



Article

A 150 year-old mystery solved: Transfer of the rheophytic endemic liverwort *Myriocolea irrorata* to *Colura*

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Abstract

Myriocolea irrorata is an endemic rheophytic liverwort known from a few localities in the Eastern Andes of Ecuador. Morphologically it belongs to the Cololejeunea-Tuyamaella clade of Lejeuneaceae, however, due to its exclusively Radula-type branching, transversely inserted, hollow leaves, large size, and an extremely high number of clustered gynoecia it has often been regarded as an isolated element of this group. Phylogenetic analyses of a molecular dataset consisting of three markers (nuclear ribosomal ITS region, plastidic *trnL-F* region and *rbcL* gene) and 20 accessions resolved *Myriocolea* in one of the main clades of *Colura*, sister to the generitype *Colura calyptrifolia*. Based on the molecular topology and a reinterpretation of morphological traits, *Myriocolea irrorata* is transferred to *Colura*, as *Colura irrorata*. The example *Myriocolea/Colura* adds to growing evidence that rheophytic liverworts may develop unusual morphologies that hamper their classification using exclusively morphology.

Key words: Lejeuneaceae, liverwort, Porellales, taxonomy

Introduction

In 1857, the English botanist Richard Spruce collected a previously unknown rheophytic liverwort along the Río Topo of the Eastern Andes of Ecuador. Describing it in a new genus of Lejeuneaceae, *Myriocolea* Spruce (1884: 305), he stated that “anything more alien from the aspect of a *Lejeunea* cannot well be imagined” (Spruce 1884: 307). Using a very wide genus concept for *Lejeunea* Libert (1820: 372), Spruce only accepted two genera in Lejeuneaceae, *Lejeunea* with several hundreds of species, and the monospecific *Myriocolea* with its single representative *Myriocolea irrorata* Spruce (1884: 305). This species differs from all other Lejeuneaceae by the exclusive presence of Radula-type branches, transversely inserted leaves without well delimited lobuli, presence of numerous antheridia per bract, and an extraordinary high number of clustered gynoecia (Thiers 1983, Gradstein et al. 2004). By its up to ca. 5 cm long, stiff, protruding leafy shoots it is easily recognizable in the field, however, was not redetected until 2002. It grows on twigs of shrubs occurring along the Topo and Zuñac rivers, especially on the periodically submerged riverbanks (Gradstein & Nöske 2002, Gradstein et al. 2004).

The systematic position of the monospecific genus *Myriocolea* has been subject to controversy. Schuster (1963a: 93) set up a new subfamily Myriocoleoideae to include *Myriocolea* and *Cladocolea* Schuster (1963a: 155), hom. illeg. [= *Schusterolejeunea* Grolle (1980: 105)], a taxon treated as a synonym of Lejeuneoideae by Gradstein (1994). The latter treatment was also supported by cladistic analyses of morphological character states of Lejeuneaceae (Gradstein et al. 2003) where *Myriocolea* was resolved in an unsupported *Cololejeunea-Tuyamaella* clade. This relationship was also recovered by several molecular phylogenies based on one or two molecular markers (Wilson et al. 2004; Gradstein et al. 2006). Phylogenetic analyses of a comprehensive four molecular marker set of Lejeuneaceae consistently located *Myriocolea* in a clade with *Macrocolura* Schuster (1994: 233) and *Colura* (Dumortier 1831: 32) Dumortier (1835: 12) (Wilson et al. 2007a). However, the lack of taxonomic sampling of these two genera hampered the discovery of the true relationships of *Myriocolea*. Addition of further *Colura* sequences to the published ones pointed to a possible close relationship of the genera *Colura* and *Myriocolea* (Yu et al., unpublished). Here we present the results of phylogenetic analyses of a dataset comprising two chloroplast genome regions and one nuclear genome region and a comprehensive taxonomic sampling of *Macrocolura*, *Myriocolea* and 15 accessions of *Colura*.

Materials and Methods

Taxa studied, including GenBank accession numbers and voucher details, are listed in Table 1. Besides availability of material, ingroup taxa were selected to represent the morphological variation and geographical distribution of *Colura*. Multiple accessions of several species were used to explore intraspecific genetic variation. Based on the analyses of Wilson et al. (2007a), *Cololejeunea laevigata* (Mitten 1855: 157) Schuster (1963b: 241) and *Cololejeunea vitalana* Tixier (1995: 230) were designated as outgroup taxa for phylogenetic reconstruction. The ingroup includes accessions of *Colura* and *Macrocolura*.

DNA extraction, PCR amplification and sequencing

Plant tissue was isolated from herbarium collections housed at the herbaria EGR, GOET and JE. Total genomic DNA was purified using Invisorb Spin Plant Mini Kit (Invitek, Berlin, Germany) prior to amplification. Protocols for PCR were carried out as described in previous publications: *rbcL* gene and *trnL-F* region from Gradstein et al. (2006), and nrITS1-5.8S-ITS-2 region from Hartmann et al. (2006). Bidirectional sequences were generated using a MegaBACE 1000 automated sequencing machine using DYEnamic ET Primer DNA Sequencing Reagent (Amersham Biosciences, Little Chalfont, UK). Sequencing primers were those used for PCR. Thirty six sequences were newly generated for this study and 21 sequences were downloaded from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>).

Phylogenetic analyses

All sequences were aligned manually in Bioedit version 7.0.5.2 (Hall 1999). Ambiguous positions were excluded from all alignments. Maximum parsimony (MP) and maximum likelihood (ML) analyses were carried out with PAUP* version 4.0b10 (Swofford 2000). MP heuristic searches were conducted with the following options implemented: heuristic search mode, 1,000 random-addition-sequence replicates, tree bisection-reconnection (TBR) branch swapping, MULTrees option on, and collapse zero-length branches off. All characters were treated as equally weighted and unordered. Non-parametric bootstrapping values (Felsenstein 1985) were generated as heuristic searches with 1,000 replicates, each with ten random-addition replicates. Bootstrap percentage values (BPV) ≥ 70 were regarded as good support (Hillis & Bull 1983). The individual marker sets and the combined chloroplast DNA dataset vs. nrITS dataset were first analysed separately to check for incongruence. The strict consensus trees of the non-parametric bootstrap analyses were compared by eye to identify conflicting nodes supported by at least 70% (Mason-Gamer & Kellogg 1996). The trees gave no evidence of incongruence. Hence the datasets were combined.

jModeltest 0.1 (Posada 2008) was used to select a model of evolution for ML analyses of the combined dataset. A General Time Reversible (GTR) model (Tavaré 1986) was chosen with among-site rate heterogeneity modelled as discrete gamma distribution with four rate categories, and its estimated parameters (Γ) (Goldman 1993). The ML-Analysis was performed as heuristic search, MULTrees option on, collapse zero length branches off, and TBR branch swapping. The confidence of branching was assessed using 300 non-parametric bootstrap resamplings generated as heuristic searches.

TABLE 1. Taxa used in the present study, including information about the origin of the studied material, voucher information, and the herbarium where the voucher is deposited, as well as GenBank accession numbers. Sequences in bold were obtained from GenBank.

Taxon	Origin	Collector and herbarium	Genbank Accession number		
			<i>rbcL</i>	<i>trnL-trnF</i>	nrITS
<i>Cololejeunea laevigata</i> (Mitt.) R.M. Schust.	New Zealand	von Konrat 81-503 (GOET)	DQ238563	DQ238571	DQ987349
<i>Cololejeunea vitalana</i> Tixier	Costa Rica	Schäfer- Verwimp & Holz SV/H-0473/A (GOET)	DQ238564	DQ238573	DQ987348
<i>Colura acroloba</i> (Mont. ex Steph.) Jovet-Ast	Fiji Isls.	Pócs 03261/BK (GOET)	DQ238565	DQ238586	DQ987306
<i>Colura acroloba</i>	Malaysia	Schäfer-Verwimp & Verwimp 18860/A (JE)	JX470966	JX470977	JX470989
<i>Colura calyptriifolia</i> (Hook.) Dumort.	Dominican Republic	Schäfer-Verwimp & Verwimp 26843 (JE)	JX470970	JX470981	JX470993
<i>Colura cylindrica</i> Herzog	Guadeloupe	Schäfer-Verwimp & Verwimp 22154/B (JE)	JX470969	JX470980	JX470992
<i>Colura cylindrica</i>	Panama	Schäfer-Verwimp & Verwimp 30741 (JE)	JX470973	JX470984	JX470996
<i>Colura digitalis</i> (Mitt.) Steph.	La Réunion	Schäfer-Verwimp & Verwimp 20134 (JE)	-	JX470988	JX471000
<i>Colura imperfecta</i> Steph.	Fiji Isls.	Pócs 03261/BA (GOET)	DQ238566	DQ238585	DQ987305
<i>Colura imperfecta</i>	Papua New Guinea	Streimann 41383/a (JE)	JX470971	JX470982	JX470994
<i>Colura inflata</i> K.I. Goebel	Malaysia	Schäfer-Verwimp & Verwimp 19010/A (JE)	JX470968	JX470979	JX470991
<i>Colura ornithocephala</i> Herzog	Ecuador, Azuay	Schäfer-Verwimp & Nebel 32854 (JE)	JX470974	JX470985	JX470997
<i>Colura ornithocephala</i>	Ecuador, Carchi	Schäfer-Verwimp et al. 24391/A (JE)	JX470975	JX470986	JX470998
<i>Colura rhynchophora</i> Jovet-Ast	Dominica	Schäfer-Verwimp & Verwimp 17755 (JE)	JX470976	JX470987	JX470999
<i>Colura tenuicornis</i> (A. Evans) Steph.	Dominican Republic	Schäfer-Verwimp & Verwimp 27039 (JE)	JX470967	JX470978	JX470990
<i>Colura tortifolia</i> (Nees & Mont.) Trevis.	Ecuador	Wilson et al. 04-12 (GOET)	DQ983671	DQ987440	DQ987338
<i>Colura tortifolia</i>	Guadeloupe	Schäfer-Verwimp & Verwimp 22441/C (JE)	JX470972	JX470983	JX470995
<i>Macrocolura sagittistipula</i> (Spruce) R.M. Schust.	Dominica	Schäfer-Verwimp & Verwimp 18014 (GOET)	DQ983707	DQ987466	DQ987367
<i>Myriocolea irrorata</i> Spruce	Ecuador	Gradstein 10033 (GOET)	AY548072	DQ238584	DQ987279
<i>Myriocolea irrorata</i>	Ecuador	Heinrichs et al. 4497	-	-	JX471001

Results

Of the 2,321 investigated molecular characters, 468 were parsimony informative and 166 autapomorphic (see Table 2 for character state distributions within the single markers). The MP analysis resulted in a single tree of 304 steps with a consistency index of 0.696 and a retention index of 0.774 (not depicted). The ML analysis likewise resulted in a single tree (Figure 1) that confirmed the MP topology. *Macrocolura* was placed sister to the well supported *Colura*-clade. *Colura* comprised two main clades with ML/MP bootstrap percentage values of 99 and 100 respectively. One main clade included only Neotropical accessions of *Colura* [*Colura calyptrifolia* (Hooker 1813: pl. 43) Dumortier (1835: 12), *C. ornithocephala* Herzog (1952a: 107), *C. rhynchophora* Jovet-Ast (1948: 27), *C. tenuicornis* (Evans 1900: 455) Stephani (1916: 942)] as well as the monospecific genus *Myriocolea*. The generitype of *Colura*, *C. calyptrifolia*, was placed sister to a clade with two accessions of *Myriocolea*. This sister relationship achieved a ML bootstrap percentage value of 100 and a MP value of 99. The second main clade of *Colura* separated into a robust Asian-Australasian clade comprising *C. inflata* Goebel (1928: 11) and *C. acroloba* (Stephani 1890: 97) Jovet-Ast (1953: 297) and a clade with Australasian [*C. imperfecta* Stephani (1916: 938)], African [*C. digitalis* (Mitten 1886: 325) Stephani (1916: 931)] and tropical American accessions [*C. cylindrica* Herzog (1952a: 106), *C. tortifolia* (Montagne 1843: 265) Trevisan de Saint-Léon (1877: 402)]. The African accession was placed sister to the Neotropical clade in a robust sister relationship. Multiple accessions of several morphologically circumscribed species formed robust monophyletic lineages with bootstrap percentage values between 93 and 100.

TABLE 2. Distribution of constant and phylogenetically informative sites for aligned positions of the three genomic regions.

	<i>rbcL</i>	<i>trnL-F</i>	ITS1-5.8S-ITS2	Total
Number of sites in matrix	935	431	955	2,321
constant	823	324	540	1,687
autapomorphic	42	34	90	166
parsimony informative	70	73	325	468

Based on the molecular topology and adopting a monophyly approach we transfer *Myriocolea irrorata* to *Colura*:

Colura (Dumortier 1831: 32) Dumortier (1835: 12)
= *Myriocolea* Spruce (1884: 305), *syn. nov.*

***Colura irrorata* (Spruce)** Heinrichs, Y.Yu, Schäf.-Verw. & Pócs, *comb. nov.*

Basionym: *Myriocolea irrorata* Spruce (1884: 305).

Type:— ECUADOR, Tungurahua, Río Topo, Spruce s.n. (isotype, G).

Discussion

Classification: General trends

Morphology based classification systems often include small or even monospecific genera. However, modern broad scaled taxonomical studies tend to the acceptance of larger genera and incorporation of many small taxonomic entities, especially when molecular data are included and a monophyly approach is adopted (Humphreys & Linder 2009). Although molecular studies may lead to the division of large genera such as *Jungermannia* Linnaeus (1753: 1131) (Hentschel et al. 2007b), there is a general trend towards larger genus concepts. Phylogenetic analyses repeatedly demonstrated that the character states separating small genera

may in fact represent autapomorphies of taxa nested in larger genera (e.g., Heinrichs et al. 2003). Small genera of liverworts that have recently been synonymized with larger genera based on phylogenetic evidence and reinterpretation of morphological character states include *Rhodoplagiochila* Schuster (1978: 247), *Stereochila* Inoue (1987: 279), *Szweykowskia* Gradstein & Reiner-Drehwald (1995: 33) [= *Plagiochila* (Dumortier 1831: 42) Dumortier (1835: 14), Heinrichs et al. 2004], *Metzgeriopsis* Goebel (1888: 54) [= *Cololejeunea* (Spruce 1884: 291) Schiffner (1893: 121), Gradstein et al. 2006], *Pachyglossa* Herzog & Grolle (1959: 150) [= *Chiloscyphus* Corda (1829: 651), Hentschel et al. 2007a] and *Perssoniella* Herzog (1952b: 268) [= *Schistochila* Dumortier (1835: 15), He & Glennly 2010]. The transfer of *Myriocolea* to *Colura* thus confirms a general trend in plant systematics.

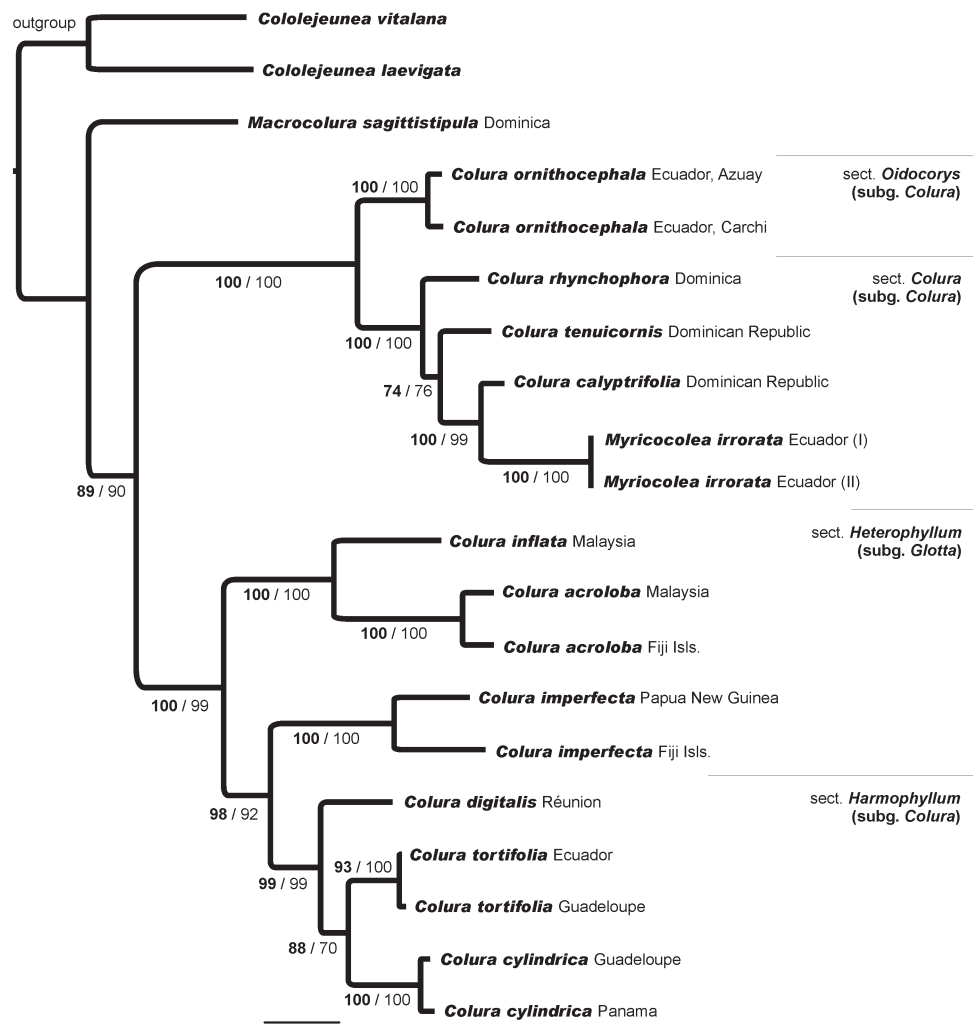


FIGURE 1. Single most likely tree (ln= -9,559.44384) derived from a cp DNA *rbcL-trnL-F* – nrITS dataset. ML bootstrap values (bold) and MP bootstrap values (not bold) are indicated at branches. Two accessions of *Myriocolea irrorata* are nested in a robust subclade of *Colura*, and are placed sister to the *Colura*-generitype, *C. calyptrifolia*.

Colura and *Myriocolea*

Colura is a large pantropical genus comprising about 70 species (Jovet-Ast 1953, Gradstein et al. 2001, Grolle & Zhu 2002) of which eleven species were investigated in our study. Species of *Colura* are typically recognized by their leaf morphology: the presence of lobules forming an apical sac with an aperture mechanism consisting of a valve and a hinge. This sac varies greatly in size and shape, and may function for water retention and in some species also for zoophagy (Barthlott et al. 2000). *Colura* species grow in well-illuminated sites from the lowlands to about 4,000 m above sea level, usually as epiphytes on twigs and trunks or on leaves, as epiphylls.

At first glance, the position of *Myriocolea irrorata* in the *Colura* sect. *Colura* clade is surprising. However, Thiers (1983) points out that the unique aspect of *Myriocolea irrorata* results from an exaggeration of conditions found elsewhere in the Lejeuneaceae whereas an exclusively *Radula*-type branching is otherwise unknown in Lejeuneaceae. However, *Radula*-type branches in combination with *Lejeunea*-type branches occur, e.g., in several species of *Lejeunea* (Schuster 1994, Reiner-Drehwald 2000a, 2005).

Morphological support for the synonymy of *Myriocolea* and *Colura* comes from the number of underleaves. Usually, Lejeuneaceae have one underleaf per leaf pair, however, a few exceptions prove the rule (Gradstein et al. 2003). The presence of one underleaf per leaf characterizes *Colura*, *Diplasiolejeunea* (Spruce 1884: 301) Schiffner (1893: 121), *Macrocolura* and *Myriocolea*, the latter being here identified as an element of *Colura*. *Colura (Myriocolea) irrorata* does not produce well-developed lobules; however, the ventral part of the hollow leaves may be interpreted as an incompletely inrolled, large lobule. Other members of *Colura* sect. *Colura* are characterized by a lobule consisting of a sac terminated by a long, conico-cylindric, elongate horn (Grolle & Zhu 2002). *Colura irrorata* is not the only species of *Colura* without a well-developed lobule. In *Colura* sect. *Heterophyllum* Jovet-Ast (1983: 213) there is also a tendency to lose the lobule, which is very much reduced or completely missing in *Colura corynephora* (Nees, Lindenberg & Gottsche in Meyen 1843: 474) Trevisan de Saint-Léon (1877: 402), a phenomenon that parallels the situation in *Colura irrorata*. This fact also raises the classification problem of *Calatholejeunea* Goebel (1928: 8), which generally resembles *Colura* by its pendular segmentation and *Colura irrorata* by its transversely inserted, hollow leaves with unsharply defined lobules. *Calatholejeunea* was morphologically compared to *Colura* by Mizutani (1984) but its molecular phylogeny has not yet been studied.

Colura irrorata is a rheophilous liverwort growing on twigs of shrubs on the periodically inundated riverbanks of the Río Topo. The rheophytic, nutrient-rich habitat may explain the untypical leaf development of *Colura irrorata* because a structure for water storage or zoophagy is not needed in such an environment. Rheophytes from different taxonomic groups exhibit a parallel development, because running waters and regular flooding shape them into flood resistant plants (Van Steenis 1981). Accordingly, *Colura irrorata* shares its long, robust stems, pinnate branching, and the presence of numerous small gametoeical branches with other rheophytic Lejeuneaceae, namely *Myriocoleopsis* Schiffner (1944: 234), *Lejeunea* subg. *Neopotamolejeunea* (Reiner-Drehwald 2000b: 449) Gradstein & Reiner-Drehwald (2007: 484) (Wilson et al. 2007a) and *Cololejeunea stotleriana* (Gradstein et al. 2011: 13). Non-rheophytic *Colura* species have a smaller size, grow usually attached to the substratum, have sac-like lobules, a lower number of gametoeica, and less robust stems. However, the phylogenetic distance between *Colura irrorata* and other members of *C.* sect. *Colura* is low (Fig. 1), indicating that the rheophytic species *C. irrorata* originated in rather recent times. This scenario is also supported by the derived position of *C. irrorata*. It is likely that the Ecuadorian endemic *C. irrorata* evolved from a local population of a species close to *C. calyptrifolia* and *C. tenuicornis*, and that the morphological rearrangements of the gametophyte took place in a short period of time. A rapid reorganization of gametophytical traits has also been demonstrated for some epiphytic representatives of *Plagiochila* sect. *Hylacoetes* Carl (1931: 50) (Heinrichs et al. 2003), providing some evidence that an occurrence in extreme habitats may occasionally lead to considerable changes in morphology. The molecular control procedures of such rapid rearrangements and their contribution to plant evolution are still incompletely understood (Stern 2000, Carroll 2008, Theissen 2009, Frankel et al. 2011). However, rheophytic plants appear to be an excellent group on which to study the impact of selection on the establishment of rapid growth habit changes. Research on Japanese occurrences of the terrestrial fern *Osmunda japonica* Thunberg (1780: 209) and the rheophytic *O. lancea* Thunberg (1784: 330) showed that habitat conditions may lead to dramatic changes of the leaf morphology in sister taxa that otherwise share more or less the same genetic information (Imaichi & Kato 1992; Yatabe et al. 2009).

Species concepts and supraspecific classification of *Colura*

Colura species have so far been described using a morphological-typological approach. Multiple accessions of several morphologically circumscribed species form monophyletic lineages (*C. acroloba*, *C. cylindrica*, *C.*

imperfecta, *C. irrorata*, *C. ornithocephala*, *C. tortifolia*, Fig. 1), indicating congruence of morphological and phylogenetic species concepts.

Grolle & Zhu (2002) provided the most recent classification of *Colura*. They split *Colura* into two subgenera and six sections, and accepted the monospecific genus *Macrocolura* based on its asymmetrically shaped, only weakly bifid underleaves with rhizoid fascicles originating from their central part. *Colura*, in contrast, has symmetrical, deeply bifid underleaves with rhizoid fascicles originating at the base of the underleaves. Earlier, *Macrocolura* was treated as *Colura* sect. *Lingua* Jovet-Ast ex Thiers (1987: 177). In our study, *Macrocolura* is placed sister to *Colura*, hence both treatments appear consistent with the recovered phylogeny. Based on the molecular topology and the morphological disparities, we tentatively accept *Macrocolura* as a genus. This taxonomic position, however, should be scrutinized by an extension of the *Colura* taxon sampling.

The classification of *Colura* into subg. *Colura* with hinged valves and subg. *Glotta* Grolle & Zhu (2002: 187) having valves without a distinct hinge (Grolle & Zhu 2002), is not confirmed in our study because *C.* subg. *Glotta* (represented in our study by *C.* sect. *Heterophyllum*) nests in *C.* subg. *Colura* (Fig. 1). In total, we included members of four out of six *Colura* sections in our sampling. Sections *Colura* (following inclusion of *Myriocolea*), *Harmophyllum* Grolle (1965: 44) and *Oidocorys* Jovet-Ast ex Grolle (1969: 140) were resolved as monophyletic entities. *Colura* sect. *Heterophyllum* is paraphyletic because *C.* sect. *Harmophyllum* nests in it. Grolle & Zhu (2002) included species with rounded valves in *Colura* sect. *Heterophyllum*, however, according to our topology this section should be split in two entities, of which the *C. imperfecta* clade represents *C.* sect. *Heterophyllum* s. str. (Grolle & Zhu 2002). Our sampling, however, is too sparse to reinterpret morphological traits in *C.* sect. *Heterophyllum* s. l. The formal denomination of the *C. acroloba*-*C. inflata* clade should thus await a more comprehensive study, which should also consider representatives of *C.* sect. *Gamolepis* Jovet-Ast (1983: 207) and *Glotta* Grolle & Zhu (2002: 187).

Biogeography

A phylogenetic analysis of the largely epiphyllous genus *Diplasiolejeunea* revealed a remarkably clear geographical structure with several Neotropical and Paleotropical lineages (Dong et al. 2012). A similar structure is seen in the epiphytic-epiphyllous genus *Colura*, containing Asian-Australasian, Neotropical and African lineages (Fig. 1). The occurrence of a relatively high number of *Colura* species on isolated and young oceanic islands such as Fiji (Pócs & Eggers 2007) is best explained by long-distance dispersal through propagules; however, the clear geographical pattern within the genus seems to indicate that long distance dispersal occurs only occasionally. The range of the Cenozoic genus *Colura* (Wilson et al. 2007b) is possibly the result of rare long distance dispersal, frequent short-distance dispersal, local diversification, extinction and recolonization, processes that likely shaped the ranges of numerous other genera of leafy liverworts (Heinrichs et al. 2009). A more detailed biogeographic reconstruction needs a considerable extension of the taxon sampling and inclusion of multiple accessions of species with broad ranges.

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