. cuneifolius* #2), especially when compared with the interspecific divergence found between *L. cuneifolius* and *L. amphibolius* (3.5% of absolute sequence divergence between *L. cuneifolius* #8 and *L. amphibolius* #5). Such an accumulation of molecular divergence within a morphologically constant lineage, known as cryptic speciation, has been increasingly documented in bryophytes (Odrzykoski and Szweykowski 1991; Shaw 2001; McDaniel and Shaw 2003; Feldberg et al. 2004; Fernandez et al. 2006; Wachowiak et al. 2007). The underlying reasons for such long periods of stasis are, however, largely unknown. As emphasized by McDaniel and Shaw (2003), the influence of any phylogenetic constraint on bryophyte morphology remains an open question.

Globally, however, our analyses suggest that morphological divergence gradually accumulated in the genus. Simulating punctuated morphological evolution by performing appropriate branch length transformations resulted in a significantly lower log likelihood than when actual branch length, or linear transformations thereof, were used to predict shifts in morphological character states. This is shown by the posterior probability distribution of the scaling parameter κ, which was always centred, on average, around 1.23 ± 0.05, and never encompassed 0. In bryophytes, morphological evolution has traditionally been assumed to be punctuated, with periods of fast evolution followed by long periods of stasis (Newton et al. 2000). Perhaps one of the reasons for such an interpretation is that morphological transformations in mosses have been assumed to be associated with dramatic ecological transitions. This is especially true for the transition between terrestrial and epiphytic habitats, which has evolved independently many times in pleurocarpous mosses and has been accompanied by a suite of homoplastic transformations in the sporophyte (Buck and Crum 1990; Buck 1991; Hedenäs 1997; Vanderpoorten et al. 2002; Huttunen et al. 2004).

Overall, our analyses thus suggest that molecular and morphological evolution in *Leptoscyphus* has been gradual. This contrasts with the rapid diversification documented in some tropical trees (Richardson et al. 2001b) and suggests that the current diversity of rainforest bryophytes and angiosperms may not have been achieved in a similar fashion.

CONCLUSIONS

Despite the extreme scarcity of explicitly time-calibrated molecular phylogenies in bryophytes, most recent studies, which have used other source of evidence such as gene flow, relative rates of nucleotide substitution, or clock-like tempo of molecular evolution, have rejected the vicariance hypothesis as an explanation for the transoceanic disjunct distributions observed both in the southern and northern hemisphere (Shaw et al. 2003; McDaniel and Shaw 2005; Hartmann et al. 2006; Heinrichs et al. 2006; Huttunen et al. 2008). Based on a time-calibrated phylogeny, our study reinforces the idea that dispersal has played an important role in shaping the distribution patterns of bryophytes. During the last 10 years, an increasing number of studies have shown that vicariance alone could not explain the disjunctions observed in a variety of plants and animals. It is widely admitted today that dispersal and extinction events have shaped most of the biogeographic patterns observed in angiosperms (Sanmartin and Ronquist 2004; de Queiroz 2005). Bryophytes are the only organisms for which biogeographic patterns were still believed to be the result of ancient vicariance events not overlaid and obscured by long-distance dispersal, mainly because bryophytes were believed to be extremely old organisms with a low dispersal ability and evolutionary potential (Schaumann et al. 2003; Pfeiffer et al. 2004; Schaumann et al. 2004; Stech and Frey 2004; Blöcher et al. 2006). Our data suggest instead that, as in Angiosperms, most of the transcontinental disjunctions observed are the results of long-distance dispersal across oceanic barriers. In fact, the lack of apparent morphological differentiation has long obscured our recognition of what are actually distinct lineages. In this context, recent developments in molecular dating and comparative methods allow to revisit previous hypotheses of dispersal and evolutionary capacity in organisms for which, like bryophytes, the fossil record and morphological variation among extant taxa alone does not allow for a satisfactory reconstruction of their evolutionary history.

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