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Phylogenetic relationships among diploid species of *Symphyotrichum* (Asteraceae: Astereae) based on two nuclear markers, ITS and GAPDH

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ABSTRACT

The mostly North American subtribe Symphyotrichinae (Asteraceae: Astereae) comprises *Canadanthus*, *Ampelaster*, *Psilactis*, *Almutaster*, and *Symphyotrichum*. Intergeneric and interspecific relationships within the subtribe have been investigated in the past, particularly by Nesom [Nesom, G.L., 1994. Review of the taxonomy of *Aster* sensu lato (Asteraceae: Astereae), emphasizing the new world species, Phytologia 77, 141–297] and Semple [Semple, J.C., 2005. Classification of *Symphyotrichum*. Available from: <http://www.jcsemple.uwaterloo.ca/Symphyotrichumclassification.htm/>], using morphological and cytological approaches. *Symphyotrichum* is the largest and most complex genus within the subtribe and includes four subgenera: *Symphyotrichum* ($x = 7, 8$), *Virgulus* ($x = 4, 5$), *Astropolium* ($x = 5$), and *Chapmaniani* ($x = 7$). In this study we used two nuclear markers, the nrDNA internal transcribed spacer (ITS) and the low-copy nuclear gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), to resolve intergeneric and interspecific relationships within the subtribe at the diploid level, and to determine whether our phylogenies validate the classifications of Nesom or Semple. Our results confirm the distinct generic status of *Canadanthus* and *Ampelaster*, whereas *Psilactis* and *Almutaster* form a polytomy with *Symphyotrichum*. Within *Symphyotrichum*, subg. *Virgulus* is monophyletic based on ITS and appears polyphyletic based on GAPDH. Neither the ITS nor the GAPDH analyses support a distinct status for subg. *Astropolium*, which groups within subg. *Symphyotrichum*. In general, interspecific relationships within *Symphyotrichum* are unresolved. Lack of resolution may be interpreted as a case of recent and rapid evolutionary radiation.

1. Introduction

With 215 genera including more than 3000 species worldwide, the Astereae is the second largest tribe of the Asteraceae (Funk et al., 2005). Nesom and Robinson (2007) divided the tribe into 18 subtribes based in part on the nrDNA (ITS) phylogenetic study of Noyes and Rieseberg (1999).

One of these subtribes, the Symphyotrichinae, is defined as monophyletic (Xiang and Semple, 1996; Brouillet et al., 2001), and includes five genera: *Symphyotrichum* Nees ($x = 4, 5, 7, 8$), *Canadanthus* G.L. Nesom ($x = 9$), *Ampelaster* G.L. Nesom ($x = 9$), *Almutaster* Á. Löve and D. Löve ($x = 9$), and *Psilactis* A. Gray ($x = 3, 4, 9$). All genera are North American. Phylogenetic analyses of nrDNA (ITS) sequence data and of cpDNA restriction sites have shown that the last four genera form a grade to *Symphyotrichum* (Morgan, 1993, 1997, 2003; Lane et al., 1996; Xiang and Semple, 1996; Semple et al., 2001). Genus *Symphyotrichum* is mostly distributed in North America and extends into South America. More than half of the 91 species belonging to this genus are polyploid (Semple, 1985; Semple and Chmielewski, 1987; Semple et al., 1989, 1992, 1993, 2001; Semple and Cook, 2004).

The taxonomic history of *Symphyotrichum* has been complex. Two major classifications have been proposed recently for the genus by Nesom (1994a) and Semple (2005) (Table 1). Nesom subdivided the genus into two subgenera, *Symphyotrichum* and *Virgulus*, and 12 sections, based on morphological and cytological evidence. Semple subdivided the genus into five subgenera, *Symphyotrichum* (with three sections), *Virgulus* (with five sections), *Ascendentes*, *Astropolium*, and *Chapmaniani*, based on morphological, cytological, and, to some extent, nrDNA ITS phylogenetic data.

The study of interspecific relationships within *Symphyotrichum* has been limited to morphometric and cytological approaches (Allen et al., 1983; Jones and Young, 1983; Labrecque and Brouillet, 1996; Owen et al., 2006). High levels of morphological plasticity and extensive interspecific hybridization have resulted in difficulties in delimiting species within the genus in both diploids and polyploids (Jones and Young, 1983; Brouillet and Labrecque, 1987; Labrecque and Brouillet, 1996; Allen and Eccleston, 1998; Semple et al., 2002; Owen et al., 2006). Previous ITS-based studies of representatives of Astereae and *Symphyotrichum* resulted in unresolved phylogenies (Noyes and Rieseberg, 1999; Brouillet et al., 2001). This lack of resolution, in combination with a high

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Table 1
Comparison of the two classifications proposed by Nesom (1994a) and Semple (2005) for genus *Symphyotrichum*. Double lines separate genera, continuous lines subgenera, and broken lines sections.

Nesom 1994a		Semple 2005	
Subgenus	Section	Section	Subgenus
Symphyotrichum (x = 5, 7, 8, 13, 18)	*Symphyotrichum*	*Symphyotrichum*	*Symphyotrichum* (x = 7, 8)
	Cordifolii		
	Dumosi		
	Occidentales		
	Concinni (in part)		
	Concinni (in part)	*Turbinelli*	
	Conyzopsis	*Conyzopsis*	
	Oxytripolium		*Astropolium* (x = 5)
	Ascendentes		*Ascendentes* (x = 13, 18)
Virgulus (x = 5, 4)	*Grandiflori*	*Grandiflori*	*Virgulus*
		Polyliguli	
		Patentes (in part)	
	Patentes	*Patentes* (in part)	
	Concolores	*Concolores*	
	Ericoidei	*Ericoidei*	
Eurybia subg. *Heleastrum*	*Chapmaniani* (x = 7)		*Chapmaniani*

number of hybrid species (e.g., 27 polyploid species may be allopolyploid) within the genus, agrees with models of recent adaptive radiation (Kim et al., 1996; Baldwin, 1997; Seehausen, 2004; Al-Shehbaz et al., 2006; Wiens et al., 2006).

Recent progress in the development of molecular markers has facilitated inferring the evolutionary history of a set of taxa and representing it in a phylogenetic tree (Takahata, 1996; Cann, 2001; Beilstein et al., 2006). Despite the fact that molecular markers may provide powerful tools for delimitating species boundaries (e.g., Brouat et al., 2004; Joly et al., 2006), the different modes of inheritance of these markers may cause phylogenetic incongruence between cytoplasmic and nuclear DNA in both plants and animals (Soltis and Kuzoff, 1995; Sota and Vogler, 2001; Okuyama et al., 2005).

The ITS region is among the most widely used molecular markers for inferring phylogenetic history at different taxonomic levels (Baldwin et al., 1995; Soltis and Soltis, 1998; Volkov et al., 2007). Despite its biparental mode of inheritance, easy amplification, and availability of universal primers, which explains the popularity of the ITS region as a phylogenetic marker, concerted evolution, which may cause homogenization of sequences within individuals or species, and insufficient resolution at low taxonomic levels, in particular, have sometimes limited the use of this marker in species delimitation and hybrid detection (Brochmann et al., 1996; Grundt et al., 2004; Volkov et al., 2007). Although ETS proved to be slightly more variable than ITS and slightly increased resolution and support in a phylogenetic analysis of the related eurybioid asters (e.g., Selliah and Brouillet, 2008), ETS and ITS are both part of the nuclear ribosomal DNA cistron that occurs in tandem repeats and is subject to concerted evolution and, potentially, to some of the same evolutionary constraints. For this analysis, we wanted a region that had been subject to independent evolutionary pressures. Since chloroplast DNA had low variability in the Symphyotrichinae and related genera of the Astereae (L. Brouillet, pers. obs.), low-copy nuclear markers appeared preferable.

Therefore, single- or low-copy nuclear markers with relatively rapid evolutionary rates appear necessary to infer the phylogenetic history of species groups such as *Symphyotrichum* that were probably subject to recent radiation (Maddison, 1997; Funk and Omland, 2003; Buckley et al., 2006). In a group of recently evolved taxa, such molecular markers present other difficulties in phylogeny reconstruction, however, such as the retention of ancestral polymorphisms (Doyle et al., 1999; Lihová et al., 2006), which may lead to a lack of concordance between gene trees derived from different nuclear markers (Page and Charleston, 1997; McCracken and Sorenson, 2005; Buckley et al., 2006; Lihová et al., 2006; Fehrer et al., 2007).

In the current study, our objectives are to investigate the phylogenetic relationships among diploid species of *Symphyotrichum*, and to determine whether our phylogenetic inferences validate the classifications of Nesom (1994a) and Semple (2005). Diploids were used exclusively because preliminary analyses showed the ITS to be unreliable to untangle the relationships of polyploids in the genus due to recombination of parental types and other complications, and because most polyploids are the result of reticulate evolution, which is not adequately analyzed using tree reconstruction algorithms. To address these goals we used the ITS region and the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) nuclear marker. The latter is a low-copy nuclear gene (Strand et al., 1997) that has been widely used in molecular studies (e.g., Olsen and Schaal, 1999; Camara et al., 2002; McCracken and Sorenson, 2005; Buckley et al., 2006; Joly et al., 2006; Buhay et al., 2007; Fauvelot et al., 2007). It is a tetramer (molecular weight 143,000) that catalyzes the NAD-mediated oxidative phosphorylation of glyceraldehyde 3-phosphate to 1,3-diphosphoglyceric acid (Buehner et al., 1973).

2. Materials and methods

2.1. Taxon sampling

One individual per diploid species for a total of 32 species of *Symphyotrichum* were included in the phylogenetic analyses (Table 2), including 17 of the 21 strictly diploid species (4 excluded due to technical difficulties), and 15 diploid specimens from species with polyploid series (of 25 species; total number of species in the genus ca. 90). These are representative of all subgenera and most sections of the genus according to the classification of Semple (2005). Among these, we included 19 species of sect. *Symphyotrichum*, the three of sect. *Conyzopsis*, seven of subg. *Virgulus*, two of subg. *Astropolium*, and *S. chapmanii* of subg. *Chapmaniani*. We did not include representatives of sections *Concinni* (sensu Nesom), *Turbinelli* (sensu Semple), and subgenus (sensu Semple) or section (sensu Nesom) *Ascendentes*. Sections *Concinni* and *Turbinelli* comprise only polyploid species, and *Ascendentes* includes two allopolyploid species of hybrid derivation (Allen and Eccleston, 1998). To investigate phylogenetic relationships among the genera of Symphyotrichinae (*Ampelaster*, *Almutaster*, *Psilactis*, *Canadanthus*, and *Symphyotrichum*), we included one species each of the first four genera in the phylogenetic analyses (note that *Almutaster, Ampelaster,* and *Canadanthus* are monospecific). We rooted our phylogenetic trees with a representative of a closely related genus, *Oreostemma*, and one of the distantly related *Heterotheca* (Xiang and Semple, 1996; Noyes and Rieseberg, 1999; Brouillet et al., 2001).

Table 2
Voucher specimens included in the study; herbarium acronyms follow the Index Herbariorum (Holmgren and Holmgren, 1998). The base chromosome number (*x*) and GenBank Accession numbers for each species are provided in the last three columns. Classification of the taxa is based on Semple (2005).

Taxon	Locality	Collector(s)	*x*	GenBank Accession #	
				ITS[a]	GAPDH
Outgroups					
Heterotheca monarchensis D.A. York, Shevock & Semple	Kern Co./Calif.	Shwock & York, 109 (WAT)	9		EU708562–EU708563
Oreostemma alpigenum (T. & G.) Greene	Mt. Hood/Oreg.	Semple, 10271 (WAT)	9	EU200219[c]	EU708564–EU708565
Canadanthus modestus (Lind.) G.L. Nesom	Swift Current/Sask.	Hudson, 3997 (SASK)	9	EU781457	EU708567
Ampelaster carolinianus (Walter) G.L. Nesom	Davenport/Fla.	Semple, 5354 (WAT)	9	EU200185[c]	EU708566
Almutaster pauciflorus (Nuttall) Á. Löve & D. Löve	Oakburn/Man.	Marchand, 1983 (SASK)	9	EU781462	EU708569
Psilactis tenuis S. Watson	Jeff Davis Co./Tex.	Semple, 8201 (WAT)	9		EU708568
Subg. *Symphyotrichum*					
Sect. *Symphyotrichum*					
Subsect. *Symphyotrichum*					
S. elliottii (T. & G.) G.L. Nesom	Onslow Co./N.C.	Semple, 10538 (WAT)	8	EU853710[b]	EU708514
S. puniceum (L.) Á. Löve & D. Löve	Marion Co./N.C.	Semple, 10853 (WAT)	8	EU781142–EU781143	EU708512–EU708513
S. firmum (Nees) G.L. Nesom	Lake Co./Mont.	Gerdes, 4945 (NM)	8	EU781250	EU708540–EU708541
Subsect. *Heterophylli*					
S. anomalum (Engel. ex T. & G.) G.L. Nesom	Carroll Co./Ark.	Semple & Suripto, 9950 (WAT)	8	EU781321–EU781326	EU708547
S. cordifolium (L.) G.L. Nesom	Carleton Co./N.B.	Semple & Keir, 4670 (MT)	8	EU781411–EU781412	EU708552–EU708553
S. drummondii (Lind.) G.L. Nesom	Newton Co./Tex.	Semple, 10049 (WAT)	8	EU781140–EU781141	EU708554
S. shortii (Lind.) G.L. Nesom	Adair Co./Ky.	Semple & Suripto, 9449 (MT)	8	EU781413–EU781414	EU708555–EU708556
S. undulatum (L.) G.L. Nesom	Orangeburg Co./S.C.	Semple & Chmielewski, 6133 (MT)	8	EU781415–EU781416	EU708557–EU708558
S. urophyllum (Lind. ex de Cand.) G.L. Nesom	Elgin Co./Ont.	Semple, 10594 (WAT)	8	EU781138–EU781139	EU708510–EU708511
Subsect. *Occidentales*					
S. foliaceum (Lind. ex de Cand.) G.L. Nesom	Missoula Co./Mont.	Semple, 10310 (WAT)	8	EU781463–EU781464	EU708545–EU708546
Subsect. *Dumosi*					
S. dumosum (L.) G.L. Nesom	Amite Co./Miss.	Semple & Suripto, 10102 (MT)	8	EU781402–EU781406	EU708560–EU708561
S. lateriflorum (L.) Á. Löve & D. Löve	Prince Edward/Ont.	Brouillet & Brammall, 587 (MT)	8	EU781418	EU708537–EU708538
S. nahanniense (Cody) Semple	Nahanni N.P.R./N.W.T.	Semple, 11161 (WAT)	8	EU781252–EU781253	EU708543–EU708544
S. racemosum (Elliott) G.L. Nesom	Wayne Co./Miss.	Semple, 9895 (WAT)	8	EU853715[b]	EU708533–EU708534
S. tradescantii (L.) G.L. Nesom	Lévis/Que.	Bouchard & Cuerrier, K-11 (MT)	8	EU853717[b]	EU708548–EU708549
S. welshii (Cronquist) G.L. Nesom	Lake Co./Mont.	Semple, 11374 (WAT)	8	EU781407–EU781408	EU708542
Subsect. *Porteriani*					
S. depauperatum (Fern.) G.L. Nesom	Nottingham/Pa.	Semple, 7681 (WAT)	8	EU200226[c]	EU708531–EU708532
S. parviceps (E.S. Burgess) G.L. Nesom	Adams Co./Ill.	Semple & Brouillet, 7378 (MT)	8	EU781417	EU708559
S. porteri (A. Gray) G.L. Nesom	Clear Creek Co./Colo.	Semple, 10470 (WAT)	8	EU853714[b]	EU708529–EU708530
Sect. *Conyzopsis*					
S. frondosum (Nuttall) G.L. Nesom	Lake Co./Oreg.	Houle & Legault, 45 (MT)	7	EU853711[b]	EU708525–EU708526
S. ciliatum (Ledeb.) G.L. Nesom	Manitoulin/Ont.	Morton & Venn, 9942 (MT)	7	EU781410	EU708528
S. laurentianum (Fern.) G.L. Nesom	Ile de la Madeleine/Que.	Houle & Brouillet, 81 (MT)	7	EU853712[b]	EU708527
Subg. *Virgulus*					
Sect. *Concolores*					
S. plumosum (Small) Semple	Franklin Co./Fla.	Semple, 10929 (WAT)	4	EU853713[b]	EU708517–EU708518
S. concolor (L.) G.L. Nesom	Laurens Co./Ga.	Semple, 4040 (MT)	4	EU781460–EU781461	EU708515–EU708516
S. sericeum (Ventenat) G.L. Nesom	Rainy river/Ont.	Semple & Heard, 8787 (WAT)	5	EU200232[c]	EU708519–EU708520

Sect. *Grandiflori*					
S. oblongifolium (Nuttall) G.L. Nesom	Webster Co./Nebr.	Semple & Brouillet, 7337 (MT)	5	EU781459	EU708521
S. yukonense (Cronquist) G.L. Nesom	Kluane Lake/Yukon	Semple, 10624 (WAT)	5	EU200234[c]	EU708524
Sect. *Polyliguli*					
S. novae-angliae (L.) G.L. Nesom	Tenton/Ca.	Semple, 11001 (WAT)	5	EU200229[c]	EU708539
Sect. *Ericoidei*					
S. ericoides (L.) G.L. Nesom	Mound City/S.Dak.	Semple, 6664 (WAT)	5	EU200227[c]	EU708522–EU708523
Subg. *Astropolium*					
S. subulatum (Michaux) G.L. Nesom	Marengo Co./Ala.	Semple & Chmielewski, 6362 (MT)	5	EU781409	EU708535
S. tenuifolium (L) G.L. Nesom	Cedar Run/N.J.	Semple, 9519 (WAT)	5	EU200233[c]	EU708536
Subg. *Chapmaniani*					
S. chapmanii (T. & G.) Semple & Brouillet	Choctawhatchee R./Fla.	Semple, 10560 (WAT)	7	EU200223[c]	EU708550–EU708551

[a] GenBank Accessions of *Heterotheca fulcrata* (Greene) Shinners (U97615) and *Psilactis asteroides* A. Gray (U97640) (Morgan, 1997) are used in this study.
[b] Accession numbers submitted by Brouillet et al. (in preparation).
[c] Accession numbers from Selliah and Brouillet (2008).

2.2. Molecular methods

2.2.1. DNA extraction

Silica-dried and herbarium leaves of all species were used for DNA extraction using the Doyle and Doyle (1987) CTAB protocol followed by a modification (Joly et al., 2006), or with the QIAgen DNeasy Plant Mini Kit (Qiagen, Mississauga, Ontario, Canada), following the instructions of the manufacturer.

2.2.2. Primer design

In a preliminary phase of this study, the ITS region was amplified using the universal primers 17SE-26SE (Sun et al., 1994), also named AB101-AB102 (Douzery et al., 1999). The amplified band on the electrophoresis gel was unique but after sequencing, chromatograms were often difficult to read; it was not always possible to distinguish single nucleotide polymorphisms (SNPs) from noise. Therefore, the PCR products of three individuals were cloned (see below) and the aligned sequences of the most conserved 5′ and 3′ end regions were used to design a new set of more internal primers: ITSvF (5′-AGGAAGGAGAAGTCGTAACAAGG-3′) and ITSvR (5′-GATATGCTTAA ACTCAGCGG-3′). Direct sequencing using the more specific primers showed clearly the singleton polymorphisms and noise was eliminated.

For the GAPDH gene, we designed a primer pair by blasting partial mRNA sequence of *Scaevola procera* (GenBank Accession No. AY894500) with similar sequences of Asteridae followed by alignment with a complete GAPDH sequence of *Arabidopsis thaliana* (GenBank Accession No. AC068324) using the MegAlign software package (Lasergene, DNASTAR Inc). The alignment was refined manually. Subsequently, two conserved 5′ → 3′ regions between the 3rd and 6th exons were selected as a primer pair: GAPDHx3F (5′-TTGAGGGTCTTATGACTACAGT-3′) and GAPDHx6R (5′-GGTGTA TCCCAAGATACCCTTGAGC-3′). After amplification and sequencing (see below) we obtained ambiguous and unreadable chromatograms. Cloning (see below) was done to identify alleles. After aligning the alleles with the complete sequence, we identified some pseudogenes which lacked the 4th exon. To prevent amplification of these pseudogenes, we designed a new primer within the 4th exon (GAPDHx4F: 5′-AGGACTG GAGAGGTGGAAGAGC-3′). The primers were designed using the Amplify program version 3.1.4 (Engels, 2005).

2.2.3. PCR amplification, sequencing and cloning

Amplification of the ITS1-5.8S-ITS2 region was done in 25 μl reactions containing 2.5 μl 10× PCR buffer (Roche Diagnostics, Indianapolis, IN, USA), 0.5 μl $MgCl_2$ (25 mM, Promega, Madison, WI, USA), 100 μmol/L of each dNTP, 1 μl DMSO, 2 U of Taq Polymerase, ca. 200 ng genomic DNA and 1 mmol/L of each primer. An initial denaturation step at 94 °C for 3 min was followed by 35 cycles of denaturation (30 s at 94 °C), annealing at 52 °C for 30 s, elongation at 72 °C for 2 min, and final extension at 72 °C for 10 min. A long elongation step (2 min) was used to reduce potential PCR recombinants (Judo et al., 1998; Cronn et al., 2002). The GAPDH gene was amplified using the same conditions as for the ITS region with the exception that the amplification was performed in 40 cycles and the annealing temperature was set at 64 °C. PCR products were purified according to PEG purification (see modified protocol of Joly et al., 2006). Sequencing cycles were performed by adding 'Big Dye' Terminator chemistry v1.1 kit (Applied Biosystems, Foster City, California, USA) following the manufacturer instructions, except that 0.25 μL of dye terminator were used in a total mix volume of 10 μL. For each polyploid individual, five to 11 clones were sequenced. Approximately 60 ng of PCR sequencing products were precipitated using a sodium acetate solution and ethanol (70%). For each amplicon, double-stranded sequences were generated using an ABI 3100 – Avant automated DNA sequencer (Applied Biosystems).

Direct sequences with two or more SNPs were cloned using the pGEM-T vector (Promega Corporation, WI, USA) and transformed into competent *Escherichia coli* DH5-α at 42 °C. The transformed bacteria were screened on a selective and solid LB Petri dish media containing 50 mg/ml kanamycin, 100 mg/ml ampicillin, 50 mg/ml X-gal, and 0.5 M of IPTG (isopropyl β-D-1-thiogalactopyranoside) at 37 °C overnight. Twelve to 15 positive colonies were selected and cultivated overnight in 1.5 ml Eppendorf containing LB liquid as well as the two antibiotics. Positive cultures were amplified and sequenced using the same protocol as for direct sequencing.

2.3. Data analyses

2.3.1. Phylogenetic analyses

The ITS and GAPDH sequences were aligned using Clustal W (Thompson et al., 1994) as implemented in BioEdit Sequence Alignment Editor (Hall, 1999); the results were manually adjusted to maximize the number of homologous characters and minimize the number of insertions and deletions (indels). After alignment, almost half of the GAPDH sequences each had three stop codons at the 5′ end of the 5th exon. These sequences were considered pseudogenes and were excluded from the matrix. Such pseudogenes were found in most of the diploid species. Identical sequences resulting from cloning within a given individual were removed using the Collapse program v.1.2. (Posada, 2004). Indels were coded using the simple gap-coding method (Simmons and Ochoterena, 2000) as implemented in SEQSTATE (Müler, 2005).

Before determining the best-fit substitution model of sequence evolution, the ITS and GAPDH sequences were partitioned as follows: ITS: ITS1, 5.8S, and ITS2; GAPDH: 4th intron, 5th exon, and 5th intron. For each data partition, the Akaike information criterion (AIC; Akaike, 1973) and the likelihood ratio test (LRT; Felsenstein, 1988) were used to identify best-fitting models as implemented in MrModeltest 2.2 (Nylander, 2004) with executable MrModelblock file in PAUP* version 4.10b (Swofford, 2002). The choice between the two model selection criteria has been controversial in phylogenetic studies (Savill et al., 2001; Posada and Buckley, 2004; Domonicus et al., 2006). Some authors prefer to use both criteria in phylogenetic inference (Beilstein et al., 2006; Fehrer et al., 2007), whereas others apply either LRT or AIC (e.g., Goldman et al., 2000; Rabosky, 2006). We used Bayes factors (Kass and Raftery, 1995) to evaluate which criterion (LRT or AIC) provided the best phylogenetic estimation. Such an approach has been applied to select single versus multiple DNA data partitions (Nylander et al., 2004; McGuire et al., 2007). Many methods have been proposed (reviewed in Nylander et al., 2004) to calculate Bayes factors. We used the method proposed by Newton and Raftery (1994), which applies the harmonic mean of likelihood values as provided by MrBayes from MCMC analysis of the posterior distribution after burn-in. We accepted Bayes factors greater than two (considered to be "positive" according to the Kass and Raftery (1995) rule) in support of a criterion with higher harmonic mean log likelihood.

Bayesian analyses were performed for two million generations for both data sets, each with two replicates (LRT and AIC) using MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001). We used four Markov Chains at default temperature setting for each run and trees were sampled every 100 generations. To assess convergence of the topologies, we compared the posterior probabilities of different splits between pairs of identical runs using TRACER version 1.3 (Rambaut and Drummond, 2003). Convergence occurred after 2,000,000 generations. After excluding the 2000 trees of the burn-in phase, the 50% majority rule consensus tree was computed. Tree visualization was carried out using Tree View version 1.6.6 (Page, 2001).

2.3.2. Incongruence length difference test

One of the most frequently used strategies to infer species phylogeny from multiple genes is to combine data (Kluge, 1989), although many authors have indicated that this strategy is not suitable for genes with different histories (Bull et al., 1993; DeQueiroz et al., 1995). To determine whether the genes under study have had similar evolutionary histories, we used the incongruence length difference (ILD) test (Farris et al., 1994) coupled with the BIONJ algorithm (ILD-BIONJ) (Gascuel, 1997) to detect the presence of strongly supported character conflict among individual data sets within a combined analysis. Cunningham (1997) concluded that the ILD test performed the best in predicting when data should be combined, compared with the tests of Templeton (1983) and Rodrigo et al. (1993). ILD test is not only sensitive to variations of substitution rates (Darlu and Lecointre, 2002) but also its *P*-values correlate poorly with improvement in phylogenetic resolution (Barker and Lutzoni, 2002). Zelwer and Daubin (2004) has shown that ILD-BIONJ test is faster with higher performance than ILD test. This test requires two matrices of identical sizes. The number of sequences in the two matrices differs (44 for the ITS and 62 for the GAPDH, Table 2). To combine the two matrices for a species where a single ITS ribotype corresponds to two alleles of GAPDH, the ribotype sequence was duplicated so that each GAPDH allele had a corresponding ITS sequence. One hundred replications with heuristic searches and TBR branch swapping were used to assess the congruence between the two data sets. The test was implemented in PAUP* version 4b 10 (Swofford, 2002) using the partition homogeneity test.

2.3.3. Gene trees in species trees: a simulation approach

A gene tree which is constructed from nucleotide variation is an illustrational method to represent not only the evolutionary history of a gene but also to more or less reflect the evolutionary history of the species, particularly when a single allele is sampled from each species. In contrast, when two or more alleles from each species are sampled, inconsistency between a gene tree and the species tree is likely because of the retention and arbitrary sorting of ancestral polymorphisms at shallow time depths. This may lead to incorrect inferences about the relationships among species (Pamilo and Nei, 1988; Doyle, 1992; Lyons-Weiler and Milinkovitch, 1997; Maddison and Knowles, 2006).

To consider a gene tree as evidence of the species tree, the gene tree can be embedded within the species tree. An optimal species tree is that in which the correspondent gene tree can be embedded with the least cost (Page, 1998). The numbers of duplications, losses, and deep coalescences are the estimators by which we can evaluate the cost (Maddison, 1997). A straightforward use of this strategy here is to topologically compare the classifications of Nesom (1994a) and Semple (2005). We do not have species trees representing the interspecific relationships of *Symphyotrichum* species based on these classifications. Therefore, we traced two species trees according to each of these classifications, using Mesquite version 1.11 (Maddison and Maddison, 2006). Having fully resolved species trees is a prerequisite for using Mesquite. For this reason, we had to resolve the polytomies present in the two classifications when drawing the species trees. To draw the species trees, we used the classification structure to create the basic hierarchy of the trees (subgenera with embedded sections, subsections, and series when available; subsections are provided in Table 2). When more than one representative species was present at the lowest level, end branches were resolved using available information on relationships from the classification authors, when available. To avoid biasing the tree in favor of one or the other classification, species relationships were drawn similarly in both species trees when such information was not available. The ITS and GAPDH phylogenetic trees were embedded into the species

trees using the deep coalescence criterion (Maddison, 1997). Mesquite is able to embed a gene tree within a species tree under the lineage sorting assumption. Counting deep coalescences, one can determine the level of discordance between a gene tree and a species tree due to incomplete lineage sorting.

3. Results

3.1. ITS analysis

The ITS matrix comprises 44 sequences and 633 aligned characters excluding coded indels. The Bayes factors positively supported the evolutionary models suggested by the AIC criterion (Table 3). Of the total characters in the aligned matrix, 437 (69.3%) were constant, 141 (22%) were parsimony uninformative, and 55 (8.7%) were parsimony informative (for *Symphyotrichum* alone: 32,5%). The phylogenetic tree (Fig. 1) supported (posterior probability (PP) = 0.84) the monophyly of subtribe Symphyotrichinae with respect to the outgroups used, from which it is discriminated by two transitions and two transversions. *Canadanthus* is sister to all other Symphyotrichinae (PP = 1.00; 1 transition, 2 transversions, 3 indels), and *Ampelaster* to the remaining genera (PP = 0.92). *Almutaster* and *Psilactis* form a polytomy with *Symphyotrichum* subg. *Virgulus* and a clade comprising the subgenera *Chapmaniani*, *Symphyotrichum*, and *Astropolium*. Subgenus *Virgulus* forms a well-supported monophyletic group (PP = 1.00; 1 transition, 3 indels). The other three subgenera are grouped in a strongly supported clade (PP = 0.93). Within it, *Symphyotrichum chapmanii* is sister to the subgenera *Symphyotrichum* and *Astropolium* with strong support (PP = 0.95). The representatives of subg. *Astropolium* are grouped within subg. *Symphyotrichum*. Within subg. *Symphyotrichum*, species mostly form a large polytomy and neither sect. *Conyzopsis* ($x = 7$) nor sect. *Cordifolii* ($x = 8$) are shown to be monophyletic.

3.2. GAPDH analysis

The alignment of the GAPDH data set includes 60 sequences and 691 aligned characters excluding coded gaps, of which 346 (50.3%) were constant, 224 (32%) were variable but parsimony uninformative, and 122 (17.7%) were parsimony informative (for *Symphyotrichum* alone: 89, 13%). The Bayes factors very strongly supported the evolutionary models suggested by the AIC criterion (Table 3). The phylogenetic tree (Fig. 2) supports (PP = 1.00; 1 transition, 1 transversion, 1 indel) the monophyly of subtribe Symphyotrichinae with respect to the outgroups used. *Canadanthus* is sister to the other genera of Symphyotrichinae with moderately strong support (PP = 0.72). *Ampelaster*, *Psilactis asteroides*, *Almutaster*, and *Symphyotrichum* form a polytomy. *Almutaster* is grouped with members of subg. *Virgulus*, both being sister to the remaining *Symphyotrichum*. Within *Symphyotrichum*, the subgenera and sections (sensu Semple, 2005) do not form distinct clades. In general, infraspecific variation appears to be greater than interspecific: the two alleles of a species are not monophyletic, but instead each groups with homologous alleles from other species. For instance, the two alleles of *S. urophyllum* group with their homologs of *S. cordifolium* in two independent clades. The only exceptions appear to be alleles of members of subg. *Virgulus* forming a monophyletic clade (P = 1.00; 1 transversion), though other alleles of the subgenus are scattered within the large clade of *Symphyotrichum* alleles, and the sect. *Conyzopsis* pair of *S. laurentianum* and *S. ciliatum* (P = 1.00; 1 indel).

3.3. ILD test

The ILD-BIONJ congruence test rejected the null hypothesis of congruence (*P*-value = 0.01) between the ITS and GAPDH data sets.

3.4. Nesting the gene trees within the species tree

Under the criterion of minimizing deep coalescences, 43 deep coalescences, 14 duplications, and 59 losses are needed to fit the ITS gene tree within the species tree derived from the classification of Semple (2005) (Fig. 3a), whereas these values are 51, 19, and 77, respectively, for the classification of Nesom (1994a) (Fig. 4a). Similarly, 151deep coalescences, 34 duplications, and 178 losses are needed to fit the GAPDH phylogenetic tree within the species tree derived from the classification of Semple (Fig. 3b), whereas these values are 208, 38, and 235, respectively, for the Nesom classification (Fig. 4b). We also illustrated the two classifications on the inferred gene trees (Figs. 1 and 2) by using symbols corresponding to the subgenera and sections of each. These trees show incongruence between the two classifications in the placement of some taxa.

4. Discussion

4.1. Introgression or incomplete lineage sorting?

The amount of variation was greater among GAPDH alleles (26% variable sites, 13% parsimony informative) than among ITS ribotypes (12% and 5%) for *Symphyotrichum* (Table 3). In GAPDH, a major proportion of the variation appears to be intraspecific rather than interspecific. For instance, the GAPDH alleles of *S. sericeum*, *S. firmum*, *S. dumosum*, *S. undulatum*, and *S. cordifolium* group with

Table 3
Statistical information of each partition for the two molecular markers, ITS and GAPDH, and DNA substitution models selected by Bayes factors. HML, harmonic mean log likelihood; LRT, likelihood ratio test; AIC, Akaike information criterion.

Marker	Length	*Symphyotrichum*				All species				Evolutionary model (LRT/AIC)	HML (LRT)	HML (AIC)	2 ln Bayes factors
		Variable		Informative		Variable		Informative					
		No.	%	No.	%	No.	%	No.	%				
ITS													
ITS1	255	47	18	31	12	76	30	31	12	K80+G/SYM+G			
5.8S	164	3	1.8	1	0.6	3	1.8	1	0.6	JC/JC			
ITS2	214	36	17	15	7	72	34	30	14	SYM+G/SYM+G			
Total	633	74	12	32	5	141	22	55	8.7		2428.73	2427.19	3.08
GAPDH													
Intron 4	369	101	27	58	16	130	35	77	21	HKY+G/HKY+G			
Exon 5	151	27	18	9	6	33	22	11	7.3	K80/K80			
Intron 5	171	53	31	22	13	61	36	34	20	HKY/HKY+I			
Total	691	181	26	89	13	224	32	122	17.7		3336.24	3328.85	14.78

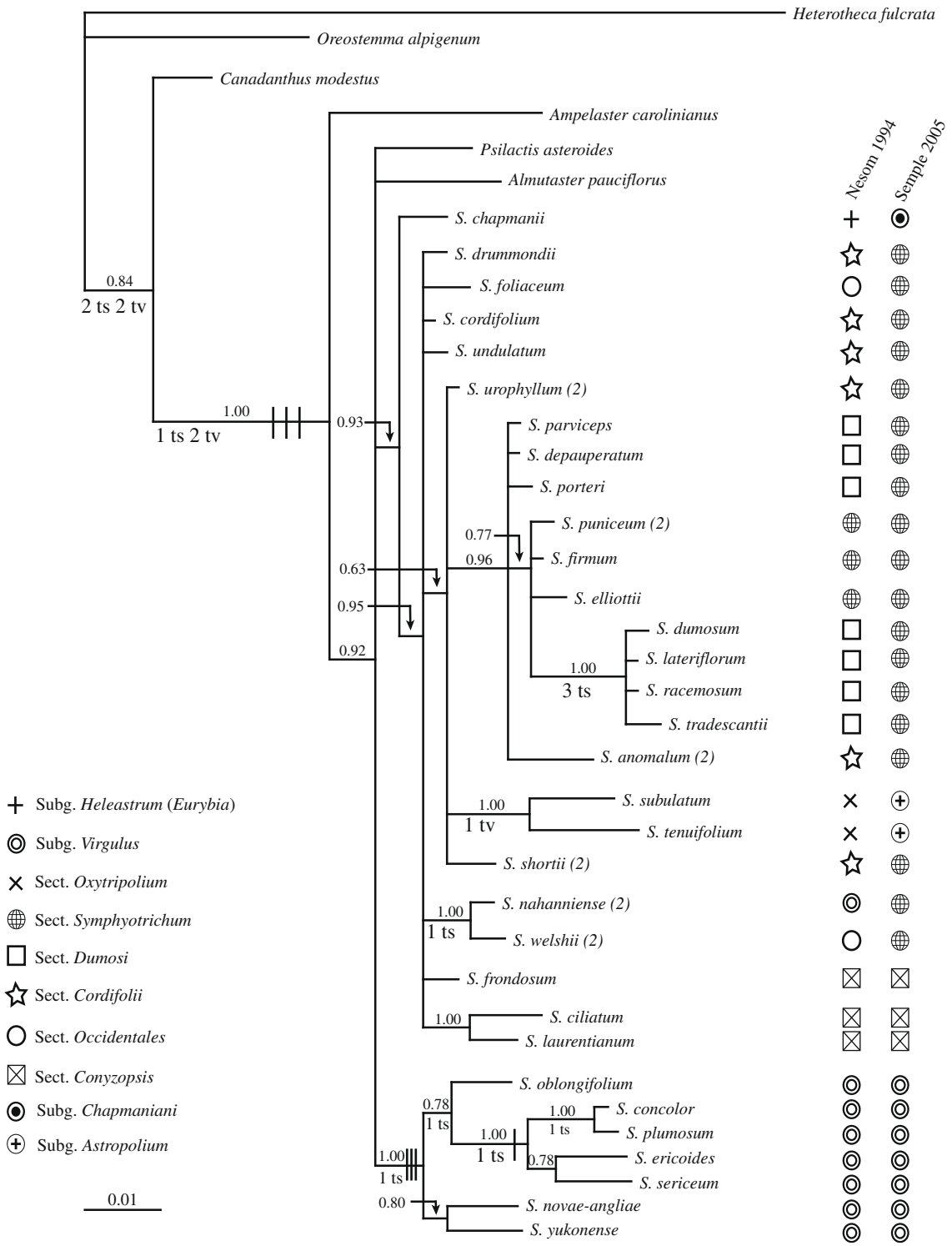

Fig. 1. Bayesian 50% majority rule consensus phylogram of the ITS data set. The classifications of Nesom (1994a) and Semple (2005) are indicated using symbols for each subgenus or section (all sections belong to subg. *Symphyotrichum*). Posterior probabilities are provided above the branches. The number and type of substitutions (ts, transition and tv, transversion) are given below the branches. Bars on branches indicate indels. When more than one ribotype grouped together for a single species, the number of ribotypes is indicated in parentheses after the species name.

homologs of other species (Fig. 2). The alleles of a given species are therefore not monophyletic. Two processes might be invoked to explain this lack of monophyly in a low-copy number nuclear gene such as GAPDH: introgression or incomplete lineage sorting (Lyons-Weiler and Milinkovitch, 1997; Page and Charleston, 1997; Wendel and Doyle, 1998). Introgression would result in a particular allele occurring at an unexpected position (incongruence) in the gene tree with respect to the position of the taxon in the species tree (based on morphology, for instance) or in another gene tree (e.g., cpDNA), whereas incomplete lineage sorting would result in a more or less random disposition of alleles in a gene tree with respect to the species tree; introgression in the presence of

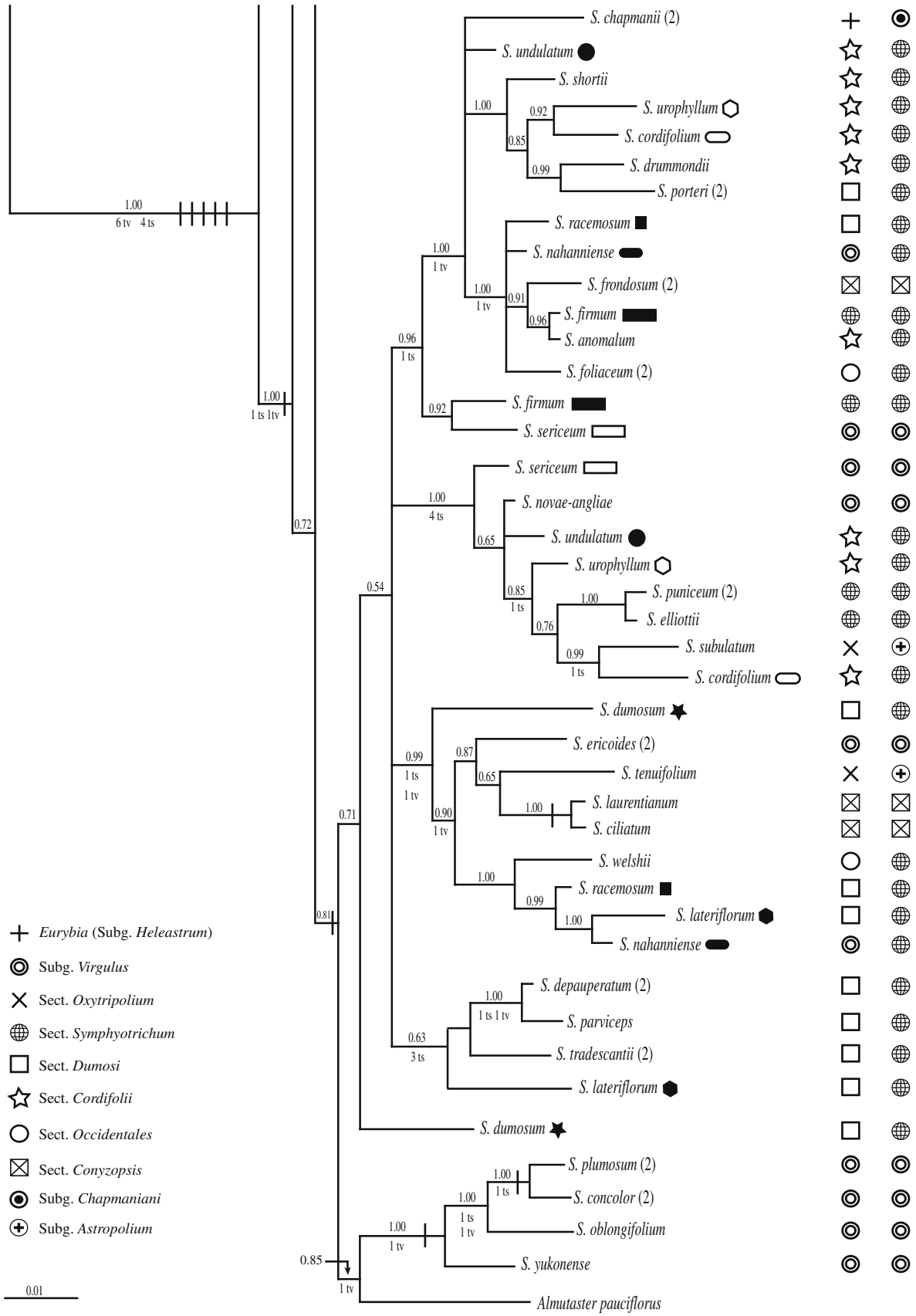

Fig. 2. Bayesian 50% majority rule consensus phylogram of the GAPDH data set. The classifications of Nesom (1994a) and Semple (2005) are indicated using symbols for each subgenus and section (all sections belong to subg. *Symphyotrichum*). Posterior probabilities are provided above the branches. The number and type of substitutions (ts, transition and tv, transversion) are given below the branches. Bars on branches indicate indels. When more than one allele grouped together for a single species, the number of alleles is indicated in parentheses after the species name. The symbols immediately after species name indicate that two non-monophyletic alleles were found for the species.

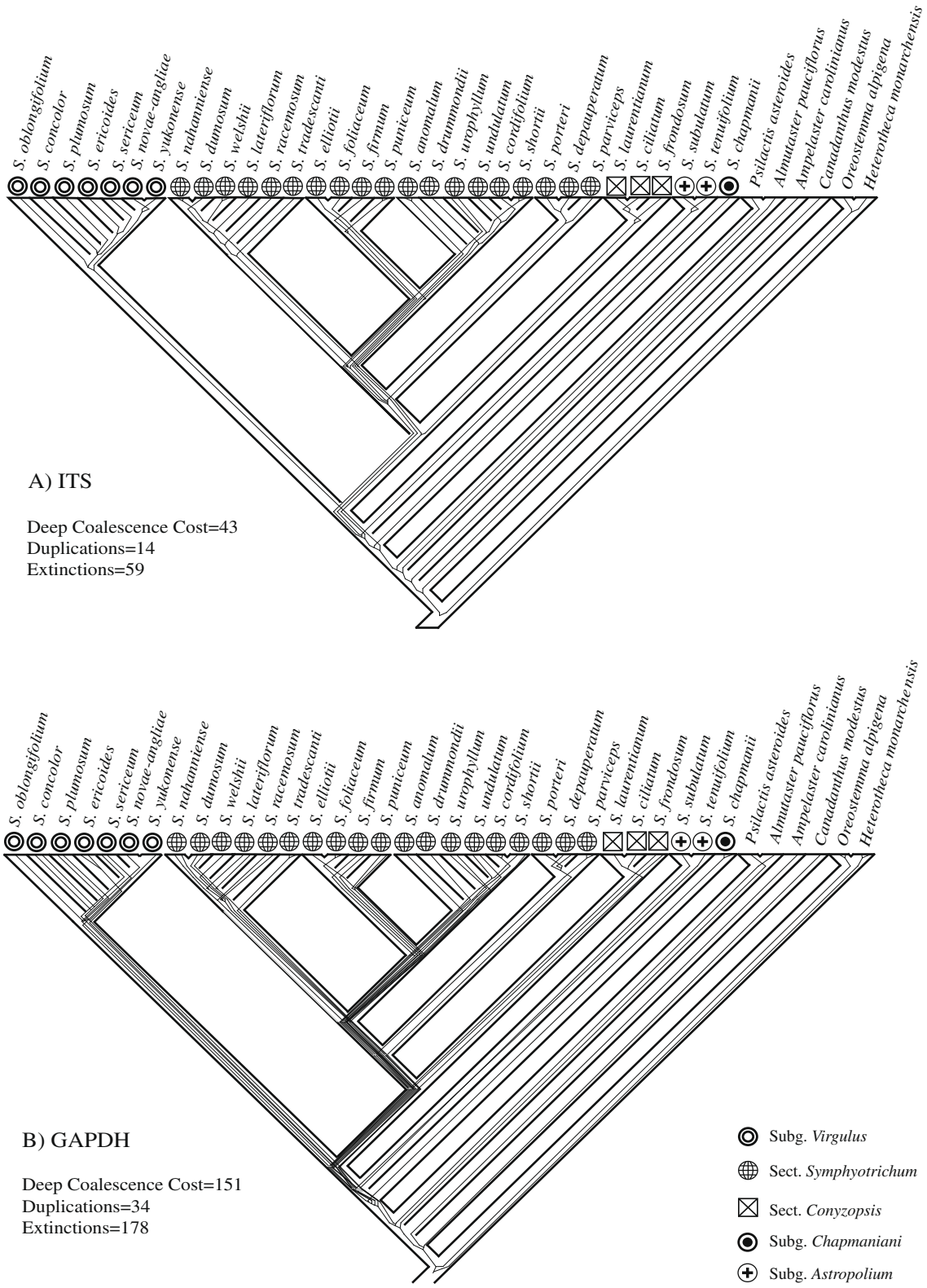

Fig. 3. Gene trees embedded within the species tree derived from the classification of Semple (2005) under the criterion of minimizing deep coalescences. The number of deep coalescences, duplications, and extinctions are given for each gene tree. (A) ITS gene tree. (B) GAPDH gene tree. The symbols below the species names are the same as those used in Figs. 1 and 2.

incomplete lineage sorting would be difficult to detect with only nuclear datasets, as is the case here. Indeed, in the nrDNA dataset, concerted evolution may have rapidly eliminated possible traces of introgression (e.g., Franzke and Mummenhoff, 1999; Fuertes-Aguilar et al., 1999), preventing its detection, even though the GAPDH marker, unlikely to have experienced concerted evolution, could

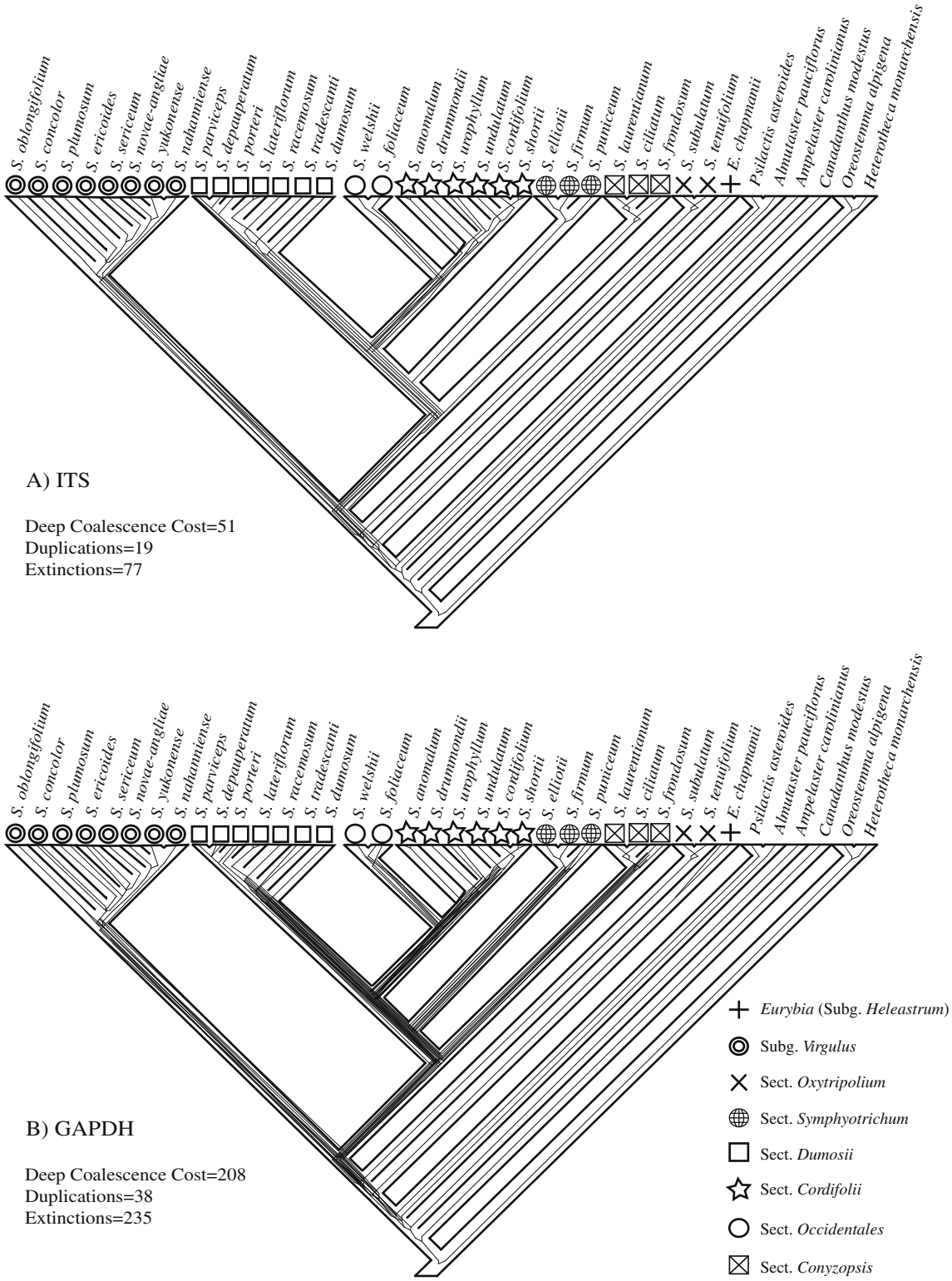

Fig. 4. Gene trees embedded within the species tree derived from the classification of Nesom (1994a,b) under the criterion of minimizing deep coalescences. The number of deep coalescences, duplications, and extinctions are given for each gene tree. (A) ITS gene tree. (B) GAPDH gene tree. The symbols below the species names are the same as those used in Figs. 1 and 2.

have kept traces of it. The two nuclear datasets used here are incongruent according to the ILD test, which is not surprising given their different evolutionary histories. This incongruence, however, cannot be ascribed readily to introgression since the ITS data show little variation and resolution within *Symphyotrichum*, while variation in GAPDH appears to be random. Thus, in this group of closely related diploid species, incongruence is more likely to be interpreted as incomplete lineage sorting rather than introgression (Comes and Abbott, 2001; Fehrer et al., 2007), even though hybridization has been documented in the genus. Incomplete lineage

sorting may contribute to phylogenetic incongruence at lower taxonomic levels (Rieseberg and Soltis, 1991; Soltis and Kuzoff, 1995) and thus render more difficult the resolution of phylogenetic relationships.

4.2. Intergeneric relationships of Symphyotrichinae

The ITS phylogenetic tree fits better within the species trees derived from each classification than the GAPDH tree (Figs. 3 and 4) based on the number of deep coalescences, which is higher in the GAPDH tree (151 and 208, respectively, for the Semple and Nesom classifications) than in the ITS tree (43 and 51). The incongruence revealed by the ILD test may reflect this difference in the number of deep coalescences when embedding the gene trees within the species trees. Given that the number of deep coalescences is higher for the Nesom (1994a) classification, it appears that both phylogenetic trees generally fit better with that of Semple (2005). This may be explained in part by the fact that the latter benefited from the molecular work done since 1994. Neither of these classifications perfectly reflects the molecular data, however. This could be due in part to the lack of resolution and incomplete lineage sorting of the latter.

Both the ITS and GAPDH phylogenetic trees support the monophyly of subtribe Symphyotrichinae relative to the outgroups, as seen in previous studies (Xiang and Semple, 1996; Brouillet et al., 2001). The disposition of monospecific *Canadanthus* and *Ampelaster* as distinct genera, first proposed by Nesom (1994a), is better resolved and supported in the ITS than in the GAPDH tree. *Canadanthus* appears as the earliest diverging member of the Symphyotrichinae in both analyses, but this relationship is well supported only in the ITS tree. *Ampelaster* appears sister to the remaining Symphyotrichinae in the ITS tree, but is in a polytomy with *Psilactis*, *Almutaster* and *Symphyotrichum* in the GAPDH tree; the position of this genus remains to be confirmed, even though it is strongly supported in the ITS tree. In contrast, the distinction of *Almutaster* and *Psilactis* from *Symphyotrichum* appears to be equivocal in both trees. The status of these two genera has not been stable in previous studies. A more complete taxic survey of *Psilactis* and data from additional genes may help resolve the position of these two genera.

4.3. Infrageneric relationships within Symphyotrichum

Subgenus *Virgulus* is morphologically (Nesom, 1994a) and cytologically (Semple and Brouillet, 1980b) distinct relative to the other subgenera of *Symphyotrichum*. It has had a controversial position. Semple and Brouillet (1980a) treated it as an independent genus, *Lasallea* (a synonym of *Virgulus*), close to the *Machaeranthera* lineage, based on morphological and cytological similarities. Nesom (1994a) rejected this hypothesis and placed it within *Symphyotrichum* based on morphological similarities and natural hybridization between members of subg. *Virgulus* and subg. *Ascendentes* (Jones, 1977; Allen, 1986; Allen and Eccleston, 1998). The distinct status of this subgenus as a monophyletic group based on the ITS analysis, with one substitution and three indels (Fig. 1), supports findings from previous studies (Jones, 1980; Semple and Brouillet, 1980a; Nesom, 1994a). The GAPDH results (Fig. 2), however, show that alleles of the subgenus have not yet reached monophyly; a unique allele of *Almutaster* groups with an apparently monophyletic clade of *Virgulus* alleles. Our results do not support the hypothesis of a close relationship between the members of subg. *Virgulus* and *Ampelaster*, as suggested by Xiang and Semple (1996) based on cpDNA restriction site analyses. Within subg. *Virgulus*, *S. concolor* and *S. plumosum*, recently segregated by Semple et al. (2002), are strongly supported as closely related in both analyses. Placement of *S. nahanniense* within subg. *Virgulus* as a synonym to *S. falcatum* var. *commutatum* (Nesom, 1994a), was strongly rejected based on both analyses (see also Owen et al., 2006). Within subg. *Virgulus*, *S. novae-angliae* (the single member of sect. *Polyliguli* sensu Semple) is closely related to *S. yukonense* (sect. *Grandiflori*). This is more in agreement with the classification of Nesom than with that of Semple (Table 1). Both classifications placed *S. sericeum* ($x = 5$) within sect. *Concolores* ($x = 4, 5$), but in the ITS analysis, it appears closer to *S. ericoides*. Data and taxic sampling are insufficient, however, to definitely conclude on lower-level relationships.

Subgenus *Chapmaniani* (Semple, 2005) is monospecific (*S. chapmanii*) and has a base chromosome number of $x = 7$. Semple (1982) hypothesized it belongs in *Eurybia* subg. *Heleastrum* and had an aneuploid origin from an $x = 9$ ancestor. Jones and Young (1983) hypothesized a hybrid origin for the species, derived from a cross between a species of *Eurybia* subg. *Heleastrum* ($x = 9$) and of *Symphyotrichum* subg. *Astropolium* ($x = 5$) (both as *Aster* subgenera). Nesom (1994a) treated *S. chapmanii* within *Eurybia* subg. *Heleastrum* based on similarities in leaves and capitulescences; the chromosome number was interpreted as a reduction from $x = 9$ to 7. Brouillet et al. (2001), however, found that this species did not belong in *Eurybia* but in *Symphyotrichum*, a fact used by Semple (in Semple et al., 2002) to transfer the species to the latter. The current ITS phylogenetic analysis confirms the treatment of subg. *Chapmaniani* within *Symphyotrichum*, as sister to the subgenera *Symphyotrichum* and *Astropolium*. The GAPDH tree does not support this sister position but strongly supports inclusion of *S. chapmanii* within *Symphyotrichum*.

Section *Oxytripolium* ($x = 5$), placed within subg. *Symphyotrichum* ($x = 7, 8$) by Nesom (1994a), was upgraded to subgeneric level as subg. *Astropolium* by Semple (2005) based on the different base chromosome numbers and karyotypes. Semple and Brouillet (1980a) suggested a close relationship between this taxon and subg. *Virgulus* due to the similar base chromosome number of $x = 5$, though they differ in the morphology of the chromosome bearing the nucleolar organizer region (NOR) (Semple and Brouillet, 1980b). Our results, especially the ITS tree, reject this idea. This taxon, represented by *S. subulatum* and *S. tenuifolium*, forms a well-supported monophyletic group (PP = 1.00) within subg. *Symphyotrichum* based on the ITS tree and it is characterized by a single transversion. The position of this taxon as sect. *Oxytripolium* within *Symphyotrichum* appears to fit better with our results; no support was obtained for the subgeneric level proposed by Semple. Further molecular data and a more complete taxic sampling are needed to settle this issue.

Nesom (1994a) and Semple (2005) placed sect. *Conyzopsis* ($x = 7$) within subg. *Symphyotrichum* based on their similarities in morphology (reduced vestiture, lack of glands, and unkeeled phyllaries; Nesom, 1994a) and NOR morphology (Semple and Brouillet, 1980b). Nesom (1994a) hypothesized that the base chromosome number of $x = 7$ is derived from ancestors with $x = 8$. This section of three species, *S. ciliatum*, *S. laurentianum*, and *S. frondosum,* is distinguished from other members of subg. *Symphyotrichum* by having 2–3 series of ray florets, pappi longer than disc florets, and its base chromosome number. Both phylogenetic analyses strongly supported (PP = 1.00) a close relationships between *S. ciliatum* and *S. laurentianum*. These species have a single deletion of 152 nucleotides in length within the 4th intron of the GAPDH gene. In contrast, the two GAPDH alleles obtained from *S. frondosum* do not have this long deletion. However, despite its distinctive features, our results do not currently support the monophyly of the section as suggested previously (Houle and Brouillet, 1985; Houle and Haber, 1990; COSEWIC, 2004). More molecular data are needed to confirm the affinities of these three species.

Section *Symphyotrichum* (sensu Semple, 2005) comprises the majority of the species (ca. 52) of the genus. A high proportion

of these species is polyploid (Semple and Brammall, 1982; Allen et al., 1983; Dean and Chambers, 1983; Brouillet and Labrecque, 1987; Nesom, 1994b; Semple and Cook, 2004 and references therein) and these were excluded here. In both phylogenetic analyses, the insufficient resolution or incomplete lineage sorting make it impossible to determine whether the sections as defined by Nesom (1994a) or by Semple (2005) (Table 1) would be supported.

Abundant interspecific hybridization, particularly within subg. *Symphyotrichum* (e.g., Brouillet and Labrecque, 1987; Allen and Eccleston, 1998; Semple et al., 2002) and lack of phylogenetic resolution within *Symphyotrichum* based on the ITS analysis (5% informative sites; Table 3) may be interpreted as the occurrence of a recent radiation in the evolution of the genus. Moreover, incomplete lineage sorting was documented in the GAPDH tree, resulting in a lack of monophyly of the ITS-based clades within *Symphyotrichum* (e.g., subg. *Virgulus*) in that tree. Many studies have shown that incomplete lineage sorting is characteristic of recently and rapidly radiating groups with short terminal branches (e.g., Ballard and James, 2002; Shaw, 2002; Broughton and Harrison, 2003; Hughes and Volger, 2004; Buckley et al., 2006). All these elements reflect a recent diversification of genus *Symphyotrichum* in North America.

In order to better resolve phylogenetic relationships within *Symphyotrichum*, it will be necessary to sample more individuals and more populations per species in order to increase the probability of sampling alleles more completely and provide more accurate information on incomplete lineage sorting events (Maddison and Knowles, 2006), as well as possibly retrieving more phylogenetic signal from subsets of alleles. Also, to resolve relationships among groups of recently diverged species, markers with higher mutation rates in the terminal branches of the phylogeny may be necessary. Because of their higher evolutionary rate, such markers would increase the probability of complete lineage sorting within branches leading to species, and thus would be useful in obtaining a gene tree that might better reflect the species tree.

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