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Phylogenetic Analyses of Timmiaceae (Bryophyta: Musci) Based on Nuclear and Chloroplast Sequence Data

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ABSTRACT. Nuclear ribosomal (26S) and chloroplast (*trnL-trnF* and *atpB-rbcL* spacer) genomic regions were sequenced from 29 exemplars of Timmiaceae and five outgroup taxa. Phylogenetic hypotheses were tested from analyses of the individual regions and a combined dataset, using both parsimony and Bayesian inference methods. Estimates of branch support were established using bootstrap analyses and Bayesian posterior probabilities. The analyses were used to test the monophyly of the species and the relationships among taxa in Timmiaceae, previously based on morphology. Phylogenetic inferences suggest that *Timmia austriaca*, *T. megapolitana* s.s., *T. megapolitana* subsp. *bavarica*, and section *Norvegica* represent monophyletic taxa. *Timmia norvegica* s.s., *T. norvegica* var. *excurrens*, and *T. sibirica* were non-monophyletic, but together form a single clade. We, therefore, recommend recognizing them as a single taxon, *T. norvegica*. A key to the taxa is presented.

KEYWORDS: *atpB-rbcL*, phylogeny, systematics, *Timmia*, *trnL-trnF*, 26S.

Timmiaceae are a monogeneric family of mosses comprising four species (Brassard 1979, 1980, 1984) in *Timmia* Hedw., named in honor of the German botanist Joachim Christian Timm (Hedwig 1801). All species are distributed across the Northern Hemisphere in arctic-montane regions, with only *T. megapolitana* Hedw. subsp. *megapolitana* occurring in temperate regions. In the Southern Hemisphere, *Timmia* is known only from three New Zealand populations of *T. norvegica* Zett. (Horton and Bartlett 1983). Despite *Timmia*'s broad distribution, populations are locally rare. They are restricted to highly calcareous substrates, such as marble and limestone outcrops where the bryophyte layer is constantly or intermittently wet due to seepage, running water, or poor drainage in low-lying areas (Miller and Ireland 1978; Horton 1981).

Timmiaceae are diagnosed by the architecture of teeth that line the sporangium mouth (i.e., the peristome). Composition of the outer peristome (exostome) resembles those of other arthrodontous mosses (i.e., 16 teeth composed of two columns of cells on the outer surface), whereas the unique endostome consists of a tall basal membrane topped by 64 narrow cilia. In all other taxa with ciliate endostomes (i.e., Bryineae sensu Vitt 1984), broad segments alternate with groups of one to three cilia. The homology of the *Timmia* endostome is ambiguous (Vitt 1984; Cox et al. 2000).

Morphological similarities between peristome teeth have been used to hypothesize relationships among bryophyte families (Fleischer 1904–23; Brotherus 1924–25; Vitt 1984). Based on similarities between endostomes, Timmiaceae were considered members of Bryineae (Taylor 1962; Vitt 1984). The advent of molecular phylogenetic analysis has allowed these morphology-based hypotheses of familial relationships to be tested. Phylogenetic inferences from nucleotide data suggest that Timmiaceae do not share a unique ancestry with Bryales but are instead more closely related to Funariales and Encalyptales; thus the origin of Timmiaceae occurs within the early radiation of mosses with joined peristomes (i.e., arthrodontous mosses; Cox et al. 2000; Newton et al. 2000; Goffinet et al. 2001; Cox et al. 2004).

Brassard (1979, 1980, 1984), the monographer of Timmiaceae, defined taxa based on morphological characters of both the sporophyte and gametophyte. Variation in the degree of appendiculation of endostome cilia is the major sporophytic character (Brassard 1979). Gametophytic characters of taxonomic significance include the hyaline versus chlorophyllose basal cells of the leaves, the dioicous or monoicous distribution of sex organs, the ornamentation type on dorsal and ventral surfaces of limb laminal cells, and width of mid-limb laminal cells (Brassard 1979). Brassard (1979, 1980, 1984) recognized four species, two subspecies, and two varieties and placed 15 previously recognized species names (Wijk et al. 1969) into synonymy. He also divided the genus into three sections based on morphological characteristics: sect. *Timmiaurea* (*T. austriaca*), sect. *Timmia* (*T. megapolitana* subsp. *bavarica* and subsp. *megapolitana*), and sect. *Norvegica* (*T. norvegica* var. *excurrens*, var. *norvegica* and *T. sibirica*). Both infraspecific taxa were previously recognized as *T. comata* Lindb. & Arnell (= *T. norvegica* var. *excurrens*) and

T. bavarica Hessl. (= *T. megapolitana* subsp. *bavarica*; Nyholm 1960; Abramova et al. 1961). The rank of these taxa remains a point of contention among authors. Frey et al. (1995) and Ignatov and Ignatova (2003) recognized both infraspecific taxa at the species level, whereas Crum and Anderson (1981) and Crum (1994) followed Brassard's taxonomic concepts and recognized these taxa at the infraspecific level.

Brassard developed his taxonomic concepts based on morphological characters. Whether these taxa are monophyletic, and hence whether the diagnostic characters are apomorphic, has never been tested. The objectives of the present study are to 1) reconstruct the phylogeny within Timmiaceae based on nucleotide sequences of chloroplast and nuclear markers obtained for multiple accessions per taxon and 2) examine whether the monophyletic groups identified can be diagnosed by traditional morphological characters.

Materials and Methods

Taxon Sampling. All taxa of Timmiaceae recognized by Brassard (1979, 1980, 1984) were included in this study, and for each taxon, several populations were sampled, for a total of 29 ingroup exemplars and five outgroup taxa. Outgroup exemplars were selected among a) Encalyptales and Funariales, which together with Timmiales comprise Funariidae, b) the putative sister-group, Dicranidae, and c) Diphysciidae, the sister-group to arthrodontous mosses (Goffinet and Buck 2004). For the ingroup, exemplars were sampled from all continents on which *Timmia* is known to occur, except for the disjunct populations in New Zealand. Morphological characteristics were utilized to confirm specimen identities. Voucher and collection information are detailed in Appendix 1.

DNA Extraction, Amplification, and Sequencing. DNA was extracted from dried herbarium specimens using DNeasy Plant Mini Kit (Qiagen, Chatsworth, CA). The DNA was recovered in 50 µL of the elution buffer. Three genomic regions were targeted: the $trnL_{UAA}$- $trnF_{GAA}$ region (chloroplast DNA), which includes the *trnL* intron, the 3'exon and the $trnL_{UAA}$-$trnF_{GAA}$ intergenic spacer, the spacer region between *atpB* and *rbcL* (cpDNA) and a portion of the nuclear ribosomal 26S region. The loci were amplified using the following primer pairs: trnC & trnF (Taberlet 1991), atpB & rbcL (Chiang et al. 1998), and LS0F & LS8R (Shaw 2000), respectively. Amplifications were performed in a 25 µL reaction volume containing: 2.5 µL HotMaster Taq Buffer (Eppendorf, Westbury, NY), 0.75 units HotMaster Taq DNA Polymerase (Eppendorf), 2.5 mM of each dNTP, 1.0 µL of each primer at 10 mM solution, and 1.0 µL of genomic DNA. Polymerase Chain Reactions (PCR) were carried out on MJ Research 200 and 220 Peltier Thermal Cyclers using the following parameters: an initial denaturation (95°C, 1 min), then 30 cycles of denaturation (95°C, 1 min), annealing (*trnL-trnF* region = 52°C, *atpB-rbcL* spacer = 50°C, 26S = 55°C, 1 min), extension (72°C, 1 min), and then a single final extension (72°C, 7 min). PCR products were cleaned using either QIAquick (Qiagen) purification columns or Nucleospin Purification Kit (BD Biosciences, Franklin Lake, NJ).

Sequencing reactions contained the following: 2 µL of purified PCR product, 2 µL of BigDye Terminator v1.1 (Applied Biosystems, Foster City, CA), and 1 µL of one of the primers used for amplification, then adjusted to a final volume of 10 µL with water. These sequencing reactions were carried out using the following conditions: 24 cycles of denaturation (96°C, 30 sec), annealing (50°C, 15 sec), extension (60°C, 4 min), preceded by an initial denaturation (96°C, 2 min). The products of cycle sequenced DNA were purified using Sephadex columns (Amersham Biosciences, Uppsala, Sweden). Sequences were determined using an ABI PRISM 3100 (Applied Biosystems) automated sequencer. Sequences were submitted to GenBank (Appendix 1).

Sequence Editing and Alignment. Sequences were edited and assembled using Sequencher v3.1.1 (Gene Codes Corporation, Ann Arbor, MI). Sequences were aligned using Se-Al v.2.0a7b (Rambaut and Charleston 2001). The sequences of the *trnL* intron were aligned against a secondary structure model proposed by Quandt and Stech (2004) to facilitate homology assessments. The amplified fragment of the 26S corresponds to the B13-1 to D4 region following the secondary structure model of Capesius and Van de Peer (1997), which was used to identify homologous stem regions in the sequence. Regions of ambiguous alignment were excluded. For ingroup and outgroup taxa 9–18 and 44–259 nucleotides were excluded per exemplar, respectively. These regions accounted for 0.76–1.66% of the total nucleotides for the ingroup taxa and 2.95–15.97% for the outgroup taxa. Aligned data matrices were submitted to TreeBASE (study number S1465).

Phylogenetic Analyses. Phylogenetic inferences were made under the optimality criterion of maximum parsimony (MP) in PAUP* version 4.0b10 for OSX (Swofford 2002) and using Bayesian approaches in MrBayes v3.0B4 (Huelsenbeck and Ronquist 2001). Individual loci were first analyzed separately to assess congruence. Loci were considered incongruent if alternative relationships were each supported by bootstrap proportions higher than 70% (Hillis and Bull 1993) or posterior probabilities exceeding 0.95 (Kauff and Lutzoni 2002). Parsimony analyses of each data set used the following conditions: 200 heuristic search replicates were carried out with sequences added at random, branch-swapping via nearest-neighbor interchange (NNI), steepest descent in effect, and no more than 10 trees of length greater than or equal to one were saved per replicate. Using the trees saved from the NNI, branch-swapping via tree bisection and reconnection (TBR) was carried out. For this branch-swapping, the maxtrees was set to 10,000. Support for the nodes was determined using 500 non-parametric bootstrap pseudoreplicates and TBR branch-swapping. All trees were rooted using *Diphyscium foliosum* (Hedw.) D. Mohr, an exemplar of Diphysciideae, whose phylogenetic origin likely precedes diversification of arthrodontous mosses (Cox et al. 2004). DNA substitution models and models for among site rate variation implemented in the Bayesian searches were determined using the Akaike Information Criterion (AIC; Posada and Buckley 2004) in MrModeltest v2.2 (Nylander 2004). The following models were selected for their respective Bayesian analyses: HKY+I (Hasegawa et al. 1985; *atpB-rbcL* spacer), HKY+G (*trnL-trnF* region), GTR+I+G (Rodriguez et al. 1990; 26S), and HKY+G (combined dataset). Bayesian inferences used default settings for the priors except for the shapepr = uniform (0.05, 50), for which the area was expanded to avoid an effect of the lower boundary on the posterior probabilities.

One million generations were run, with trees sampled every 50 generations. A total of four Metropolis-coupled Markov chains (MCMC) were run. Consensus trees of the type halfcompat were created from the saved trees. Based on examination of the p-files, a stationary likelihood value was reached after the first 15,000 generations, and thus, the first

TABLE 1. Variation in sequences for the genomic regions and subregions for all exemplars of both the ingroup and outgroup. The numbers of nucleotide characters are described for the ranges in length of the generated sequences (total length), the length of the aligned matrix (matrix), the total number of characters included in the analyses (analyzed), the number of constant characters (constant), the number of parsimony informative characters (PI), the percentage of PI in relation to the number of characters included in the analysis per region (%PI), and the percentage of PI per region in relation to the total number of PI (PI/total PI). Only members of the ingroup (Timmiaceae) were included to calculate the total length. Characters with ambiguous alignments were excluded.

Genomic regions	Total length	Matrix	Analyzed	Constant	PI	%PI	PI/total PI
trnL intron	249–361	412	323	233	38	11.8	18.3
trnL 3′exon	52	52	52	48	1	1.9	0.5
intergenic spacer	63–67	227	58	27	19	32.8	9.1
trnL- trnF region	364–419	691	433	308	58	13.4	27.9
atpB-rbcL spacer	532–569	828	642	427	96	14.6	46.2
26S	504–619	676	621	489	54	8.7	26.0
Combined	-	2195	1696	1224	208	12.3	100.0

300 recorded trees were excluded from each analysis. Based on these consensus trees the posterior probabilities (PP) of the respective clades were determined.

RESULTS

Sequences. We obtained sequences for the three loci for all specimens with the following exceptions: *atpB-rbcL* spacer for *Timmia austriaca* #2 and 26S for *T. megapolitana* subsp. *bavarica* #7 (Appendix 1). A comparison of sequence attributes is presented in Table 1. The non-coding *trnL-trnF* intergenic spacer yielded the highest percentage (32.8%) of parsimony informative (PI) characters. However, the other non-coding region (*atpB-rbcL* spacer) represented the largest total percentage of informative characters (46.2%). The remainder of the informative characters was evenly distributed between the *trnL-trnF* region (27.9%) and 26S (26.0%).

Parsimony Analyses. Tree statistics for the most parsimonious reconstructions obtained from inferences based on individual partitions and the combined data set are presented in Table 2. The number of most parsimonious trees (MPT) ranged widely among analyses from three for the *atpB-rbcL* spacer to 4174 for the combined analysis.

Separate analyses of the three genes did not reveal topological conflict among the nodes that had greater than 70% bootstrap support (Fig. 1), thus, the partitions were considered congruent and were combined in subsequent analyses. Inferences from the combined data set supported *Timmia* as monophyletic. Furthermore, *T. austriaca* and *T. megapolitana* were monophyletic sister taxa (Fig. 1). Within *T. megapolitana*, both subsp. *bavarica* and subsp. *megapolitana* were monophyletic (Fig. 1). In contrast, *T. norvegica* was paraphyletic due to the nested position of *T. sibirica* (Fig. 1). The two samples of *T. norvegica* var. *excurrens* were not closely related, and *T. sibirica* was also non-monophyletic (Fig. 1).

Bayesian Analyses. Inferences from individual partitions did not lead to conflict using the 0.95 PP as a level of significance, hence all data were merged into a combined dataset (Fig. 1). Four replicates of the combined analysis were generated. Excluding the burnin, all trees sampled from the four analyses were combined to produce a consensus tree (Fig. 2). The genus *Timmia* as a whole, *T. austriaca*, *T. megapolitana* and subsp. *bavarica* were monophyletic (Fig. 2). As with parsimony analyses, *T. norvegica* var. *norvegica*, var. *excurrens*, and *T. sibirica* were non-monophyletic. Comparisons between maximum parsimony and Bayesian consensus trees did not result in topological incongruence for clades with support values greater than 70% BS and greater than 0.95 PP (Figs. 1, 2).

TABLE 2. Summary of tree statistics for the parsimony analyses of the three genomic regions in independent and combined analyses. The data include the length of the most parsimonious tree (tree length), the number of most parsimonious trees found in the analyses (MPT), consistency index (CI), consistency index excluding uninformative characters (CI ex.), retention index (RI), and rescaled consistency index (RC).

Analysis	Tree length	MPT	CI	CI ex.	RI	RC
trnL- trnF region	183	54	0.792	0.670	0.871	0.690
atpB- rbcL spacer	313	3	0.840	0.727	0.900	0.756
26S	235	1022	0.745	0.575	0.814	0.606
Combined	710	4174	0.799	0.660	0.864	0.690

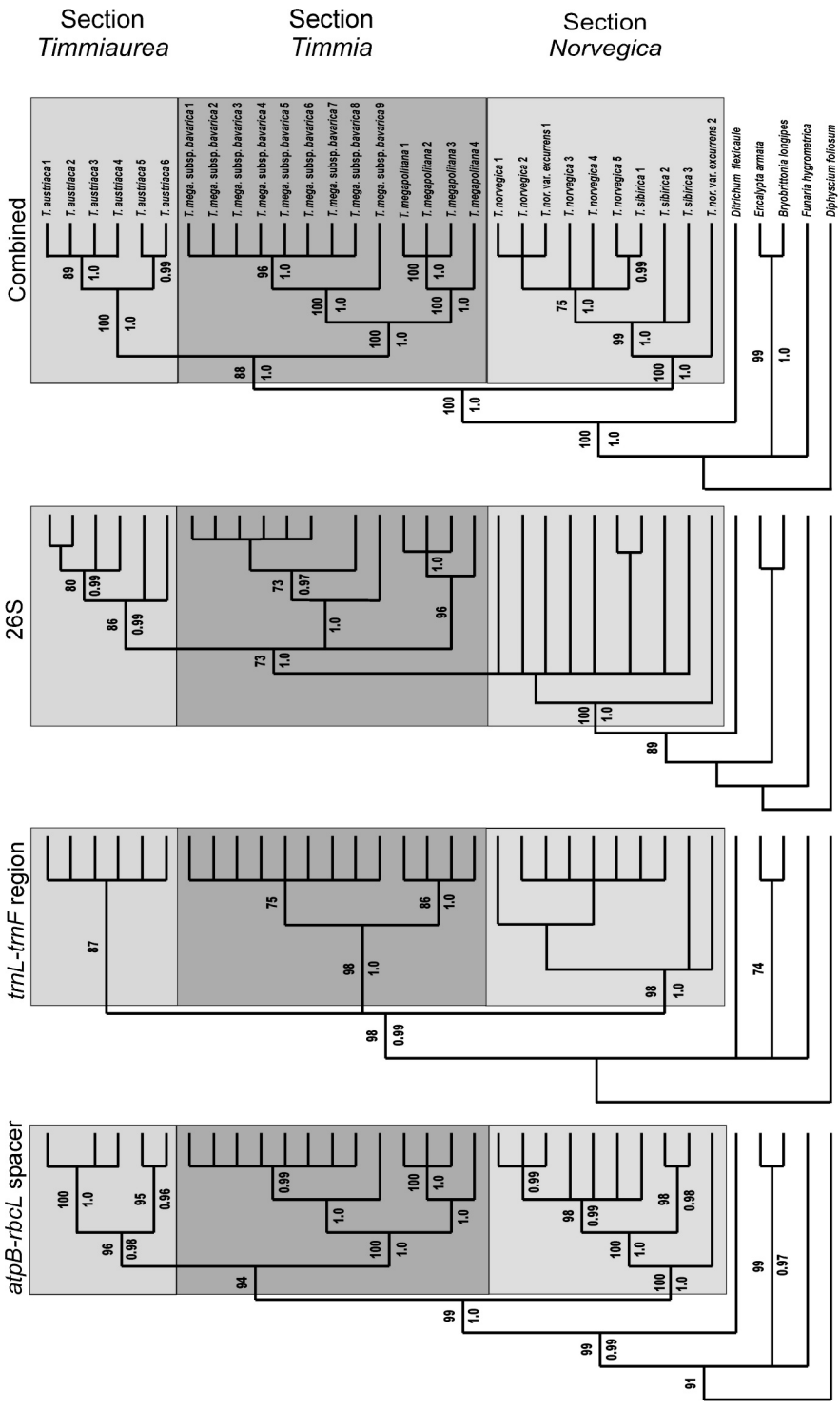

FIG. 1. 50% majority rule consensus trees from TBR parsimony analyses for individual genomic regions and the combined dataset. Values above branches = bootstrap support greater than 70%; values below branches = posterior probability support greater than 0.95 from Bayesian analyses. Shaded regions indicate the three sections defined in Timmiaceae.

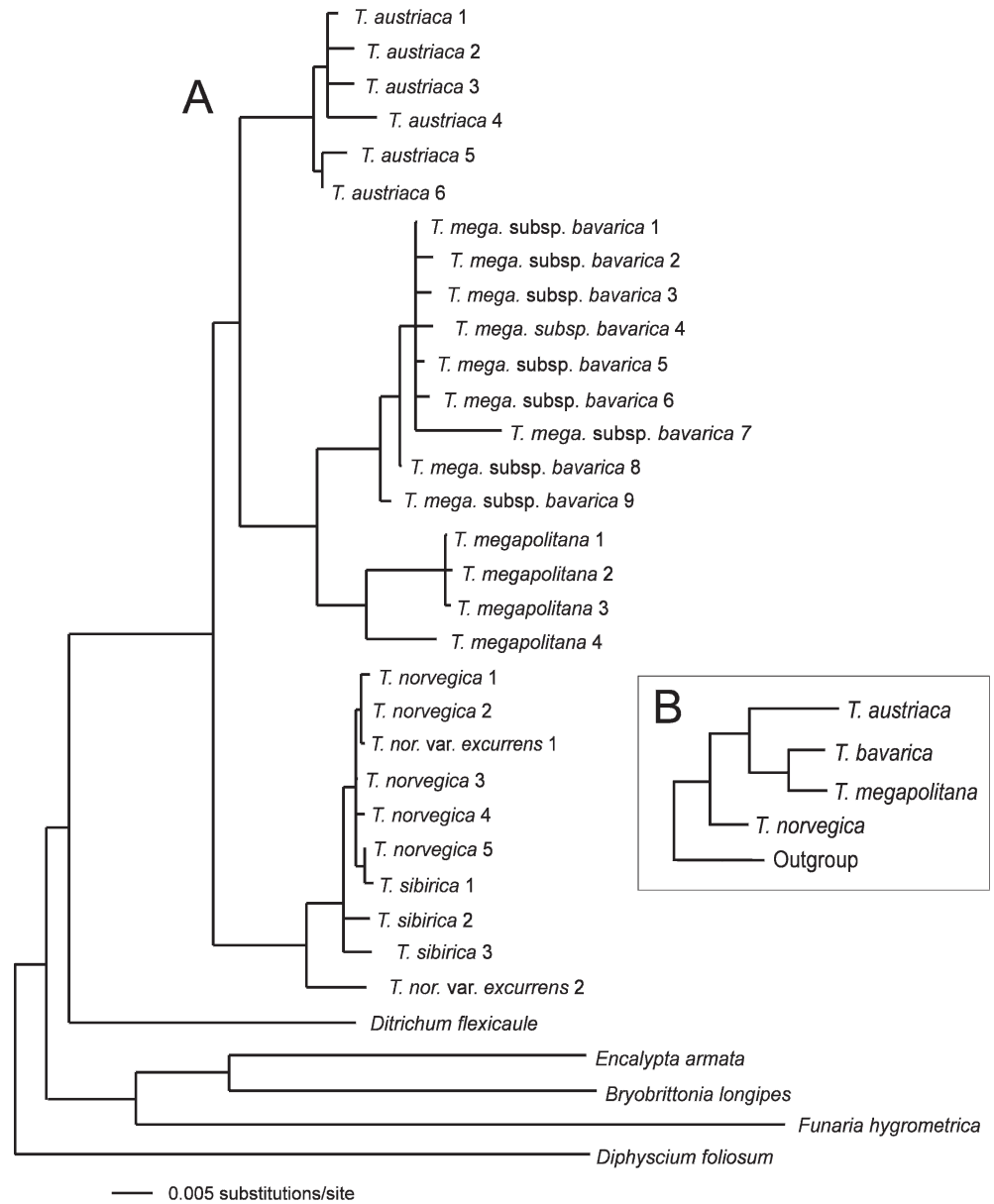

FIG. 2. A. 50% majority rule phylogram consensus tree from four Bayesian analyses of 1,000,000 generations each for the combined dataset, excluding burnin trees. B. Summary phylogeny of relationships among the four monophyletic groups in Timmiaceae.

Insertions and Deletions. Sequences of the *trnL* intron and the *atpB-rbcL* spacer varied in length among exemplars. Insertions and deletions were not scored as binary characters, but an a posteriori examination of their distribution suggests that two deletions and one insertion could serve as phylogenetic markers. A six-nucleotide deletion (TTTTCT) within the *trnL* intron characterized samples of sect. *Norvegica*, except *T. norvegica* var. *excurrens* #2. In the *atpB-rbcL* spacer, a deletion of six nucleotides (ATATAT) occurred in all exemplars of subsp. *bavarica*. Additionally in the *atpB-rbcL* spacer, a nine-nucleotide insertion (GTATATATA) defined all members of *T. megapolitana* s.l.

DISCUSSION

Phylogenetic inferences from variation in sequences of two chloroplast and one nuclear loci resolve lineages that are congruent with most taxonomic concepts within Timmiaceae (Brassard 1979, 1980, 1984). Except for *T. sibirica* and *T. norvegica* var. *excurrens*, all taxa are well supported

as monophyletic. *Timmia* is composed of four main lineages: the initial divergence is between sect. *Norvegica* (i.e., *T. norvegica*, *T. sibirica*) and the clade containing the sister groups *T. austriaca* and *T. megapolitana*; within *T. megapolitana* the varieties *bavarica* and *megapolitana* are both monophyletic.

Brassard (1980) hypothesized that *T. austriaca* is more closely related to sect. *Norvegica* than to *T. megapolitana* s.l. based on the dioicous sexual condition shared by *T. austriaca* and sect. *Norvegica*. Within our phylogenetic hypothesis the distribution of sex organs is informative only with regard to the monophyly of *T. megapolitana* s.l. A transition from dioicy to monoicy has been reported in other lineages of mosses (e.g., Amblystegiaceae, Vanderpoorten et al. 2002; *Macromitrium* Brid., Ramsay and Vitt 1986), further supporting the derived monoicous condition in *T. megapolitana* s.l. Other characters shared by *T. austriaca*, *T. norvegica*, and *T. sibirica* are the sinuose exothecial cells of the sporangium and the papillose dorsal surfaces of the leaf sheaths (Brassard 1979), which also may be convergent or ancestral. These characteristics are too homoplasious to support a close relationship between *T. austriaca* and the members of sect. *Norvegica*.

The inner endostomial surface of the capsule (sporophyte) provided one of the most important taxonomic characters in *Timmia* (Brassard 1979, 1980, 1984). However, most populations of dioicous species are only known in the gametophytic stage: sporophytes of *T. norvegica* have been documented from only three populations of var. *norvegica*, and for *T. sibirica*, only capsules with immature spores are known (Brassard 1979). Consequently, morphological characteristics of the gametophyte must be used for species identification.

Brassard (1979) accommodated *T. norvegica*, including var. *excurrens*, and *T. sibirica* in sect. *Norvegica*. The monophyly of this section is supported by our phylogenetic inferences. However, recognition of two species and distinction of two varieties for *T. norvegica*, based on the morphological characters used by Brassard (1979), is not supported: both *T. sibirica* and *T. norvegica* var. *excurrens* are non-monophyletic. Thus, gametophytic characters traditionally used to diagnose taxa within sect. *Norvegica* do not define monophyletic entities.

Timmia sibirica is distinguished from *T. norvegica* and other species by the multiple papillae per cell on ventral and dorsal sides of the costa and leaf lamina (Brassard 1979; Horton 1981). Evaluating Siberian populations, Arnell (1913) noted morphological variability and concluded that *T. sibirica* represented a form of *T. norvegica*. Subsequently, Horton (1981) investigated the taxonomic status of *T. sibirica* and supported its recognition as a distinct species. The three accessions of *T. sibirica* included in this study are characterized by multiple papillose cells and are, thus, representative of the species (Brassard 1979). Our analyses resolve *T. sibirica* as non-monophyletic and nested within *T. norvegica* (Figs. 1, 2), suggesting that the presence of multipapillose cells is homoplastic. Therefore, our results support Arnell's (1913) assessment of *T. sibirica* as a form of *T. norvegica*.

Varieties of *T. norvegica* are distinguished by dorsal papillosity of the costa, the costa apex, and width of the lamina cells. In var. *norvegica*, costae are papillose from the leaf shoulders upward and percurrent, whereas in var. *excurrens* costae are obscurely papillose only at the apices but otherwise smooth and excurrent (Crum 1967; Brassard 1979; Crum and Anderson 1981). Furthermore, laminal cells are narrower in var. *excurrens* than var. *norvegica* (6–9(10) μm vs. (8)9–14 μm; Brassard 1979). *Timmia norvegica* var. *excurrens* has also been recognized by a number of authors (Nyholm 1960; Abramova et al. 1961; Frey et al. 1995; Ignatov and Ignatova 2003) as a distinct species, *T. comata*, based on these morphological characters. However, Nyholm (1960) noted that juvenile plants of this taxon could be difficult to distinguish from *T. norvegica*. Although these differences appear unambiguous, and hence could be used to diagnose the varieties (Horton and Bartlett 1983), populations with intermediate morphologies also exist. For example, the only New Zealand populations of *T. norvegica* are characterized by individuals with laminal cell widths ranging from 7 to 9(11) μm, and costae that were percurrent and papillose only at the apices representing an intermediate morphology between the two varieties (Horton and Bartlett 1983). The two exemplars of var. *excurrens* sampled for this study fail to form a monophyletic taxon. Therefore, we consider the restricted distribution of papillae on the costa as well as the smaller cell size of var. *excurrens* to represent extremes of the variation exhibited by *T. norvegica*. The broader morphological concept for *T. norvegica* is well differentiated from other species of *Timmia*: the endostome is bluntly appendiculate on the inner surface of the cilia, plants are regularly heterophyllous, basal cells are hyaline, and leaves are deciduous due to fragile leaf bases. Consequently, we propose treating *T. norvegica* as a single, although variable, taxon.

Timmia austriaca (sect. *Timmiaurea*) is a monophyletic and well supported taxon (Fig. 1), distinguished by cilia that are finely granulose on the

inner surface and leaf sheaths with abrupt change in color as well as a sharp angle at the transition between leaf lamina and sheath, with the lamina erect-spreading when wet (Brassard 1980; Crum and Anderson 1981). This species is also diagnosed by the presence of stereids in the costa only at the transition region from sheathing base to lamina; stereids are, thus, lacking in cross section at the middle of the leaf sheath (Mastracci 1993). Within the genus these character-states are unique to *T. austriaca*.

Timmia megapolitana (sect. *Timmia*) is primarily recognized on the basis of its monoicous condition and delicately appendiculate inner surface of the cilia (Crum and Anderson 1981; Brassard 1984). Since this species also lacks stereids in the base of the leaf sheath, the character used to distinguish this species from *T. austriaca* is presence of stereids in cross section at the middle of the leaf sheath (Mastracci 1993). *Timmia megapolitana* subsp. *bavarica* and subsp. *megapolitana* differ in ecological distributions, widths of mid-laminal cells, and position of the calyptra at maturity (Brassard 1984). Subspecies *megapolitana* is the only taxon in the genus with a temperate distribution. It is also the only taxon occurring in man-made habitats, including lawns, golf courses, and cemeteries (Brassard 1984). All other taxa in the genus including subsp. *bavarica* have an arctic-montane distribution. Mid-laminal cells are, on average, wider in subsp. *megapolitana* (10.5 μm) than in subsp. *bavarica* (8.5 μm), but the ranges of cell widths for these two taxa overlap (subsp. *megapolitana* (8)9–12(14) μm and subsp. *bavarica* (6)7–10(12) μm; Brassard 1984). Additionally, subsp. *megapolitana* is the only taxon in Timmiaceae in which the calyptra remains attached (via the base) to the seta at maturity (Crum and Anderson 1981). Brassard (1984) distinguished these taxa in *T. megapolitana* as subspecies. Our data unambiguously resolve both of these subspecies as monophyletic entities, corroborating the morphological distinction between *T. megapolitana* and *T. bavarica*.

Based on the proposed phylogeny inferred from variation in the primary nucleotide sequences of one nuclear and two chloroplast loci, we recognize four groups that satisfy the diagnosable (Nixon and Wheeler 1990) and apomorphic (Mishler 1985) phylogenetic species concepts: *T. austriaca*, *T. norvegica*, *T. megapolitana*, and *T. bavarica*.

Key to the Species of Timmiaceae

Key to the monophyletic taxa that were supported in our phylogenetic analyses. Adapted from Brassard (1979, 1980, 1984) and Mastracci (1993).

1. Endostome cilia bluntly appendiculate on the inner surface; plants heterophyllous; basal cells of leaves hyaline . . . . . *Timmia norvegica* [*T. norvegica* var. *excurrens* and *T. sibirica*]
1. Endostome cilia finely granulose or delicately appendiculate on the inner surface; plants isophyllous; basal cells of leaves opaque 2
   2. Plants dioicous; endostomial cilia finely granulose on the inner surface; costae wider at the limb-sheath transition; stereids lacking in cross section at the middle of the leaf sheath . . . . . *Timmia austriaca*
   2. Plants auto(or mono)icous; endostomial cilia delicately appendiculate on the inner surface; costae are not wider at the limb-sheath transition; stereids present in cross section at the middle of the leaf sheath 3
      3. Width of the mid-laminal cells (8)9–12(14) μm; calyptra attached to the seta at maturity . . . . . *Timmia megapolitana*
      3. Width of the mid-laminal cells (6)7–10(12) μm; calyptra absent from the seta at maturity . . . . . *Timmia bavarica*

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APPENDIX 1. Ingroup taxa listed with taxon names according to Brassard (1979, 1980, 1984), followed by outgroup taxa. Numbers before voucher citations indicate multiple samples analyzed per species and correspond to numbers in Figs. 1 and 2. Voucher information is listed as follows: taxon name, collection locality, collector name and number (herbarium), GenBank accession numbers for the three loci: *trnL-trnF*, *atpB-rbcL* spacer, and 26S; sequences not obtained indicated by n.a.

Timmia austriaca Hedw. - **1** British Columbia, Canada. *Goffinet, Vitt & Hastings 1284* (CONN); DQ397165, DQ397132, DQ397099. **2** Nízké Tatry, Slovakia, *Pilous 6.VII.1980* (LG); DQ397166, n.a., DQ397100. **3** Michigan, USA, *Hermann 28700* (NY); DQ397167, DQ397133, DQ397101. **4** Haerjedalen, Sweden,

Frahm 11.VIII.1981 (NY); DQ397169, DQ397135, DQ397103. **5**. Siberia, Russia, *Hephrdebba 51* (NY); DQ397168, DQ397134, DQ397102. **6**. Svalbardia, Norway, *Bednarek-Ochyra, Godzik, & Grodzińska Bryophyta Svalbardensia Exsiccata 75-1987* (NY); DQ397189, DQ397155, DQ397122.

Timmia megapolitana subsp. *bavarica* (Hessl.) Brassard – **1** British Columbia, Canada, *Schofield & McIntosh 74733* (NFLD); DQ397170, DQ397136, DQ397104. **2** Lérida, Spain, *Brugués 5.V.1980* (NFLD); DQ397175, DQ397141, DQ397109. **3** Haerjedalen, Sweden, *Frahm 6.VIII.1981* (NY); DQ397177, DQ397143, DQ397111. **4** Karachaevo-Cherkesskaya Republic, Russia, *Onipehenko 105/95* (NY); DQ397178, DQ397144, DQ397112. **5**. Alma Atla Oblast, Kazakhstan, *Whittemore 3868* (NY); DQ397179, DQ397145, DQ397113. **6** Jbel Bouhalla, Morroco, *Cano & Ros 16.VI.1997* (NY); DQ397181, DQ397147, DQ397115. **7** Quebec, Canada, *Belland 5100* (NFLD); DQ397182, DQ397148, n.a. **8** Alaska, USA, *Brassard 13809* (NFLD); DQ397176, DQ397142, DQ397110. **9** Quinghai Providence, China, *Tan 95-1735* (NY); DQ397180, DQ397146, DQ397114.

Timmia megapolitana subsp. *megapolitana* – **1** Ontario, Canada, *Ireland 20088* (NFLD); DQ397171, DQ397137, DQ397105. **2** Archangelsk Providence, Russia, *Ignatov 3.VIII.1988* (NY); DQ397173, DQ397139, DQ397107. **3** New York, USA, *Budke 101* (CONN); DQ397174, DQ397140, DQ397108. **4**. Honshu, Japan, *Tanaka 2210* (NY); DQ397172, DQ397138, DQ397106.

Timmia norvegica var. *excurrens* Bryhn – **1** Altai Mountains, Russia, *Ignatov 22.VII.1991* (NY); DQ397188, DQ397156, DQ397123. **2**. Jämtland, Sweden, *Hakelier 18.VIII.1984* (NY); DQ397190, DQ397154, DQ397121.

Timmia norvegica var. *norvegica* – **1** Grisons, Switzerland, *Vanderpoorten 4022* (LG); DQ397184, DQ397150, DQ397117. **2** Argell, Great Britain, *Vanderpoorten 3090* (LG); DQ397183, DQ397149, DQ397116. **3**. Quebec, Canada, *Ireland 21263* (NY); DQ397185, DQ397151, DQ397118. **4** Alaska, USA, *Lewis 164* (NY); DQ397186, DQ397152, DQ397119. **5** Yakutia, Russia, *Afonina Bryophyta Rossica et Civitatum Collimitanearum Exsiccata 78-1995* (NY); DQ397187, DQ397153, DQ397120.

Timmia sibirica Lindb. & Arnell – **1** Ellesmere Island, Nanavut, Canada, *Hedderson 6819* (RND); DQ397193, DQ397159, DQ397126. **2** Northwest Territory, Canada, *Brassard 4410* (NY); DQ397191, DQ397157, DQ397124. **3** Bolshevik Island, Archipelag Severnaya Zemlya, Russia, *Safronova 24.VII.1992* (NY); DQ397192, DQ397158, DQ397125.

Bryobrittonia longipes (Mitt.) D.G. Horton – Altai Mountains, Russia, *Ignatov 197* (NY); DQ397197, DQ397163, DQ397130. *Diphyscium foliosum* (Hedw.) D. Mohr – North Carolina, USA, *Goffinet 4595* (CONN); DQ397195, DQ397161, DQ397128. *Ditrichum flexicaule* (Schwägr.) Hampe – Ellis Basin, New Zealand, *Bartlett 15091* (NY); DQ397194, DQ397160, DQ397127. *Encalypta armata* Broth ex. Dusén – Santiago, Chile, *Goffinet 5613* (CONN); DQ397196, DQ397162, DQ397129. *Funaria hygrometrica* Hedw. – Bio-Bio, Chile, *Goffinet 5576* (CONN); DQ397198, DQ397164, DQ397131.