Molecular Ecology (1999) **8**, 1037–1042

Phylogeography of North American populations of the moss species *Hylocomium splendens* based on the nucleotide sequence of internal transcribed spacer 2 of nuclear ribosomal DNA

T. Y. CHIANG* and B. A. SCHAAL†
*Department of Biology, Cheng-Kung University, Tainan, Taiwan 701, †Department of Biology, Washington University, St. Louis, MO 63130–4899, USA

Abstract

Sequence variation of the internal transcribed sequence 2 (ITS2) region of nuclear ribosomal DNA (nrDNA) was investigated in 10 North American populations of *Hylocomium splendens*. Cladistic analyses supported the monophyly of this moss species, rooted at *Hylocomiastrum* and *Neodolichomitra*. Three geographically based groups (Great Lakes Forest, Appalachian Mountains, and Pacific Northwest) were identified by a minimum spanning network. Significant genetic differentiation was detected (F_{ST} = 0.197 – 0.390) among three geographical regions in North America. Although high genetic divergence exists within *H. splendens*, these results do not suggest sufficient divergence for designating sibling species.

Keywords: *Hylocomium splendens*, internal transcribed spacer; minimum spanning network, monophyly, phylogeography, populations, ribosomal DNA

Received 20 August 1998; revision received 11 February 1999; accepted 17 February 1999

Introduction

Phylogeny estimation has been a central theme of macro- and microevolutionary studies ever since cladistic and nucleotide sequencing techniques became available (Castelloe & Templeton 1994). During the last decade, most research has focused on phylogeny above the species level using various molecular markers of nuclear as well as organelle genomes (Avise 1994). Recently, increasing numbers of evolutionary biologists have turned their attention to the subspecies level (e.g. Kooistra *et al*. 1992; Vogler & DeSalle 1994). Gene trees provide powerful data to investigate population-level phenomena such as gene flow, founder events and the history of lineages (cf. Castelloe & Templeton 1994).

Internal transcribed spacers of nuclear ribosomal DNA (nrDNA) have been widely used for resolving phylogenetic relationships among closely related species of angiosperms (Baldwin *et al*. 1995). Little to no variation within species is reported in many cases (cf. Baldwin *et al*. 1995), although high variation of ITS among populations has been documented in a few organisms (O'Donnell 1992; Baldwin 1993). Vogler & DeSalle (1994) showed that the variation between sequences of ITS1 could separate distant populations of tiger beetles. In many species, ITS regions of nrDNA have frequent insertions/deletions which can be phylogenetically informative. For example, populations of malaria mosquitoes in Colombia and Venezuela are distinguished from populations of other areas by indels in the ITS2 region (Fritz *et al*. 1994).

Here, we examine an ancient species of mosses, *Hylocomium splendens* Hedw., a conspicuous moss with a tower-like habit and deer-horn (branched) paraphyllia (Noguchi 1972; Rohrer 1985), to detect the mode of molecular divergence and evolution of the nrDNA ITS2 region. The earliest fossil records of this monotypic genus are found in Poland and Russia and can be traced back to the Miocene (Miller 1984). Since then, *H. splendens* has undergone little morphological evolution; no significant differences in morphological characters between fossils

Correspondence: T. Y. Chiang. Fax: + 886 6-2742583, E-mail: tychiang@mail.ncku.edu.tw

and extant plants of *H. splendens* can be identified (cf. Delcourt & Delcourt 1991).

Morphological characters of mosses may be highly conservative due to natural selection as well as developmental constraints. However, molecular evolution may have followed a different course during the long evolutionary history of *Hylocomium*. Genetic variation, especially in noncoding DNA sequences, may have accumulated within lineages. If molecular evolution is more rapid than morphological evolution, cryptic species could evolve and the monophyly of all populations within a species would be invalidated. Cryptic species have been discovered in the liverwort, *Conocephalum conicum*, based on extensive isozyme data (Kim *et al*. 1996).

The purpose of this investigation is: (i) to reconstruct the phylogeny of North American populations of *Hylocomium splendens* to determine whether geographical differentiation occurs among regions; (ii) to test the hypothesis of a molecular clock within *Hylocomium*; and (iii) to test the hypothesis of cryptic species.

Materials and methods

Plant materials

Four populations of *Hylocomium splendens* from the Appalachian Mountains of the United States and three populations in the Pacific Northwest of America were collected (Table 1). Specimens were air-dried. Three herbarium specimens from the Missouri Botanical Garden (MO) herbarium, collected in Pennsylvania, Michigan, and Newfoundland, were also sampled. *Hylocomiastrum pyrenaicum* and *Neodolichomitra yunnanensis* were chosen as outgroups based on a previous systematic study (Chiang 1994). Voucher specimens were deposited in the Missouri Botanical Garden (MO).

DNA extraction and sequencing

Leaf tissue from single individuals was frozen in liquid nitrogen and ground in Eppendorf tubes with a metal dounce. Genomic DNAs were extracted from the powdered tissue in 600 mL of 2× CTAB buffer (Doyle & Doyle 1987) with 0.4% (v/v) β–mercaptoethanol and incubated for 1 h at 65 °C. After adding equal volumes of 24:1 chloroform:isoamyl alcohol, the tissue mixture was centrifuged at 17 968 ***g*** for 15 min at room temperature. The supernatant was transferred to an Eppendorf tube followed by addition of 1.2 mL of absolute ethanol. After overnight incubation at 4 °C, DNA was recovered by centrifuging the mixture at 17 968 ***g*** for 15 min at 4 °C. The brown to black DNA pellet was rinsed in 70% ethanol and centrifuged for 5 min at 9168 ***g***. The DNA pellet was resuspended in 20 mL of TE.

The extracted genomic DNA was purified on a low-melt agarose gel to remove secondary compounds and RNAs. The band on the gel containing the DNA of the correct size was cut out and transferred into an Eppendorf tube. Distilled water of equal volume was added to the gel block containing the purified DNAs. Prior to use of the DNAs for PCR, the gel was heated in a 65 °C water bath for 3 mins.

Two primers, ITS2–3 (5′-GCATCGATGAAGAACGTCGC-3′) and ITS2–4 (5′-TCCTCCGCTTATTGATATGC-3′) (Baldwin 1992), were used for amplifying and sequencing the ITS2 of nrDNA. PCR reactions were carried out using *Taq* polymerase (New England BioLab) and were optimized at annealing temperatures of 55 °C. PCR products were polyacrylamide gel purified and sequenced by a dideoxy-mediated chain-termination method (Sanger *et al*. 1977). The fmol™ DNA Sequencing System (Promega), which uses *Taq* polymerase, was used for sequencing. The detergent NP-40 was added to assist sequencing through

Table 1 Plant materials for DNA isolation and sequencing

Localities	Acronyms	Vouchers	EMBL Accession no.
Hylocomium splendens			
Smoky Mt., N. Carolina, USA	Smoky Mt. 1	Chiang 31091	AJ010345
Smoky Mt., N. Carolina, USA	Smoky Mt. 2	Chiang 31094	AJ010426
Appalachian Mt., N. Carolina, USA	Appalachian	Chiang 32092	AJ010427
N. Carolina, USA	N Carolina	Redfearn 36380	AJ010428
Glacier National Park, Idaho, USA	Glacier	Chiang 31093	AJ010430
N. Cascade Nat. Park, Washington, USA	Cascade	Chiang s.*n*.	AJ010429
Olympia National Park, Washington, USA	Olympia	Chiang s.*n*.	AJ010434
Michigan, USA	Michigan	Allen 13283	AJ010431
Newfoundland, Canada	Newfoundland	Redfearn 37557	AJ010432
Pennsylvania, USA	Pennsylvania	Allen & Pursell 13219	AJ010433
Outgroups			
Hylocomiastrum pyrenaicum:	British Columbia, Canada	Vitt 34097	AJ010435
Neodolichomitra yunnanensis:	Yunnan, China	He 30880	AJ010436

G + C-rich regions and secondary structure (Wang *et al*. 1992). Up to 24 samples were electrophoresed on each 6% acrylamide gel at 60 W. Both strands of DNA were sequenced with approximately 50 bp of overlap.

Data analysis

Sequence alignment. The boundary of ITS-2 was determined by comparing the aligned sequence with sequences of other mosses and liverworts registered in GenBank. Sequences were aligned by multiple alignments without weighting transversions or transitions using the CLUSTAL V program (Higgins *et al*. 1992) and later adjusted visually. The fixed gap penalty was 35 and the floating penalty was 4.

Phylogenetic analyses. The cladistic analysis of aligned sequences was performed by maximum parsimony using the program PAUP (version 3.1.1., Swofford 1993) and a neighbour-joining (NJ) method using MEGA (version 1.01, Kumar *et al*. 1993). Parsimony analyses were conducted using branch-and-bound searches. Neighbour-joining analyses were conducted by calculating Kimura's (1980) 2-parameter distance. Phylogenetic relationships among the nucleotide sequences were also inferred from a minimum spanning network with the aid of the MINSPNET (Excoffier & Smouse 1994).

Relative rate tests. The hypothesis of a molecular clock was tested by relative rate tests (Wu & Li 1985). The data on number and ratio of transversions to transitions between taxa were obtained from MEGA. The difference of nucleotide substitution (K) between sequences, which is the number of transitional and transversional substitutions per site, was calculated using *Hylocomiastrum pyrenaicum* as the reference species. The null hypothesis of a molecular clock suggests that the number of nucleotide substitutions between two lineages would be the same. Based on the assumption of a normal distribution of nucleotide substitutions (Wu & Li 1985), the hypothesis of molecular clock will be rejected with 95% significance when the difference of substitution rates between two lineages is greater than 1.96-times the standard error.

Results

Nucleotide sequences and intraspecific divergence

A total of 412 bp of nrDNA ITS2 nucleotide sequence from *Hylocomium* populations and outgroups was aligned. Alignment is available from the authors upon request. The length of ITS2 in *Hylocomium splendens* varied from 382 to 386 bp. Outgroup spacer lengths were 384 bp in *Neodolichomitra yunnanensis* and 392 bp in *Hylocomiastrum pyrenaicum*. Both the ITS2 of *Hylocomium splendens* and the outgroup are G + C rich with an average of 60.9%.

Most of the variation between populations of *H. splendens* was nucleotide substitutions, except for several indels: such as a 4-base deletion (GGCG, 187–190) in the Glacier National Park population, and a 3-base 'GAA' insertion (275–278) in the Smoky Mt population. Pairwise comparisons of transitions vs. transversions indicated that in 841 substitution events, 434 were transitions and 407 were transversions between populations of *H. splendens* (transitions/transversions = 1.07). Within *H. splendens*, 77 of 412 bases (18.7%) are variable. Among them, 15 bases (3.6% of total) were phylogenetically informative and the rest occurred only in single populations (autaptomorphy).

Kimura's (1980) 2-parameter distance was calculated both within and between geographical groups. Within the Appalachian Mountains clade the ITS2 distance ranged from 0.0055 to 0.0251 (mean = 0.014); within the Great Lakes Forest clade ITS2 distance ranged from 0.00 (between Pennsylvania and Newfoundland) to 0.0308 (mean = 0.0205); and within the Pacific Northwest clade ITS2 distance ranged from 0.0138 to 0.0481 (mean = 0.0357). Populations distributed in the Pacific Northwest had higher ITS2 sequence divergence. As expected, higher genetic divergence occurs between clades than within clades. Genetic divergence ranges from 0.00 to 0.0691 between populations, with averages of 0.0351 between the Appalachian Mountains and the Pacific Northwest, 0.0284 between the Appalachian Mountains and the Great Lakes Forest, and 0.405 between the Great Lakes Forest and the Pacific Northwest.

Phylogenetic reconstruction

Thirteen equally parsimonious trees, rooted at *Neodolichomitra yunnanensis* and *Hylocomiastrum pyrenaicum*, with 119 steps, a confidence interval (CI) of 0.95 ($P \leq 0.01$; Klassen *et al*. 1991), and a *g1* statistic of –1.28 ($P \leq 0.05$; Hillis & Huelsenbeck 1992), were recovered by PAUP. The monophyly of *H. splendens* was significantly supported (Fig. 1A) by a bootstrap value of 91% (cf. Hillis & Bull 1993). Two nodes were also significantly supported: the clade of all populations except that of Glacier National Park with 96% bootstrap value; and the clade of populations from Pennsylvania and Newfoundland with 100% bootstrap value. MEGA analysis identified identical topology to PAUP trees based on Kimura's 2-parameter genetic distances among populations (Table 2).

Although two subclades within *H. splendens* were identified, the cladogram did not reveal clear biogeographical patterns either between Appalachian populations or populations of Pacific Northwest (Fig. 1A). For example, the population of Olympia National Park was more related to the clades of Great Lakes Forest and Appalachian

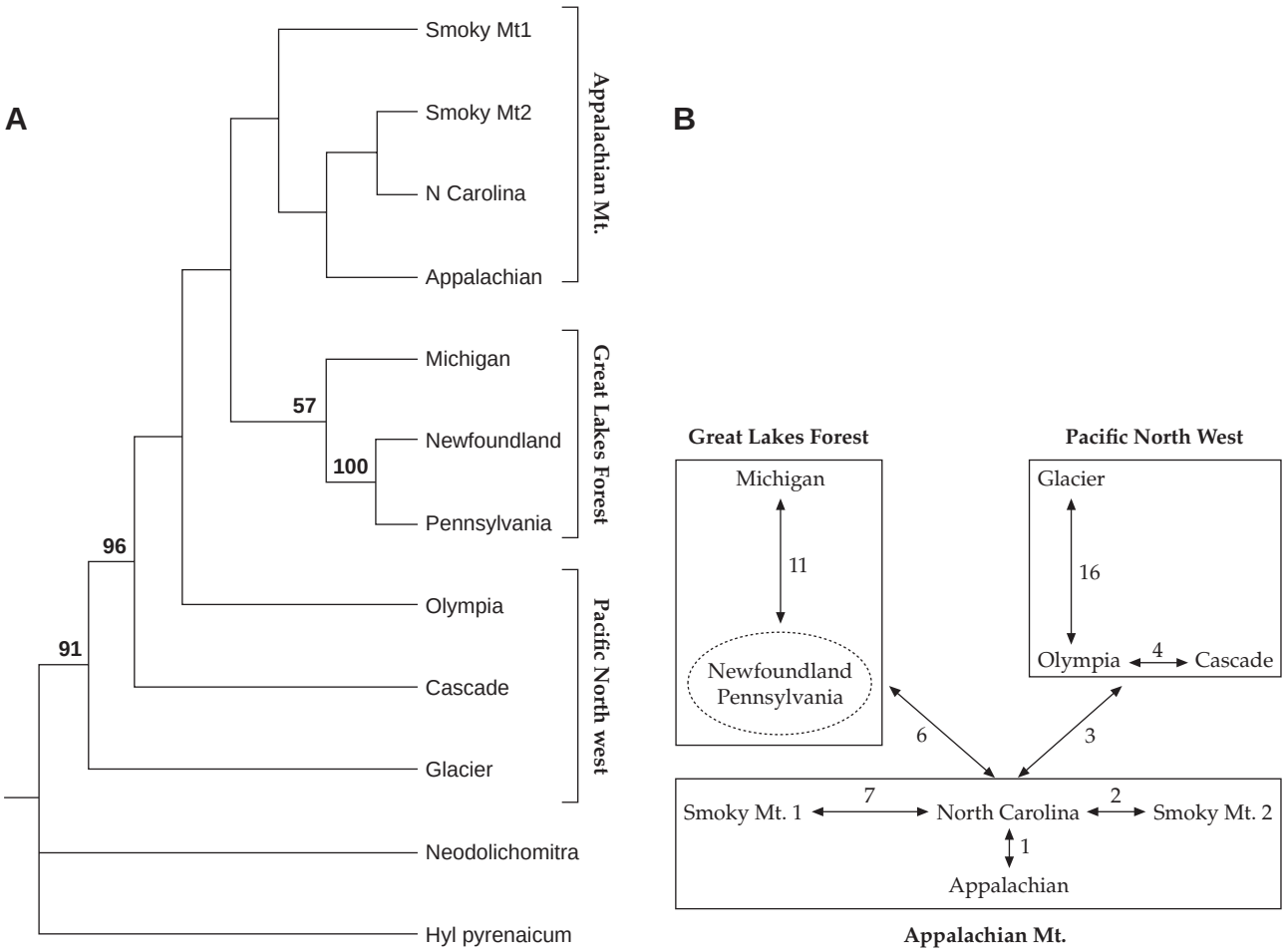

Fig. 1 (A) One of the parsimonious trees reconstructed by PAUP rooted at *Hylocomiastrum pyrenaicum* and *Neodolichomitra yunnanensis* indicating the phylogeny of populations of *Hylocomium splendens* based on ITS2 sequences. Numbers at nodes are bootstrap values. (B) Minimum spanning network generated using the method of Excoffier & Smouse (1994) for ITS2 sequences of North American populations of *H. splendens*. Numbers at nodes indicate the number of nucleotide changes between haplotypes.

Table 2 Kimura 2-parameter distance (above the diagonal) and the absolute value of K/SE (SE = standard error) (below the diagonal) among populations (1–10) of *Hylocomium splendens* and outgroups (11, 12). Genetic distances are in the upper-right matrix and the standard errors are in lower-left matrix

OTUs 1	2	3	4	5	6	7	8	9	10	11	12
1	0.0251	0.0194	0.0222	0.0279	0.0689	0.0365	0.0510	0.0365	0.0365	0.0871	0.0964
2	0.67	0.0055	0.0083	0.0138	0.0540	0.0166	0.0365	0.0222	0.0222	0.0659	0.0840
3	2.27*	0.002	0.0027	0.0083	0.0482	0.0166	0.0308	0.0166	0.0166	0.0659	0.0779
4	1.78	0.003	1.00	0.0110	0.0511	0.0194	0.0336	0.0194	0.0194	0.0688	0.0810
5	1.58	0.004	5.08**	0.00	0.0452	0.0138	0.0336	0.0194	0.0194	0.0688	0.0810
6	1.10	0.013	0.013	0.014	0.013	0.0481	0.0749	0.0599	0.0599	0.0779	0.0934
7	0.85	0.004	0.79	0.73	0.004	0.63	0.0423	0.0279	0.0279	0.0659	0.0841
8	1.89	0.010	3.86**	3.48**	0.009	2.5*	2.72*	0.0308	0.0308	0.0996	0.1122
9	0.00	0.004	2.43*	1.87	0.004	1.17	0.94	2.45*	0.0000	0.0840	0.0964
10	0.00	0.004	2.43*	1.87	0.004	1.17	0.94	2.45*	0.00	0.0840	0.0964
11	—	—	—	—	—	—	—	—	—	—	0.0932

1, Smoky Mt1; 2, Smoky Mt2; 3, N Carolina; 4, Appalachian; 5, Olympia; 6, Glacier; 7, Cascade; 8, Michigan; 9, Newfoundland; 10, Pennsylvania; 11, *Neodolichomitra*; 12, *Hylocomiastrum pyrenaicum*.
*$P \leq 0.05$; ** $\Pi \leq 0.01$.

Table 3 Pairwise estimates of *Nm* (above diagonal) and F_{ST} (below diagonal) (Hudson *et al*. 1992) using DnaSP (Rozas & Rozas 1997)

	Appalachian Mt.	North-west Pacific	Great Lake Forests
Appalachian Mt.	—	1.02	0.39
North-west Pacific	0.197	—	0.58
Great Lake Forests	0.390	0.302	—

Mountains than to Cascade or Glacier National Park. The population of Glacier National Park is the basal clade on the cladogram rooted at *Hylocomiastrum* and *Neodolichomitra*.

Discussion

ITS sequences are insufficient for resolving the population phylogeny of *Hylocomium splendens* based on the trees recovered by PAUP which are rooted at *Hylocomiastrum* and *Neodolichomitra*. For a better picture of the evolution among populations of *H. splendens*, an unrooted network based on all variable sites was constructed (Fig. 1B). The network reveals a hierarchical relationship among populations and identifies three major geographical groups based on nucleotide substitutions: the Appalachian Mountains, the Great Lakes Forest, and the Pacific Northwest. The latter two regions are connected to the clade of Appalachian Mountains by six and three substitutions, respectively. This nested network suggests a model of 'isolation by distance' among geographical groups. The relationships within each clade were also resolved with nucleotide changes, except for the Newfoundland and Pennsylvania populations, which share identical ITS2 sequences. In the Pacific Northwest clade, populations of Cascade National Park and Glacier National Park are linked to Olympia National Park with 4 and 16 substitutions, respectively; and in the clade of Appalachian Mountains, three populations diverged from the North Carolina population. Similarly, populations of Newfoundland and Pennsylvania were distinguished from the Michigan population by 11 nucleotide substitutions.

Based on pairwise sequence comparisons the genetic divergence among North American populations of *H. splendens* was higher than for most angiosperm species (e.g. *Lopezia* species, O'Kane 1993), species in the Winteraceae (Suh *et al*. 1993), or races of oca (Emshwiller & Doyle 1998). The high intraspecific divergence may be associated with the long evolutionary history of *H. splendens*, which allows genetic variation to accumulate within lineages. However, we find little support for the hypothesis of sibling speciation. Populations from three regions in North America and an additional one in China (data not shown; cf. Chiang 1994) constitute a monophyletic group which is a single lineage.

The nucleotide sequences of the ITS2 region reveal genetic differentiation among populations of *H. splendens* in North America, with F_{ST} ranging from 0.197 to 0.390 (Table 3). Gene flow may be estimated from F_{ST}; *Nm* ranges from 0.39 to 1.02, suggesting that gene dispersal is somewhat limited by physical constraints. However, values of *Nm* calculated for F_{ST} values should be interpreted with caution, because few plant populations conform to the implicit assumption of genetic equilibrium. Shared polymorphisms may be due to lineage sorting as well as gene flow. In a study of allozyme variation in *H. splendens*, Cronber *et al*. (1997) found low levels of genetic differentiation (G_{ST} = 0.073) and correspondingly high estimates of gene migration. The different estimates of population structure may be due to different geographical ranges in the two studies (Scandinavia vs. North America) or to a difference in the specific evolution of the markers (allozymes vs. noncoding spacer region).

Finally, we examine the rate of ITS2 sequence evolution in *H. splendens*. Relative rate tests (Wu & Li 1985) indicate that the ITS sequence is not consistent with the hypothesis of a molecular clock. Heterogeneity in the substitution rates of the ITS2 region was detected in the clade of Michigan, Pennsylvania, and Newfoundland and the population of North Carolina. The populations of northeast America evolved much faster than the populations of other regions, whereas the North Carolina population evolved significantly slower. The cause of this rate heterogeneity is not clear but could involve genetic drift, relaxation of natural selection, or some other factors.

H. splendens has the highest level of ITS2 variation found for any plant species to date. The levels of variation detected within the species are sufficient for phylogeographic studies, and suggest that mosses may be ideal candidates for studies that examine broad patterns of biogeographical differentiation.

Acknowledgements

We thank Dr Brent D. Mishler for his advice on DNA extraction and Steve O'Kane for his assistance in DNA sequencing and data analysis. We thank Prof. Peter H. Raven for his encouragement in this research and two reviewers for their helpful comments.

References

Avise JC (1994) *Molecular Markers, Natural History and Evolution*. Chapman & Hall, New York.

Baldwin BG (1992) Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: an example from the Compositae. *Molecular Phylogenetics and Evolution*, **1**, 3–16.

Baldwin BG (1993) Molecular phylogenetics of *Calycadenia* (Compositae) based on ITS sequences of nuclear ribosomal DNA: chromosomal and morphological evolution reexamined. *American Journal of Botany*, **80**, 222–238.

Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS, Donoghue MJ (1995) The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Annals of Missouri Botanical Garden*, **82**, 247–277.

Castelloe J, Templeton AR (1994) Root probability for intraspecific gene trees under neutral coalescent theory. *Molecular Phylogenetics and Evolution*, **3**, 102–113.

Chiang TY (1994) Phylogenetics and evolution of the moss family Hylocomiaceae. PhD Thesis, Division of Biology and Biomedical Sciences, Washington University, St. Louis, USA.

Cronberg N, Molau U, Sonesson M (1997) Genetic variation in the clonal bryophyte *Hylocomium splendens*: a hierarchical geographical scale in Scandinavia. *Heredity*, **78**, 293–301.

Delcourt HR, Delcourt PA (1991) *Quaternary Ecology. A Paleoecological Perspective*. Chapman & Hall, London.

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, **19**, 11–15.

Emshwiller E, Doyle JJ (1998) Origins of domestication and polyploidy in oca (*Oxalis tuberosa*: Oxalidaceae): nrDNA ITS data. *American Journal of Botany*, **85**, 975–985.

Excoffier L, Smouse PE (1994) Using allele frequencies and geographic subdivision to reconstruct gene trees within a species: molecular variance parsimony. *Genetics*, **136**, 343–359.

Fritz GN, Conn J, Cockburn A, Seawright J (1994) Sequence analysis of the ribosomal DNA internal transcribed spacer 2 from populations of *Anopheles nuneztovari* (Diptera: Culicidae). *Molecular Biology and Evolution*, **11**, 406–416.

Higgins DG, Bleasby AJ, Fuchs R (1992) CLUSTAL V: improved software for multiple sequence alignment. *CABIOS*, **8**, 189–191.

Hillis DM, Bull JJ (1993) An empirical test of bootstrapping as a method assessing confidence in phylogenetic analysis. *Systematic Biology*, **42**, 182–192.

Hillis DM, Huelsenbeck JP (1992) Signal, noise, and reliability in molecular phylogenetic analyses. *Journal of Heredity*, **83**, 189–195.

Hudson RR, Slatkin M, Maddison WP (1992) Estimation of levels of gene flow from DNA sequence data. *Genetics*, **132**, 583–589.

Kim HN, Harada K, Yamazaki T (1996) Isozyme polymorphism and genetic structure of a liverwort *Conocephalum conicum*. *Genes and Genetic Systematics*, **71**, 225–235.

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, **16**, 111–120.

Klassen GJ, Mooi RD, Locke A (1991) Consistency indices and random data. *Systematic Zoology*, **40**, 446–457.

Kooistra WHCF, Stam WT, Olson JL, van den Hoek C (1992) Biogeography of *Cladophoropsis membranacea* based on comparisons of nuclear rDNA ITS sequences. *Journal of Phycology*, **28**, 660–668.

Kumar S, Tamura K, Nei M (1993) MEGA: Molecular Evolutionary Genetics Analysis, Version 1.01. The Pennsylvania State University, University Park, PA 16802, USA.

Miller NG (1984) Tertiary and Quaternary fossils. In: *New Manual of Bryology* (ed. Schuster RM), pp. 1194–1232, The Hattori Botanical Laboratory, Nichinan, Japan.

Noguchi A (1972) On the delimitation of the genera of Hylocomiaceae and Rhytidiaceae. *Journal of the Hattori Botanical Laboratory*, **35**, 155–168.

O'Donnell K (1992) Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium sambucinum* (*Gibberella pulicares*). *Current Genetics*, **22**, 213–220.

O'Kane SL (1993) Molecular systematics of *Lopezia* (Onagraceae). PhD Thesis, Division of Biology and Biomedical Sciences, Washington University, St Louis, MO, USA.

Rohrer JR (1985) A phenetic and phylogenetic analysis of the Hylocomiaceae and Rhytidiaceae. *Journal of Hattori Botanical Laboratory*, **59**, 185–240.

Rozas J, Rozas R (1997) DnaSP, Version 2.0: a novel software package for extensive molecular population genetics analysis. *Computer Application Biosciences*, **13**, 307–311.

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciencesof the USA*, **74**, 5463–5467.

Suh Y, Thien LB, Reeve HE, Zimmer EA (1993) Molecular evolution and phylogenetic implications of internal transcribed spacer sequences of ribosomal DNA in Winteraceae. *American Journal of Botany*, **80**, 1042–1055.

Swofford DL (1993) PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1.1. Computer program distributed by the Illinois Natural History Survey, Champaign, Illinois.

Vogler AP, DeSalle R (1994) Evolution and phylogenetic information content of the ITS-1 region in the tiger beetle *Cicindela dorsalis*. *Molecular Biology and Evolution*, **11**, 393–405.

Wang B, Fang Q, Williams WV, Weuber DB (1992) Double-stranded DNA sequencing by linear amplification with Taq DNA polymerase. *Biotechniques*, **13**, 527–529.

Wu CI, Li WH (1985) Evidence for higher rates of nucleotide substitution in rodents than in man. *Proceedings of the National Academy of Sciences of the USA*, **82**, 1741–1745.

This study is part of Tzen-Yuh Chiang's PhD project, which involves the sequencing and use of the ITS2 sequences of rDNA to study the genetic diversity in North American populations of *Hylocomium splendens*. This project is under the supervision of Barbara A. Schaal, Peter H. Raven, Alan Templeton, Tuan-Hua Ho, Allan Larson, Barbara Kunkel (Washington University) and Peter H. Hoch (Missouri Botanical Garden).
