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Patterns and causes of incongruence between plastid and nuclear Senecioneae (Asteraceae) phylogenies[1]

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One of the longstanding questions in phylogenetic systematics is how to address incongruence among phylogenies obtained from multiple markers and how to determine the causes. This study presents a detailed analysis of incongruent patterns between plastid and ITS/ETS phylogenies of Tribe Senecioneae (Asteraceae). This approach revealed widespread and strongly supported incongruence, which complicates conclusions about evolutionary relationships at all taxonomic levels. The patterns of incongruence that were resolved suggest that incomplete lineage sorting (ILS) and/or ancient hybridization are the most likely explanations. These phenomena are, however, extremely difficult to distinguish because they may result in similar phylogenetic patterns. We present a novel approach to evaluate whether ILS can be excluded as an explanation for incongruent patterns. This coalescence-based method uses molecular dating estimates of the duration of the putative ILS events to determine if invoking ILS as an explanation for incongruence would require unrealistically high effective population sizes. For four of the incongruent patterns identified within the Senecioneae, this approach indicates that ILS cannot be invoked to explain the observed incongruence. Alternatively, these patterns are more realistically explained by ancient hybridization events.

Key words: ancient hybridization; deep coalescence; ETS; incomplete lineage sorting; incongruence; ITS; molecular dating; plastid sequences.

Since the onset of the use of DNA sequences for phylogeny reconstruction, molecular systematics has experienced a steady increase in the number of DNA regions used to resolve evolutionary relationships (Degnan and Rosenberg, 2009). This development has significantly contributed to our understanding of the evolutionary history of numerous lineages by clarifying relationships that were previously unresolved in studies using fewer markers and less data (e.g., Kuzoff and Gasser, 2000; Pryer et al., 2004; Panero and Funk, 2008). In addition, multigene studies have enabled a more detailed understanding of macroevolution by revealing congruence or incongruence between phylogenies obtained from the analysis of different genes, genic regions, and genomes (Degnan and Rosenberg, 2009). Incongruence may, among others, indicate differences in the evolutionary histories of the DNA regions employed (i.e., gene tree–species tree discordance), which could result from hybridization or incomplete lineage sorting (ILS; Doyle, 1992; Maddison, 1997; Buckley et al., 2006; Liu and Pearl, 2007). ILS is the failure of ancestral polymorphisms to track speciation events accurately, which may result in incongruence between gene trees and species trees. Unfortunately, ILS can result in phylogenetic patterns similar to those observed for hybridization events (Doyle, 1992; Seelanan et al., 1997; Holder et al., 2001; Buckley et al., 2006; Holland et al., 2008; Joly et al., 2009). Therefore ILS and hybridization are often difficult to distinguish. Furthermore, in the absence of an effective methodology to distinguish between them (Joly et al., 2009), there are few empirical studies that present a detailed assessment of incongruent patterns and even fewer in which the specific causes for these patterns were studied (Wiens and Hollingsworth, 2000; Van der Niet and Linder, 2008; Morgan et al., 2009). There is therefore an urgent need for research that explores ways to examine incongruent patterns and to determine the causes (Buckley et al., 2006; Holland et al., 2008; Degnan and Rosenberg, 2009). In this paper, we aim to contribute to the development of new approaches to the study of incongruent phylogenetic patterns by documenting strongly supported topological incongruence in tribe Senecioneae (Asteraceae) and using a new approach to establish whether ILS can be excluded as an explanation for incongruent patterns. This coalescent-based method uses estimates of the

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duration of putative ILS events to determine if invoking ILS as an explanation for incongruence would require unrealistically high effective population sizes.

The Senecioneae are one of the largest tribes in the Asteraceae (ca. 3100 species and 155 genera) with an almost worldwide distribution, and it exhibits remarkable morphological and ecological diversity. *Senecio* is the largest genus in the tribe (ca. 1000 species) and is perceived as taxonomically difficult because of its size and extensive morphological diversity. In a recent study, ITS sequence data were used to reconstruct a phylogeny for the Senecioneae and to redefine a previously polyphyletic *Senecio* (Pelser et al., 2007). This study included 186 *Senecio* species and 114 of the 150 genera recognized by Nordenstam (2007). Because the molecular phylogeny placed several lineages outside of core *Senecio*, a new circumscription resulted with several taxa being transferred to other genera within the tribe and others to be described as new genera (Nordenstam et al., 2009c; unpublished data). Furthermore, seven genera (*Aetheolaena*, *Cadiscus*, *Culcitium*, *Hasteola*, *Iocenes*, *Lasiocephalus*, and *Robinsonia*) were deeply embedded within *Senecio* and thus, are being transferred into the genus to arrive at a monophyletic delimitation (Pelser et al., 2007; Nordenstam et al., 2009b; unpublished data). It is important to note that while this new delimitation of *Senecio* is largely based on an ITS phylogeny, the major clades are also supported by plastid sequence data composed of *ndhF*, the *trnL* intron, and the *psbA-trnH*, 5′ and 3′ *trnK*, and *trnL-F* intergenic spacers (Pelser et al., 2007). In addition, the plastid data augmented the utility and efficacy of the ITS for phylogeny reconstruction in the Senecioneae by supporting many of the resolved evolutionary relationships at the species, generic, and subtribal levels in the tribe. Furthermore, many patterns of relationships in the ITS Senecioneae phylogeny were corroborated by morphological, karyological, and/or biogeographic data and supported the taxonomic conclusions from previous Senecioneae studies (reviewed in Pelser et al., 2007; Nordenstam et al., 2009a). Although the plastid data provided additional support for the ITS phylogeny with overall congruence between the two topologies observed, some incongruence was present that prevented taxonomic conclusions for some lineages (Pelser et al., 2007). Because the plastid phylogeny was only based on a subset of the Senecioneae taxa that were sampled for the ITS analyses (73 vs. 614 species), it was not possible to sufficiently resolve these patterns for a detailed analysis of the extent and understanding of the underlying causes of the incongruence at the generic level. In the current study, we therefore have significantly increased the taxon and marker sampling for the plastid and nuclear data and conducted extensive phylogenetic analyses. The goals were (1) to identify strongly supported relationships that are incongruent between trees produced with different data sets and (2) to explore possible causes of the incongruence.

MATERIALS AND METHODS

Taxon and character sampling—The taxa selected for this study were chosen from the plastid and ITS phylogenies of Pelser et al. (2007) to represent the Senecioneae genera included in that study. In addition, 31 Senecioneae genera were added for which material was not previously available to us (Appendix S1; see Supplemental Data with the online version of this article) resulting in a sampling of approximately 94% of all Senecioneae genera. A total of 27 genera from 26 other Asteraceae tribes was included for outgroup comparisons (Appendix S1). DNA samples of representatives of the taxa not included in Pelser et al. (2007) were obtained from tissue samples taken with permission from herbarium specimens at B, BHCB, F, L, MO, MU, P, S, TENN, TEX, UC, US, WAG, and XAL, or from field-collected leaf tissue preserved on silica gel.

Phylogenetic analyses were performed with DNA sequences from eight regions: the ITS (ITS1, 5.8S, ITS2) and ETS of the nuclear genome, plus the *ndhF* gene, the *trnL* intron, and *psbA-trnH*, 5′ and 3′ *trnK*, and *trnL-F* intergenic spacers of the plastid genome. To root tribe Senecioneae in subfamily Asteroideae, partial sequences of the plastid *rbcL* gene were obtained for representatives of the major Senecioneae lineages and the other Asteraceae tribes, in addition to the above markers. However, the relationship among outgroups is not discussed since this is not a goal of this paper.

DNA extraction, PCR amplification, and sequencing—Total genomic DNA was extracted using the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA). PCR amplification of the ITS, *ndhF*, *psbA-trnH*, 5′ and 3′ *trnK*, *trnL*, and *trnL-F* regions followed Pelser et al. (2002, 2003, 2007) or minor modifications thereof. The ETS region was partially amplified with primer AST1 (Markos and Baldwin, 2001) or a modification of primer ETS2 (Bayer et al., 2002; 5′-CAA CTT CCA CCT GGC TTA CCT CC-3′) as forward primers and 18S-ETS (Baldwin and Markos, 1998) as the reverse primer. The *rbcL* gene was amplified in two parts using forward primers 1F and a modification of primer 636F (Fay et al., 1997; 5′-GCG TTG GAG AGA CCG TTT CT-3′), and reverse primers 1460R (Savolainen et al., 2000) and a modification of primer 724R (Fay et al., 1997; 5′-TCG CAT GTA CCC GCA GTA GC-3′). PCR products were purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin, USA). Cycle sequencing was carried out with the BigDye Terminator v.3.1 (Applied Biosystems, Foster City, California, USA) cycle sequencing kit. The fluorescently labeled samples were run on an ABI 3130xl or 3730 automated sequencer at the Center for Bioinformatics and Functional Genomics at Miami University (Oxford, Ohio, USA). The program Sequencher v.4.8 (Gene Codes, Ann Arbor, Michigan, USA) was used for trace file editing.

DNA sequence alignment and phylogeny reconstruction—DNA sequences were aligned with the program CLUSTALX v.1.83 (Thompson et al., 1997) using the default penalty settings of the program. This alignment was edited using the program Se-Al v.2.0a11 (Rambaut, 1996) by excluding portions of sequences that could not be unambiguously aligned (mostly of ITS and ETS accessions of tribes distantly related to the Senecioneae) and replacing those with a missing data symbol. A Python script (Richard Ree, Field Museum, Chicago, Illinois, USA) was used to code indels as binary characters using the simple indel coding method of Simmons and Ochoterena (2000).

For several species, sequences of multiple accessions were available (Appendix S1). In the first round of heuristic searches performed under maximum parsimony (MP, see below) for each of the DNA regions individually, all available sequences were included. When multiple accessions of the same species formed a clade, a consensus sequence was generated in which polymorphisms were coded as ambiguous nucleotide characters, and this consensus sequence was used for subsequent phylogenetic analyses (Pelser et al., 2007). This strategy was preferred over selecting one conspecific accession among those available to avoid subjective decisions in the process of selecting a single examplar and to be able to include all available data that potentially contribute to the phylogeny reconstruction. In addition, this approach allowed for the inclusion of longer sequence reads for taxa of which individual accessions only resulted in partial sequences. Comparisons to results of parsimony analyses did not reveal decreased resolution when multiple accessions per species were included, and analyses in which consensus sequences also were included placed these among the individual accessions of the same taxon.

The MP phylogeny was reconstructed with the program TNT 1.0 (Goloboff et al., 2008) using the Driven Search option with the default settings for Sectorial Searches (RSS, CSS, and XSS), Ratchet, Tree Drifting, and Tree Fusing, 10 initial random addition sequences, terminating the search after finding minimum length trees five times. Bootstrap support (BS; Felsenstein, 1985) was calculated with Poisson independent reweighting using 1000 replicates. Bayesian inference (BI) analyses were performed using the program MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) on the Redhawk Cluster at Miami University (EM64T Cluster with 128 dual nodes with 4 GB of memory and 150 GB of disk space per node). Prior to the BI analyses, the Akaike information criterion (AIC) in the program MrModeltest 2.2 (Nylander, 2004) was employed to select nucleotide substitution models for each of the DNA regions. These analyses selected the GTR+I+Γ model for each of the individual DNA regions and the combinations of DNA regions analyzed, which was therefore used in all BI analyses. Indel characters were included as "restriction type" data in the BI analyses. These analyses were performed using two independent simultaneous

runs. The Markov chain Monte Carlo analyses (MCMC; Geyer, 1991) were run with up to 40 chains per analysis, temperature settings ranging between 0.0001 and 0.2, and one tree per 1000 generations saved. BI analyses were run until the average deviation of split frequencies between both simultaneous analyses reached a value below 0.01. The burn-in values were determined empirically from the likelihood values. Trees were visualized using the program FigTree v.1.2.2 (Rambaut, 2009).

Identification and localization of incongruence—The incongruence length difference test (ILD; Farris et al., 1995) is one of the most widely applied methods for assessing incongruence (Darlu and Lecointre, 2002; Hipp et al., 2004). Its use has, however, been criticized, because of a high false positive rate (Cunningham, 1997; Darlu and Lecointre, 2002; Hipp et al., 2004). Because alternative methods for testing incongruence (e.g., the Templeton [Templeton, 1983], Kishino–Hasegawa [Kishino and Hasegawa, 1989], and Shimodaira–Hasegawa [Shimodaira and Hasegawa, 1999] tests) may suffer from errors as well (Cunningham, 1997; Shimodaira and Hasegawa, 1999; Buckley et al., 2001; Shimodaira, 2002; Hipp et al., 2004), incongruence is probably best studied using a combination of methods (Hipp et al., 2004). In this study, we therefore explored patterns of phylogenetic incongruence using two approaches: the ILD test and an assessment of incongruent patterns that are supported by high BS or BI values. To reduce the chance of false positives and following the recommendations of Cunningham (1997), we considered only ILD *P*-values below 0.01 as evidence of significant incongruence.

Strongly supported incongruence is here defined as incongruent patterns that are supported by BS values ≥80% and/or posterior probabilities (PP) ≥0.95 as well as ILD values of $P < 0.01$. The ILD tests were conducted in PAUP* with 500 to 10 000 replicates and 1 to 100 random addition sequences, depending on the size and complexity of the data sets analyzed. Invariant characters were removed from the data sets prior to performing ILD tests (Cunningham, 1997). This methodology revealed strong incongruence between trees obtained from the plastid and ITS/ETS data sets, but not among the plastid markers or between the ITS and ETS. Subsequent phylogenetic analyses were therefore focused on identifying lineages with strongly supported, but incongruent, topological placements in trees obtained from the plastid vs. the ITS/ETS data sets.

Lineages with strongly supported, yet incongruent, phylogenetic placements in the plastid vs. ITS/ETS trees were identified using a novel two-step approach designed to examine complex patterns involving multiple incongruent lineages for which some also have internal incongruence. First, plastid and ITS/ETS trees were visually compared to identify the largest mutually exclusive lineages that form a clade in all or some consensus trees. In this way, lineages that are resolved as closely related in both plastid and ITS/ETS trees were identified. Among these lineages are clades that are retrieved in all trees (e.g., Tussilagininae s.s.; Figs. 1, 2), but also those lineages that form a clade in some, but not all, trees. The latter category of lineages were only included when their taxa are in close phylogenetic proximity (e.g., *Lachanodes-S. thapsoides* group; Figs. 1, 2). Although *Senecio* s.s. is not resolved as monophyletic in either the plastid or the ITS/ETS trees, support for its nonmonophyly is low (Figs. 1, 2), and it comprises a single clade in trees obtained from a combined plastid-ITS/ETS data set (not shown). *Senecio* s.s. was therefore also identified as one of the major lineages present in both plastid and ITS/ETS trees.

Secondly, these identified major lineages were then examined for the presence of strongly supported internal incongruence by evaluating branch support values and then subjecting them to ILD tests that only included the taxa of the lineage under investigation. This method allowed for the identification of strongly incongruent accessions and subclades within each of these major lineages without the confounding effects of unrelated incongruence within or among other major lineages. In addition, branch support values and ILD tests were employed in a similar fashion to study the incongruence among the major lineages. Due to the large size of the data sets, one or two placeholder species were included in the latter round of ILD tests to eliminate the confounding effects of internal incongruence within the major lineages.

After strongly incongruent lineages were identified, MP and BI analyses of a combined plastid-ITS/ETS data set were performed in which each taxon in a strongly incongruent lineage was included twice: once as a plastid-only accession (ITS/ETS characters coded as missing) and once as an ITS/ETS-only accession (plastid characters coded as missing; Pirie et al., 2008). This approach was used to resolve a generic level tree without the confounding effects of taxa with strongly supported incongruence between the plastid and ITS/ETS partitions and to examine their alternative phylogenetic placements relative to a backbone composed of lineages among which relationships are not strongly incongruent. Because BI analyses of this data set using various temperature settings and numbers of chains did not reach convergence (40 chains per run, temperature setting of 0.0001), a user-defined starting tree was supplied using the MP 50% majority rule consensus topology of the combined plastid-ITS/ETS data set. Each chain was then initiated with a slightly perturbed version of this starting tree by setting the parameter nperts to 1. This approach resulted in convergence of the chains.

ITS orthology/paralogy assessment—To determine if specimens possessed divergent ITS copies, potentially indicating that the incongruence is caused by inaccurate assessment of orthology, we cloned and sequenced the ITS region for accessions of strongly incongruent lineages. Because ITS trees were not strongly incongruent with ETS trees and both regions are adjacent, the ETS region was not cloned and sequenced. ITS PCR products were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, California, USA). Between 5 and 10 clones per accession were PCR-amplified directly from plated culture with the manufacturer's supplied M13 plasmid primers. PCR products were cycle sequenced with M13 plasmid primers. Sequencing and alignment followed the protocol outlined above.

Long-branch attraction—Because long-branch attraction (Felsenstein, 1978) is a possible explanation for topological incongruence, MP and BI phylograms were visually inspected for the presence of exceptionally long branches associated with strongly incongruent lineages. A series of phylogenetic analyses was subsequently carried out in which accessions with long branches were individually excluded in turn. This was done to determine whether the exclusion of these accessions resulted in changes in topology, which could indicate the presence of long-branch attraction.

Incomplete lineage sorting—Topological incongruence caused by hybridization and ILS can be difficult to distinguish, because both phenomena may result in similar topological patterns. Coalescent theory, however, predicts that ancestral polymorphisms are likely to coalesce within approximately $5N_e$ generations (N_e being the effective population size; Rosenberg, 2003; Degnan and Rosenberg, 2009) and that congruence between gene trees and species trees becomes highly probable. Information about generation times and estimates of the duration of an ILS event can therefore be used to calculate the minimum N_e that must be assumed to explain incongruence due to ILS. If these calculations result in N_e estimates that are much higher than observed in nature, then ILS can be excluded, and hybridization is favored as the likely explanation for the observed incongruence.

To calculate N_e estimates, we estimated molecular dates using penalized likelihood analyses performed in the program r8s 1.71 (Sanderson, 2002, 2003) on the Redhawk Cluster at Miami University. Separate analyses were carried out for the plastid and the ITS/ETS data sets using the topologies and branch lengths obtained in the BI analyses. Some accessions were pruned from the input trees to meet the r8s requirement of nonzero branch lengths. Several calibration points outside and within the Senecioneae were used based on previous age estimates in Asteraceae (Wikström et al., 2001; Kim et al., 2005; Hershkovitz et al., 2006), fossil evidence (Graham, 1996), and inferred ages for islands or archipelagos with endemic Senecioneae taxa (Baker et al., 1967; McDougall and Schmincke, 1976; McDougall et al., 1981; Stuessy et al., 1984; Cronk, 1987; Ancochea et al., 1990; Geldmacher et al., 2000; Table 1). The smoothing parameters for the penalized likelihood analyses were determined using cross-validation tests.

In addition to estimating divergence dates using penalized likelihood, molecular dating studies were performed using a Bayesian approach with the program BEAUti/BEAST v.1.4.7 (Drummond and Rambaut, 2007) on the Redhawk Cluster at Miami University. To compare the dating results of the BEAST analyses to those estimated by r8s, we also used the input trees from the r8s analyses as the starting trees in BEAST, and their topology was fixed. The BEAST analyses were performed with the GTR+I+Γ model, the uncorrelated relaxed lognormal clock, and the Yule tree prior. The calibration points used in the r8s analysis were specified using uniform distributions with an upper and lower bound. Following the instructions in the BEAST manual, the weights of the operators for the treeModel were modified to improve the efficiency of the MCMC: the upDownOperator, uniformOperator on internalNodeHeights, narrowExchangeOperator, and subtreeSlideOperator were set at 115 and the wilsonBaldingOperator and the wideExchangeOperator were set at 23. Nine (plastid data) and 11 (ITS/ETS data) independent BEAST runs totaling 136 687 000 (plastid data) and 318 672 000 (ITS/ETS data) generations were carried out to ensure that all parameters had an effective sampling size greater than 200 (after a burnin of 10% was removed). Convergence was examined using the program Tracer v.1.4 (Rambaut and Drummond, 2007).

TABLE 1. Calibration points for r8s and BEAST molecular dating analyses.

Calibration point	Age constraint used (Myr)	Age estimates from literature (Myr)	References
Root (fixed)	35	33–36.5	Wikström et al., 2001; Kim et al., 2005; Hershkovitz et al., 2006
MRCA of Cichorioideae and Asteroideae	24–38	24–38	Wikström et al., 2001; Kim et al., 2005; Hershkovitz et al., 2006
Anthemideae	≥23	Late Oligocene (fossil pollen)	Graham, 1996
MRCA of *Helianthus* and *Tagetes*	13–19	13–19	Wikström et al., 2001; Kim et al., 2005; Hershkovitz et al., 2006
MRCA of *Senecio* and *Blennosperma*	16–19	16–19	Kim et al., 2005; Hershkovitz et al., 2006
Lachanodes[a]	≤14.5	14.5 (age of St. Helena)	Baker et al., 1967; Cronk, 1987
Pladaroxylon[a]	≤14.5	14.5 (age of St. Helena)	Baker et al., 1967; Cronk, 1987
Pericallis aurita[a]	≤14.3	14.3 (age of Porto Santo)	Geldmacher et al., 2000
Pericallis[a]	≤14.3	14.3 (age of Porto Santo[b])	McDougall and Schmincke, 1976; Geldmacher et al., 2000
Bethencourtia[a]	≤11.6	11.6 (age of Tenerife[b])	Ancochea et al., 1990
Lordhowea[a]	≤6.9	6.9 (age of Lord Howe Island)	McDougall et al., 1981
Robinsonia[a]	≤4.2	4.2 (age Juan Fernández Islands)	Stuessy et al., 1984
Robinsonia masafuerae[a]	≤2.4	2.4 (age of Masafuera)	Stuessy et al., 1984

[a]Island or archipelago endemics.
[b]Oldest island in distribution area.

After obtaining age estimates of divergences, we calculated the duration of each putative ILS event as follows. First, the estimated time of onset of an ILS event was determined from both the plastid and ITS/ETS BEAST chronograms. Although the plastid and ITS/ETS BEAST chronograms resulted in different age estimates for the onset of ILS events, we assume that these estimates are of the same speciation event in the species tree. To approximate the age of each speciation event that marks the start of an ILS event, we used the overlap in the 95% highest posterior density (HPD) intervals of the plastid and ITS/ETS chronograms. For a few of the strongly incongruent lineages, r8s estimates of the onset of putative ILD events were slightly outside the ranges obtained from BEAST. In these cases, the ranges of the assumed time interval of the ILS onset calculated from the BEAST chronograms were extended with the r8s ages. Secondly, the ages of the most recent common ancestor (MRCA) of each strongly incongruent lineage and its nonincongruent sister group were recorded from the plastid and ITS/ETS chronograms. These age estimates signify the end of a putative ILS event in either data set. Again, the 95% HPD intervals of the BEAST analyses were used to account for some uncertainty in the data, and these age estimate ranges were extended with the r8s results when the r8s estimates fell outside of the ranges calculated with BEAST. Finally, the ranges of age estimates for the onset and the end of the putative ILS event were used to calculate its minimum and maximum duration.

Using these estimates of putative ILS durations and information about generation times in the Senecioneae, the effective population sizes needed to explain incongruent patterns invoking ILS were calculated. Most Senecioneae species flower in their first or second year, and generation times of one or two years are characteristic for most lineages. To test for robustness of our conclusions to violations of this assumption, effective population sizes were also calculated using generation times of 5 and 10 yr. Senecioneae populations typically range between several dozen to a few thousand individuals (e.g., Widén and Andersson, 1993; Comes and Abbott, 1998; Müller-Schärer and Fischer, 2001). N_e values of 20 000 (plastid data) and 40 000 (ITS/ETS data) were therefore selected as conservative estimates of maximum population sizes. These coalescent calculations were carried out for each of the strongly incongruent lineages, but were also performed for scenarios in which the conflicting phylogenetic positions of multiple taxa were assumed to be nonindependent (i.e., a single ILS event resulting in incongruent positions of multiple lineages). Because species-level sampling within *Senecio* was limited (48/~1000 species) and accurate estimates of the duration of putative ILS events could not be obtained, these calculations were not performed for *Senecio* species.

RESULTS

Identification and localization of incongruence—Although an ILD test comparing all five plastid markers resulted in $P = 0.005$, the consensus trees obtained from the individual markers did not reveal well-supported (BS values ≥ 80% and/or PP ≥ 0.95) incongruence. Sequence data from all plastid markers were therefore combined into a single data set, which was used for all subsequent analyses. The ITS and ETS trees (not presented) were mostly congruent, and incongruent clades were not well-supported. Further analyses were carried out with a combined ITS/ETS data set, even though an ILD test resulted in $P = 0.004$. The results of the MP and BI analyses of the combined plastid data and the combined ITS/ETS data sets are summarized in Table 2 and presented in Figs. 1 and 2. The ILD test for the plastid vs. the ITS/ETS data sets indicated significant incongruence ($P = 0.004$), and a visual comparison of their topologies and branch support values revealed well-supported incongruence (Figs. 1, 2). Therefore, further studies of incongruence within Senecioneae were confined to examining the strongly conflicting topologies of the plastid vs. the ITS/ETS data sets. Discussion in this paper is limited to relationships within the Senecioneae only and does not include outgroup relationships.

Although all analyses of plastid and ITS/ETS data resolved the core of Senecioneae (excluding *Doronicum* and *Abrotanella*) as monophyletic (Figs. 1, 2; BS 100%, PP 0.99 and 1.00) and supported *Capelio* as sister to the well-supported clade composed of the remaining genera forming the Senecioneae

TABLE 2. Character information and clade support data. In the combined plastid-ITS/ETS data set strongly incongruent taxa were included as separate plastid and ITS/ETS accessions (see text).

Data set	No. of characters	Gap characters	Variable characters	Informative characters	No. of clades with ≥50% BS	≥80% BS	PP ≥0.5	PP ≥0.95
Plastid	12494	1293	3253 (26.0%)	1406 (11.3%)	125 (55.1%)	87 (38.3%)	194 (85.5%)	132 (58.2%)
ITS/ETS	3303	914	1605 (48.6%)	1096 (33.2%)	116 (50.5%)	76 (33.0%)	195 (84.8%)	127 (55.2%)
Combined plastid-ITS/ETS	15797	207	4861 (30.7%)	2505 (15.9%)	158 (56.8%)	100 (36.0%)	248 (89.2%)	180 (64.7%)

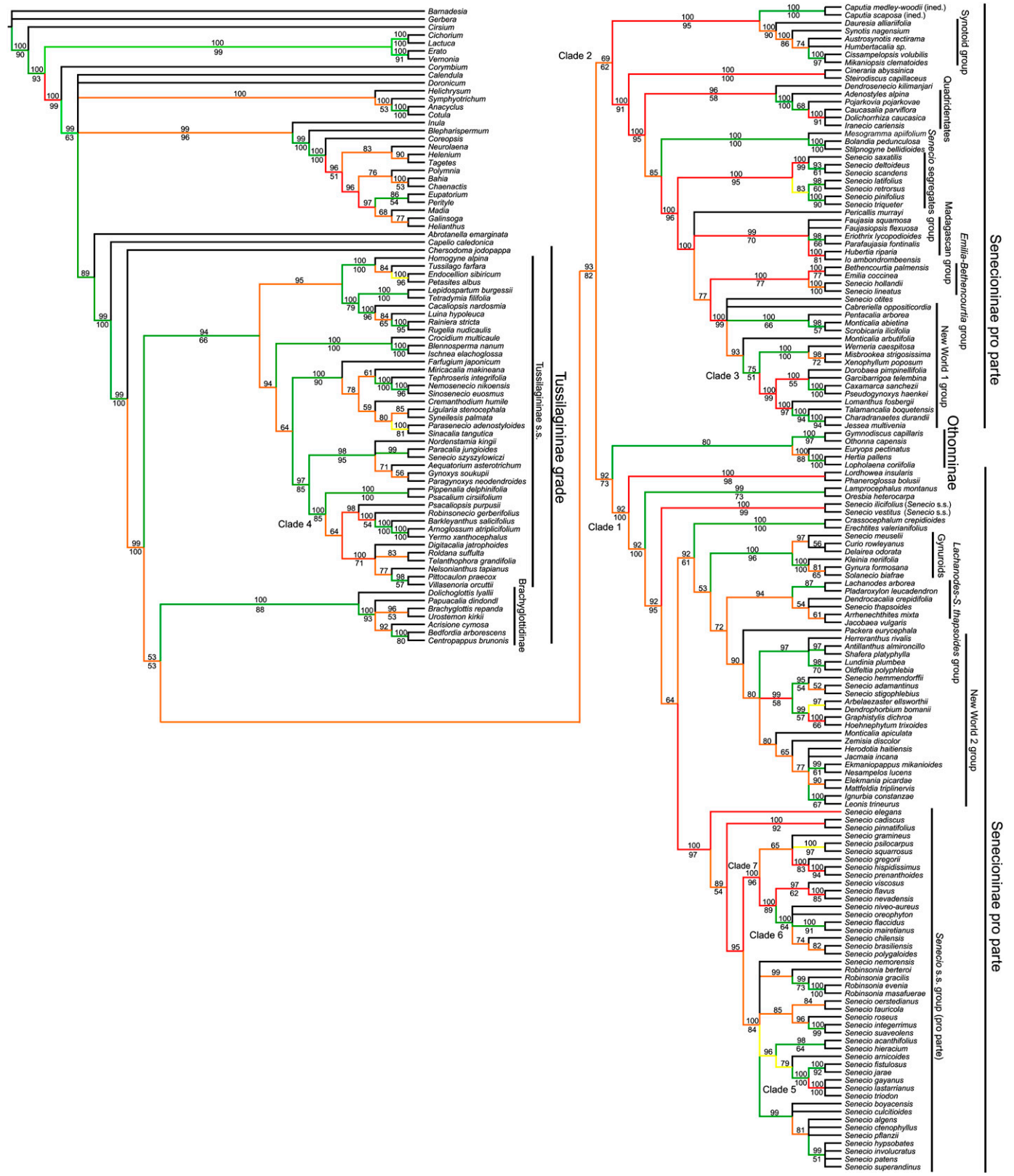

Fig. 1. Bayesian inference (BI) topology from the plastid data set. Bayesian consensus percentages (posterior probabilities × 100) are placed above branches, bootstrap support above 50% below branches. Branch color indicates congruence with ITS/ETS BI tree (Fig. 2): green: congruent; yellow: clade absent in ITS/ETS BI tree, but not contradicted; orange: clade incongruent, but not with posterior probabilities ≥0.95 in both trees; red: incongruent with posterior probabilities ≥0.95 in both trees.

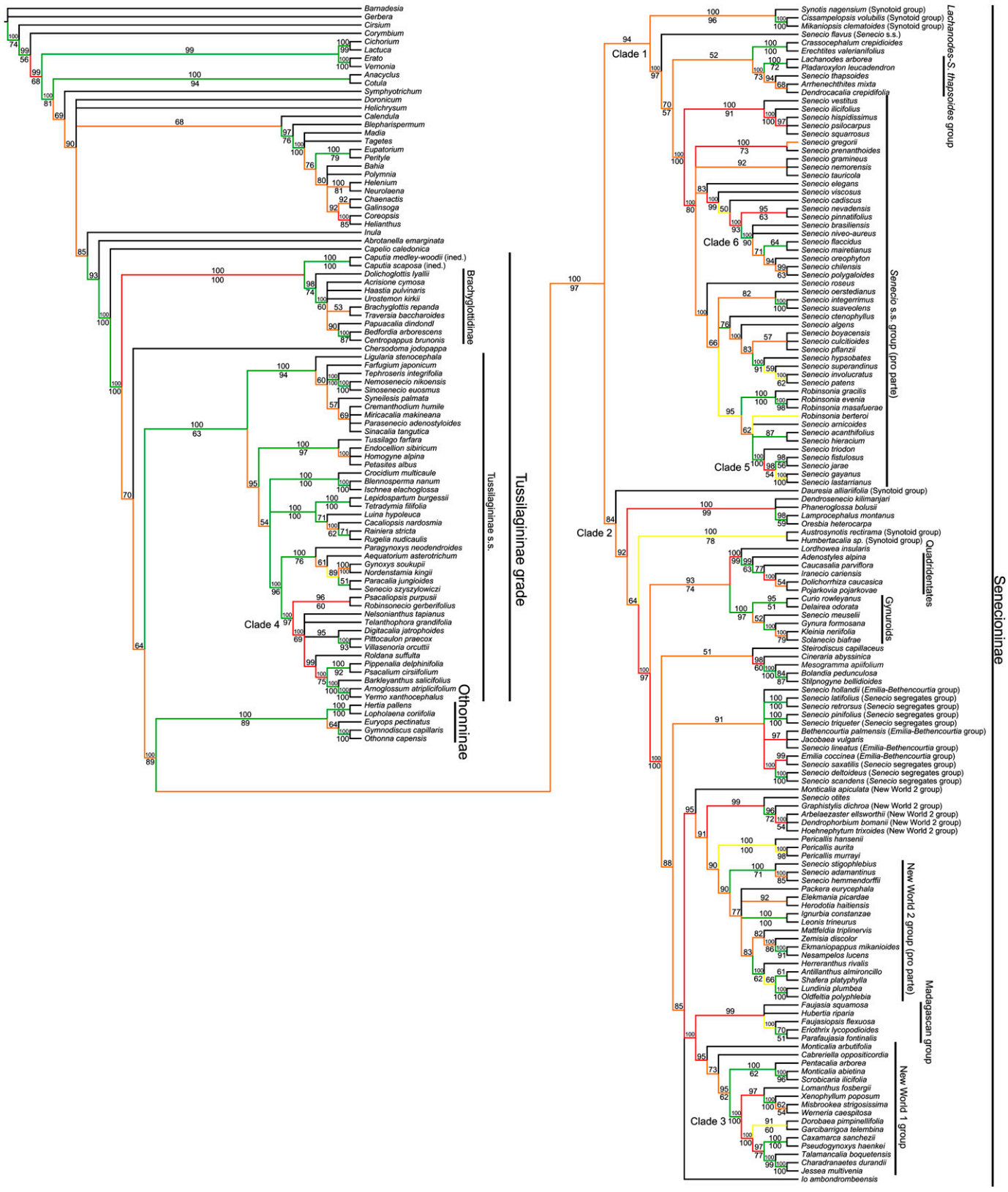

Fig. 2. Bayesian inference (BI) topology from ITS/ETS data set. Bayesian consensus percentages (posterior probabilities × 100) are placed above branches, bootstrap support above 50% below branches. Branch color indicates congruence with plastid BI tree (Fig. 1): green: congruent; yellow: clade absent in plastid BI tree, but not contradicted; orange: clade incongruent, but not with posterior probabilities ≥0.95 in both trees; red: incongruent with posterior probabilities ≥0.95 in both trees.

(BS 100%, PP 0.99 and 1.00), relationships among many of the other taxa are obscured by complex patterns of topological incongruence. Among the 29 major lineages of Senecioneae that were identified (Table 3), only two exhibit strongly supported internal incongruence: the New World 1 group and *Senecio* (Table 3).

Within New World 1 group, incongruence is located in clade 3 (Figs. 1, 2) in which *Lomanthus* and the *Caxamarca-Pseudogynoxys* clade take incongruent positions (Table 3). An ILD test of the data set that was solely composed of the accessions of clade 3 indicated significant incongruence between the plastid and ITS/ETS data ($P = 0.001$). This conflict is not significant ($P = 0.256$) if *Lomanthus* is excluded, but remains statistically significant if only the *Caxamarca-Pseudogynoxys* clade is excluded ($P = 0.001$). Similarly, in a series of MP and BI analyses of a data set in which only the accessions of clade 3 were included, incongruence supported with high BS and PP values only disappeared when *Lomanthus* was excluded (not shown).

Within *Senecio*, *S. flavus*, *S. cadiscus*, *S. pinnatifolius*, *S. hispidissimus*, *S. psilocarpus*, and *S. squarrosus* are placed in strongly supported incongruent positions (Table 3; $P = 0.001$). Removing all six taxa from the data sets resulted in trees without high BS and PP values for incongruent patterns (not shown) and an insignificant ILD value ($P = 0.06$). When any five of these six species were excluded, ILD tests indicated significant incongruence ($P = 0.001$–0.007), and incongruent patterns were supported with high support values (not shown).

In addition to documenting strongly supported internal incongruence for two of the 29 major Senecioneae lineages, 13 others exhibit well-supported incongruence with regard to their phylogenetic positions relative to the 16 remaining lineages among which well-supported incongruence was not identified (Table 4). These 13 incongruent lineages are *Caputia*, New World 2 group, *Jacobaea*, *Lordhowea*, gynuroids, *Lamprocephalus-Oresbia* clade, *Phaneroglossa*, *Cineraria*, *Steirodiscus*, *Packera*, *Emilia-Bethencourtia* group, *Senecio otites*, and *Io*. An ILD test in which placeholders for each of the 29 major Senecioneae lineages were included indicated significant incongruence between the plastid and ITS/ETS data (Table 5: test 1; $P = 0.001$). Excluding the placeholders for the 13 lineages that have well-supported incongruent phylogenetic positions still resulted in significant incongruence among the remaining lineages (Table 5: test 2; $P = 0.006$). Only when Othonninae (i.e., *Othonna*) was also excluded, an ILD test did not indicate significant incongruence (Table 5: test 3; $P = 0.052$). Adding placeholders for each of the incongruent major lineages individually to the nonsignificant incongruent data set of test 3 introduced significant incongruence for all of these groups (Table 5: tests 4–13) except for the *Emilia-Bethencourtia* group, *Senecio otites*, and *Io* (tests 14–16). Although adding all three of these placeholders (test 17), the *Emilia-Bethencourtia* group and *Senecio otites* (test 18), or *Senecio otites* and *Io* (test 19), resulted in significant incongruence (Table 5), incongruence was insignificant ($P = 0.013$) in an ILD test in which the *Emilia-Bethencourtia* group and *Io* were added to the insignificantly incongruent selection of placeholder species used in test 3 (test 20). Inspection of the BS and PP values in trees obtained from the data sets used in ILD tests 1–20 generally confirmed these findings (not shown).

In summary, after exploring incongruence within and among the major Senecioneae lineages and following the criteria outlined in the Materials and Methods, we identified the following taxa and lineages as having strongly incongruent placements: *Caputia*, *Cineraria*, *Steirodiscus*, *Phaneroglossa*, the *Lamprocephalus-Oresbia* clade, *Lordhowea*, the gynuroids, *Jacobaea*, *Packera*, *Senecio otites*, *Lomanthus*, the New World 2 group, *S. flavus*, *S. hispidissimus*, *S. psilocarpus*, *S. squarrosus*, *S. cadiscus*, and *S. pinnatifolius*. The MP and BI analyses of the combined plastid-ITS/ETS data set in which the strongly incongruent lineages were included as separate plastid and ITS/ETS accessions resulted in trees that generally have slightly higher support than the individual plastid and ITS/ETS consensus trees (Fig. 3; Table 2). There are only a few topological differences between the MP 50% majority rule consensus tree (not shown) and the BI phylogeny (Fig. 3), and all of these differences are weakly supported. The phylogenetic positions of the plastid and ITS/ETS accessions of the strongly incongruent lineages in the combined plastid-ITS/ETS trees (Fig. 3) correspond well to those in the separate plastid and ITS/ETS trees (Figs. 1, 2).

ITS orthology/paralogy assessment—Cloned ITS sequences selected from 18 strongly incongruent Senecioneae lineages (Appendix S1) revealed a relatively high level of polymorphism. Most specimens had between 3 and 24 (0.4–3.2%) polymorphic nucleotide positions; however, the cloned sequences from *Oresbia* had higher levels (7.8%) with 59 polymorphic nucleotide positions. Despite this high level of polymorphism, sequences of the ITS clones obtained from the same specimen always formed well-supported clades in MP analyses (results not shown) and with the directly sequenced ITS PCR products.

Long-branch attraction—Visual inspection of the plastid and ITS/ETS trees revealed several species in the Tussilagininae s.s. clade with relatively long branches: *Blennosperma*, *Crocidium*, *Ischnea*, *Robinsonecio*, and the *Arnoglossum-Barkleyanthus-Yermo* clade (Appendix S2). Although the relationships among *Blennosperma*, *Crocidium*, and *Ischnea* and between these and other genera in the Tussilagininae s.s. clade are not affected by well-supported incongruence, the relationship between *Robinsonecio* and the *Arnoglossum-Barkleyanthus-Yermo* clade shows incongruence that is well supported in the BI trees. In contrast to being more distantly related in the ITS/ETS phylogenies (Fig. 2), plastid data place *Robinsonecio* sister to the *Arnoglossum-Barkleyanthus-Yermo* clade (Fig. 1). Reciprocal exclusion of *Robinsonecio* and the *Arnoglossum-Barkleyanthus-Yermo* clade (results not shown) in both the ITS/ETS and plastid data sets, however, does not change the relationships in clade 4.

Within Senecioninae, *Emilia*, *Jacobaea*, and *Packera* have branches that are much longer than other genera in this subtribe (Appendix S2). These three genera form a clade in the ITS/ETS MP trees (not shown); however, *Packera* is placed more distant to the other two genera in the ITS/ETS BI trees (Fig. 2). Reciprocal exclusion of two of these three genera from MP analyses (results not presented) resulted in the phylogenetic positions of *Emilia* and *Jacobaea* remaining unchanged when the two other genera were excluded (resp. *Jacobaea* and *Packera*, and *Emilia* and *Packera*). *Emilia* and *Jacobaea* were therefore not affected by long-branch attraction, but when both are excluded, *Packera* occupies a different position in the ITS/ETS trees. Because the results of the BI analyses of the ITS/ETS data agree with the plastid trees in indicating a close relationship between the New

TABLE 3. Identification of the major Senecioneae lineages and analyses of the incongruent patterns within them. Placeholders of the major groups were selected to study incongruent phylogenetic patterns between them (see Table 5).

Major Senecioneae group[a]	Forms clade in		Conflicting relationships within group[b]		Support for internal conflict			Placeholder taxa
	Plastid trees	ITS/ETS trees	Plastid trees (Fig. 1)	ITS/ETS trees (Fig. 2)	BS	PP	ILD test (*P*)	
1. Tussilagininae s.s.	Y	Y	*Arnoglossum-Barkleyanthus-Yermo* clade sister to *Robinsonecio* and *Psacaliopsis* (BS <50%, PP 0.98)	*Arnoglossum-Barkleyanthus-Yermo* clade sister to *Pippenalia-Psacalium* clade (BS 75%, PP 1.00)	N	Y	0.114	*Tephroseris*
			Robinsonecio sister to the *Arnoglossum-Barkleyanthus-Yermo* clade (BS 54%, PP 1.00)	*Psacaliopsis* sister to *Robinsonecio* (BS 60%, PP 0.96)				
			Roldana sister to *Digitacalia*, *Telanthophora*, *Nelsonianthus*, *Pittocaulon*, and *Villasenoria* (BS 71%, PP 1.00)	*Roldana* sister to the *Arnoglossum-Barkleyanthus-Yermo* clade, *Pippenalia*, and *Psacalium* (BS <50%, PP 0.99)				
2. New World 1 group[c]	N	Y	*Lomanthus* sister to a clade formed by *Charadranaetes*, *Jessea*, and *Talamancalia* (BS 97%, PP 1.00)	*Lomanthus* sister to a clade composed of *Misbrookea*, *Werneria*, and *Xenophyllum* (BS <50%, PP 0.97)	Y	Y	0.001	*Caxamarca*
			Caxamarca and *Pseudogynoxys* sister to *Dorobaea* and *Garcibarrigoa* (BS 55%, PP 1.00)	*Caxamarca* and *Pseudogynoxys* sister to the *Charadranaetes-Jessea-Tamancalia* clade (BS 77%, PP 0.97)				
3. Quadridentates	Y	Y	*Caucasalia*, *Dolichorrhiza*, and *Iranecio* form a clade (BS 91%, PP 1.00)	*Dolichorrhiza*, *Iranecio*, and *Pojarkovia* form a clade (BS <50%, PP 1.00)	N	Y	0.022	*Caucasalia*
4. *Senecio*[d]	N	N	*Senecio flavus* is deeply nested within the core of *Senecio* s.s. and a member of a clade composed of *S. nevadensis*, *S. viscosus*, and clade 6 (BS 89%, PP 1.00)	*Senecio flavus* is placed basal to the core of *Senecio* s.s. (BS 100%, PP 1.00)	Y	Y	0.001	*S. viscosus*
			S. cadiscus and *S. pinnatifolius* are not most closely related to *S. nevadensis*, *S. viscosus*, and clade 6, which instead are more closely related to other *Senecio* species and form clade 7 with these (BS 96%, PP 1.00)	*S. cadiscus* and *S. pinnatifolius* are most closely related to *S. nevadensis*, *S. viscosus*, and members of Clade 6 (BS 99%, PP 1.00)				
			S. hispidissimus, *S. psilocarpus*, and *S. squarrosus* are members of clade 7 (BS 96%, PP 1.00)	*S. hispidissimus*, *S. psilocarpus*, and *S. squarrosus* sister to *S. ilicifolius* and *S. vestitus* (BS 91%, PP 1.00)				
Clade 5 within *Senecio* (Figs. 1, 2)	Y	Y	*S. triodon* sister to *S. gayanus* and *S. lastarrianus* (BS 100%, PP 1.00)	*S. triodon* sister to *S. fistulosus, S. gayanus*, *S. jarae*, and *S. lastarrianus* (BS 54%, PP 0.98)	N	Y	0.026	-
5. *Emilia-Bethencourtia* group	Y	N	*Senecio lineatus* sister to *S. hollandii* (BS 100%, PP 1.00) and *Bethencourtia* sister to *Emilia* (BS 77%, PP 1.00)	*Senecio lineatus* sister to *Bethencourtia* (MP: 93%) or is unresolved on a clade with *Bethencourtia* and *Jacobaea* (BI: PP 0.97)	Y	Y	0.015	*Emilia*
6. *Senecio* segregates	Y	N			N	N	0.9998	*S. deltoideus*
7. New World 2 group	Y	N			N	N	0.255	*Dendrophorbium*
8. Brachyglottidinae	Y	Y			N	N	0.709	*Centropappus*
9. Othonninae	Y	Y			N	N	1	*Othonna*
10. *Bolandia-Mesogramma-Stilpnogyne* clade	Y	Y			N	N	1	*Bolandia*
11. Gynuroids	Y	Y			N	N	0.97	*Kleinia*
12. Synotoids	Y	N			N	N	0.157	*Dauresia, Mikaniopsis*
13. Madagascan group	N	Y			N	N	1	*Faujasia*
14. *Lachanodes-Senecio thapsoides* group	N	Y			N	N	1	*Arrhenechthites*
15. *Caputia*	Y	Y			n.a.	n.a.	n.a.	*Caputia medley-woodii* (ined.)
16. *Lamprocephalus-Oresbia* clade	Y	Y			n.a.	n.a.	n.a.	*Lamprocephalus*
17. *Crassocephalum-Erechtites* clade	Y	Y			n.a.	n.a.	n.a.	*Crassocephalum*
18. *Capelio*	n.a.	n.a.			n.a.	n.a.	n.a.	*Capelio*

TABLE 3. Continued.

Major Senecioneae group[a]	Forms clade in		Conflicting relationships within group[b]		Support for internal conflict			
	Plastid trees	ITS/ETS trees	Plastid trees (Fig. 1)	ITS/ETS trees (Fig. 2)	BS	PP	ILD test (*P*)	Placeholder taxa
19. *Chersodoma*	n.a.	n.a.			n.a.	n.a.	n.a.	*Chersodoma*
20. *Cineraria*	n.a.	n.a.			n.a.	n.a.	n.a.	*Cineraria*
21. *Dendrosenecio*	n.a.	n.a.			n.a.	n.a.	n.a.	*Dendrosenecio*
22. *Io*	n.a.	n.a.			n.a.	n.a.	n.a.	*Io*
23. *Jacobaea*	n.a.	n.a.			n.a.	n.a.	n.a.	*Jacobaea*
24. *Lordhowea*	n.a.	n.a.			n.a.	n.a.	n.a.	*Lordhowea*
25. *Pericallis*	n.a.	n.a.			n.a.	n.a.	n.a.	*Pericallis*
26. *Phaneroglossa*	n.a.	n.a.			n.a.	n.a.	n.a.	*Phaneroglossa*
27. *Steirodiscus*	n.a.	n.a.			n.a.	n.a.	n.a.	*Steirodiscus*
28. *Senecio otites*	n.a.	n.a.			n.a.	n.a.	n.a.	*Senecio otites*
29. *Packera*	n.a.	n.a.			n.a.	n.a.	n.a.	*Packera*

Notes: BS = incongruent clades supported with maximum parsimony bootstrap support ≥80% in plastid and ITS/ETS trees; PP = incongruent clades supported with Bayesian inference posterior probabilities ≥0.95 in plastid and ITS/ETS trees; groups in boldface indicate strong internal incongruence according to the criteria used in this study (incongruent patterns supported by bootstrap values ≥80% *and/or* PP ≥0.95 *and* ILD values of $P < 0.01$).

[a]Species/generic composition outlined in Figs. 1 and 2.

[b]Only shown for groups in which incongruent relationships were supported with either MP bootstrap support ≥80% or BI posterior probabilities ≥0.95

[c]Internal incongruence for the phylogenetic position of *Lomanthus*.

[d]Internal incongruence for the phylogenetic positions of *Senecio flavus*, *S. hispidissimus*, *S. psilocarpus*, *S. squarrosus*, *S. cadiscus*, and *S. pinnatifolius*.

World 2 group and *Packera*, *Packera* may very well be a member of the New World 2 group.

Incomplete lineage sorting—Molecular dating analyses using r8s yielded age estimates that were often older than ages retrieved with BEAST (Figs. 4 and 5) and sometimes outside the 95% HPD interval of the BEAST estimates (Appendix S3). The 95% HPD intervals of the plastid and ITS/ETS analyses were often overlapping. Estimates of the duration of putative ILS events obtained with r8s are within or slightly outside the ranges suggested by BEAST. Using these estimates and assuming generation times of 1, 2, 5, and 10 yr, coalescent analyses indicate that for four of the 10 strongly incongruent lineages that were examined (*Caputia*, the *Lamprocephalus-Oresbia* clade, the New World 2 generic group and *Packera*, and *Jacobaea*) effective population sizes must be assumed that are higher than the selected thresholds of $N_e = 20\,000$ (plastid data) and 40 000 (ITS/ETS data) to explain their incongruent phylogenetic positions by ILS (Table 6). If multiple strongly incongruent phylogenetic positions of lineages are assumed to be the result of single ILS events, even higher effective population sizes of ancestral lineages are required (not shown).

DISCUSSION

Topological incongruence—One of the longstanding and increasingly prominent questions of phylogenetic systematics is how to address incongruence between phylogenies obtained from multiple data sets and how to determine its cause (e.g., Sullivan, 1996; Wiens and Hollingsworth, 2000; Rokas et al., 2003; Holland et al., 2008; Degnan and Rosenberg, 2009). Incongruence between phylogenetic trees inferred using different molecular markers may result from biological phenomena as well as analytical artifacts. Among the potential causes are undetected paralogous sequences in one or more data sets (Doyle, 1992; Álvarez and Wendel, 2003); however, topological incongruence may also be found when different genomes or portions of individual genomes may have different evolutionary histories (e.g., due to hybridization or ILS; Doyle, 1992; Maddison, 1997; Buckley et al., 2006; Liu and Pearl, 2007). Phylogeny reconstruction methods may also introduce error via long-branch attraction (Graybeal, 1998; Wiens and Hollingsworth, 2000), which can be particularly problematic in data sets with a relatively sparse taxon sampling and composed of highly divergent sequences. In addition, incongruence may result from errors in phylogeny reconstruction due to low numbers of informative characters (sampling error; Huson and Bryant, 2006). Bootstrap values and posterior probabilities are often used as measures of sampling error (Huson and Bryant, 2006). The presence of well-supported (BS values ≥ 80% and/or PP ≥ 0.95) incongruent patterns in the Senecioneae therefore suggests that these patterns are not due to sampling error.

Due to the multicopy nature of ribosomal DNA, differences among ITS, ETS, and plastid trees may be due to incorrect ITS/ETS homology assessments, confounding interpretations essentially through undetected paralogous copies (Álvarez and Wendel, 2003). Cloning of ITS PCR products of incongruent taxa or representatives of incongruent lineages indeed revealed base-pair differences among ITS copies within individual samples. Parsimony analyses in which both directly sequenced PCR products and sequences of ITS clones were included (results

TABLE 4. Well-supported (BS ≥ 80% and/or PP ≥ 0.95) incongruent patterns among genera and major Senecioneae generic assemblages.

Lineage	Plastid trees	ITS/ETS trees
Caputia	Sister to the synotoids (BS 95%, PP 1.00)	Sister to the Brachyglottidinae (BS 100%, PP 1.00)
New World 2 group	Nested among core members of clade 1 (BS 100%, PP 0.92), not among core members of clade 2 (BS 95%, PP 1.00)	Nested among core members of clade 2, except *Dendrosenecio* (BS 97%, PP 1.00)
Jacobaea	Nested among core members of clade 1 (BS 100%, PP 0.92), not among core members of clade 2 (BS 95%, PP 1.00)	Nested among core members of clade 2, except *Dendrosenecio* (BS 97%, PP 1.00)
Packera	Nested among core members of clade 1 (BS 100%, PP 0.92), not among core members of clade 2 (BS 95%, PP 1.00)	Nested among core members of clade 2, except *Dendrosenecio* (BS 97%, PP 1.00)
Lordhowea	Nested among core members of clade 1 (BS 100%, PP 0.92), not among core members of clade 2 (BS 95%, PP 1.00). Sister to *Phaneroglossa* (BS 98%, PP 1.00)	Nested among core members of clade 2, except *Dendrosenecio* (BS 97%, PP 1.00). Sister to the Gynuroids, *Jacobaea*, *Packera*, the New World 2 group and the core groups of clade 2 in which they are nested (BS 97%, PP 1.00)
Gynuroids	Nested among core members of clade 1 (BS 100%, PP 0.92), not among core members of clade 2 (BS 95%, PP 1.00)	Nested among core members of clade 2, except *Dendrosenecio* (BS 97%, PP 1.00)
Lamprocephalus-Oresbia clade	Nested among core members of clade 1 (BS 100%, PP 0.92), not among core members of clade 2 (BS 95%, PP 1.00). Sister to the Gynuroids, *Jacobaea*, *Packera*, the New World 2 group and the core groups of Clade 1 nested between them (BS 100%, PP 0.92)	Sister to *Dendrosenecio* (core member of clade 2) and *Phaneroglossa* (BS 99%, PP 1.00)
Phaneroglossa	Nested among core members of clade 1 (BS 100%, PP 0.92), not among core members of clade 2 (BS 95%, PP 1.00)	Most closely related to *Dendrosenecio* (core member of clade 2) and the *Lamprocephalus-Oresbia* clade (BS 99%, PP 1.00)
Cineraria	Core member of clade 2. Not placed in the clade composed of *Dendrosenecio*, the Quadridentates, New World 1 group, the Madagascan group, Io, the *Emilia-Bethencourtia* group, the *Senecio* segregates group, *Pericallis*, the *Bolandia-Mesogramma-Stilpnogyne* clade, and *Senecio otites* (BS 95%, PP 1.00)	Forms a clade with, among others, New World 1 group, the Madagascan group, *Io*, the *Emilia-Bethencourtia* group, the *Senecio* segregates group, *Pericallis*, the *Bolandia-Mesogramma-Stilpnogyne* clade, and *Senecio otites* (BS 100%, PP 1.00)
Steirodiscus	Core member of clade 2. Not placed in the clade composed of *Dendrosenecio*, the Quadridentates, New World 1 group, the Madagascan group, *Io*, the *Emilia-Bethencourtia* group, the *Senecio* segregates group, *Pericallis*, the *Bolandia-Mesogramma-Stilpnogyne* clade, and *Senecio otites* (BS 95%, PP 1.00)	Forms a clade with, among others, New World 1 group, the Madagascan group, *Io*, the *Emilia-Bethencourtia* group, the *Senecio* segregates group, *Pericallis*, the *Bolandia-Mesogramma-Stilpnogyne* clade, and *Senecio otites* (BS 100%, PP 1.00)
Emilia-Bethencourtia group	Core member of clade 2. Sister to *Pericallis*, the New World 1 group, *Senecio otites*, *Io*, and the Madagascan group (BS <50%, PP 1.00)	Core member of clade 2. Sister to species of the *Senecio segregates* group of which *S. deltoideus*, *S. scandens*, and *S. saxatilis* form a clade with *Emilia* (BS <50%, PP 1.00)
Senecio otites	Core member of clade 2. Sister to the New World 1 group (BS 99%, PP 1.00)	Core member of clade 2. Sister to *Pericallis* and the New World 2 group (BS <50%, PP 0.95)
Io	Core member of clade 2. Forms a clade with the Madagascan group (BS 70%, PP 0.99)	Core member of clade 2. Does not form a clade with the Madagascan group, which instead is sister to the New World 1 group (BS <50%, PP 1.00)

not shown), however, showed that all cloned sequences of a taxon consistently form one clade including the directly sequenced ITS products of the same taxon. Similarly, for species and genera for which ITS sequences obtained from different specimens or species were included in our data sets, these different accessions were always resolved as each other's closest relatives. Unless copies of greater divergence have been lost or remained undiscovered (a possibility that cannot be confirmed nor excluded), it is unlikely that paralogy is the cause of the widespread ITS/ETS vs. plastid incongruence found in the Senecioneae.

Long-branch attraction (Felsenstein, 1978) is another possible explanation for the topological incongruence observed. Reciprocal exclusion of accessions with long branches in MP and BI analyses showed that only the phylogenetic position of *Packera* in the ITS/ETS MP trees appears to be affected by long-branch attraction. Our results indicate that this is caused by the long branches of *Emilia* and *Jacobaea*. Long-branch attraction, however, does not explain the plastid vs. ITS/ETS incongruence regarding the position of *Packera*, which persists when *Emilia* and *Jacobaea* are excluded from the MP analyses (not shown). Although more detailed, tree-wide analyses aimed at identifying branches that may be susceptible to long-branch attraction have not been performed (e.g., Huelsenbeck, 1997; Wiens and Hollingsworth, 2000; Johnson et al., 2008), visual comparisons of MP and BI trees (the latter claimed to be less susceptible to long-branch attraction; e.g., Bergsten, 2005) did not reveal well-supported incongruence related to strongly supported incongruent patterns between plastid and ITS/ETS trees.

Hybridization and ILS are important biological explanations for incongruence between data sets and are often difficult to distinguish from each other (Doyle, 1992; Seelanan et al., 1997; Holder et al., 2001; Buckley et al., 2006; Holland et al., 2008; Joly et al., 2009). The detection of hybrids can for instance be obscured by backcrossing, introgression, extinction of parental species, and secondary hybridization (Doyle, 1992). Hybridization events may also be difficult to recognize when they result in homoploid hybrids, are ancient, or were followed by speciation or dispersal in combination with extinction in the parental distribution area. Hybridization has been demonstrated within many Senecioneae genera (e.g., *Bedfordia*, *Blennosperma*, *Brachyglottis*, *Cineraria*, *Crassocephalum*, *Dendrosenecio*, *Dolichoglottis*, *Emilia*, *Euryops*, *Farfugium*, *Hubertia*, *Jacobaea*, *Jessea*, *Ligularia*, *Packera*, *Petasites*, *Senecio*, *Traversia*; Nordenstam, 1963, 1968, 1978, 1996; Ornduff, 1964;

TABLE 5. Incongruence length difference (ILD) tests performed to study incongruent patterns among placeholders of the major Senecioneae groups (see Table 3).

ILD test	Accessions included	ILD test results (*P*)
Test 1	Placeholders of all major groups	0.001
Test 2	Placeholders of all major groups, except for those in well-supported incongruent phylogenetic positions (BS ≥80% and/or PP ≥0.95; *Caputia*, the New World 2 group, *Jacobaea*, *Lordhowea*, gynuroids, *Lamprocephalus-Oresbia* clade, *Phaneroglossa*, *Cineraria*, *Steirodiscus*, *Emilia-Bethencourtia* group, *Senecio otites*, *Io*, and *Packera*)	0.006
Test 3	Placeholders of test 2 minus placeholder of Othonninae	0.052
Test 4	Placeholders of test 3 plus placeholder of *Caputia*	0.001
Test 5	Placeholders of test 3 plus placeholder of New World 2 group	0.001
Test 6	Placeholders of test 3 plus *Jacobaea*	0.001
Test 7	Placeholders of test 3 plus *Lordhowea*	0.001
Test 8	Placeholders of test 3 plus placeholder of the Gynuroids	0.001
Test 9	Placeholders of test 3 plus placeholder of the *Lamprocephalus-Oresbia* clade	0.001
Test 10	Placeholders of test 3 plus *Phaneroglossa*	0.001
Test 11	Placeholders of test 3 plus *Cineraria*	0.002
Test 12	Placeholders of test 3 plus *Steirodiscus*	0.001
Test 13	Placeholders of test 3 plus *Packera*	0.001
Test 14	Placeholders of test 3 plus placeholder of the *Emilia-Bethencourtia* group	0.084
Test 15	Placeholders of test 3 plus *Senecio otites*	0.024
Test 16	Placeholders of test 3 plus *Io*	0.020
Test 17	Placeholders of test 3 plus placeholder of the *Emilia-Bethencourtia* group, *Io*, and *Senecio otites*	0.001
Test 18	Placeholders of test 3 plus placeholder of the *Emilia-Bethencourtia* group and *Senecio otites*	0.001
Test 19	Placeholders of test 3 plus *Io* and *Senecio otites*	0.006
Test 20	Placeholders of test 3 plus *Io* and placeholder of the *Emilia-Bethencourtia* group	0.013

Chapman and Jones, 1971; Drury, 1973; Olorode and Olorunfemi, 1973; Jeffrey, 1986; Yamaguchi and Yahara, 1989; Beck et al., 1992; Lowe and Abbott, 2000; Kirk et al., 2004; Kadereit et al., 2006; Cron et al., 2008; Pan et al., 2008; Vanijajiva and Kadereit, 2009) and is therefore a likely hypothesis for explaining incongruence between plastid and ITS/ETS phylogenies.

ILS is especially likely when species rapidly radiate and population sizes are large (Maddison, 1997). Because of the stochastic nature of the coalescence process, ILS may yield gene trees with random patterns of relationships among taxa (Buckley et al., 2006), which may result in gene tree–species tree incongruence. Among other methods, gene tree parsimony (Page and Charleston, 1997) or Bayesian hierarchical model approaches based on coalescent theory (Liu and Pearl, 2007) can be used to reconstruct species trees from gene trees that are incongruent due to lineage sorting. However, these approaches do not account for hybridization (Liu and Pearl, 2007), which can result in similar phylogenetic patterns (Buckley et al., 2006). Using coalescent-based approaches, we can distinguish ILS from hybridization by testing whether patterns of incongruence are random (ILS) or nonrandom (hybridization; Buckley et al., 2006). These studies, however, require more than two unlinked genomic data sets to distinguish between both hypotheses (Buckley et al., 2006). Because our Senecioneae data were composed of only two unlinked data sets (ITS/ETS region and the plastid markers), this approach could not be used to test whether patterns of incongruence in the Senecioneae are random or not. Instead, we used the assumption that ancestral polymorphisms coalesce within approximately $5N_e$ generations (Rosenberg, 2003; Degnan and Rosenberg, 2009) to assess whether ILS may be regarded as a plausible explanation for the observed incongruence. This approach requires information about generation times and the duration of the ILS events that could be invoked to explain incongruent patterns, as well as effective population sizes in the Senecioneae. Because of the relatively ancient nature of many of the historical events resulting in the incongruence and the diversity and enormity of the tribe, making assumptions about effective population sizes and generation times of ancestral lineages is somewhat precarious. Furthermore, calculations of the duration of putative ILS events rest on our molecular dating analyses, which resulted in large 95% HPD intervals of age estimates and substantial differences between the dating results of the BEAST and r8s analyses and between estimates obtained from plastid and ITS/ETS sequences. We therefore used a conservative approach in estimating these parameters. Coalescence calculations were performed with generation times of 1 and 2 yr, which are assumed to be typical for most Senecioneae species. In some species, however, longer generation times are expected, particularly in species with a tree-like (e.g., *Aequatorium*, *Brachyglottis*, *Dendrosenecio*, *Lachanodes*, *Nordenstamia*, *Pladaroxylon*) or succulent (e.g., *Curio*, *Kleinia*, *Othonna*) habit or those forming seed banks (e.g., *Euryops annuus*, *Jacobaea vulgaris*). Calculations were therefore also carried out for generation times of 5 and 10 yr. Senecioneae populations are generally small, with individuals countable in dozens or hundreds, or containing up to 5000 plants (e.g., Widén and Andersson, 1993; Comes and Abbott, 1998; Golden, 1999; Panero et al., 1999; Müller-Schärer and Fischer, 2001). More rarely, larger populations are observed, which may comprise several hundreds of thousands of plants (e.g., *Euryops annuus*). Although few estimates of N_e of plant populations based on genetic data have been published (Siol et al., 2007), N_e/N (N being the census number of reproductive individuals) ratios of approximately 5–10% have been suggested as typical (Siol et al., 2007). The selected values of $N_e = 40\,000$ for the ITS/ETS data and $N_e = 20\,000$ for the plastid data (due to the 1/2 reduction in effective population size necessary for the plastid markers relative to the nuclear markers in hermaphroditic individuals) are therefore conservative estimates of

Fig. 3. Bayesian inference (BI) topology from the combined plastid-ITS/ETS data set in which each strongly incongruent taxon (in bold) is included twice: once as a plastid-only accession (ITS/ETS sequences coded as missing data) and once as an ITS/ETS-only accession (plastid sequences coded as missing data). Bayesian consensus percentages (posterior probabilities × 100) are placed above branches, bootstrap support above 50% below branches. Relationships between the outgroup species are not shown.

Fig. 4. Plastid BEAST chronogram. Strongly incongruent taxa and groups of taxa in boldface.

maximum effective population sizes. To accommodate for uncertainty in the calculations of the duration of ILS events, the coalescent analyses were performed with both minimum and maximum durations using the 95% HPD intervals of the BEAST estimates extended with the occasional outliers from the r8s analyses.

Although ILS could not be eliminated as a possible explanation for the incongruent phylogenetic positions for six of the 10 strongly incongruent lineages that were examined, our coalescent analyses were able to demonstrate that ILS cannot be invoked to explain incongruence regarding the relationships of *Caputia*, the *Lamprocephalus-Oresbia* clade, the New World 2

Fig. 5. ITS/ETS BEAST chronogram. Strongly incongruent taxa and groups of taxa in boldface.

TABLE 6. Results of the coalescent analyses in which effective population (N_e) sizes were calculated for a selection of the strongly incongruent lineages assuming that the incongruent patterns were caused by incomplete lineage sorting (ILS). Coalescence was assumed to occur within $5N_e$ generations, and calculations were performed for generation times of 1, 2, 5, and 10 yr. Estimates of the duration of putative ILS events were calculated with BEAST and r8s (Figs. 4, 5).

Strongly incongruent lineage	Data set	Duration of putative ILS (Myr)	N_e (×1000) for assumed generation times			
			1 yr	2 yr	5 yr	10 yr
Caputia	Plastid	2.26–11.97	452–2394	226–1197	90.4–478.8	45.2–239.4
	ITS/ETS	3.73–14.28	746–2856	373–1428	149.2–571.2	74.6–285.6
Gynuroids	Plastid	4.22–9.3	844–1860	422–930	168.8–372	84.4–186
	ITS/ETS	1.7–8.23	340–1646	170 to 823	68–329.2	34–164.6
Lordhowea	Plastid	0–5.41	up to 1082	up to 541	up to 216.4	up to 108.2
	ITS/ETS	6.67–11.43	1334–2286	667–1143	266.8–457.2	133.4–228.6
Phaneroglossa	Plastid	0–5.41	up to 1082	up to 541	up to 216.4	up to 108.2
	ITS/ETS	5.98–13.13	1196–2626	598–1313	239.2–525.2	119.6–262.6
Lamprocephalus and *Oresbia*	Plastid	1.03–6.97	206–1394	103–697	41.2–278.8	20.6–139.4
	ITS/ETS	5.95–13.62	1190–2724	595–1362	238–544.8	119–272.4
New World 2 group and *Packera*	Plastid	4.7–9.79	940–1958	470–979	188–391.6	94–195.8
	ITS/ETS	2.84–7.77	568–1554	284–777	113.6–310.8	56.8–155.4
Jacobaea	Plastid	8.08–13.38	1616–2676	808–1338	323.2–535.2	161.6–267.6
	ITS/ETS	5.29–12.41	1058–1482	529–1241	211.6–496.4	105.8–148.2
Cineraria and *Steirodiscus*	Plastid	0–3.45	up to 690	up to 345	up to 138	up to 69.0
	ITS/ETS	0.06–8.37	12–1647	6–837	2.4–334.8	1.2–164.7
Lomanthus	Plastid	1.66–5.64	332–1128	166–564	66.4–225.6	33.2–112.8
	ITS/ETS	0–1.39	up to 278	up to 139	up to 55.6	up to 27.8
Senecio otites	Plastid	1.05–6.3	210–1260	105–630	42–252	21.0–126.0
	ITS/ETS	0–2.98	up to 596	up to 298	up to 119.2	up to 59.6

generic group and *Packera*, and *Jacobaea* (Table 6). To result in the incongruent patterns, ILS events must have continued for at least 1.03 (*Lamprocephalus-Oresbia* clade) to 5.29 (*Jacobaea*) Myr with effective population sizes never falling below 20 600 (*Lamprocephalus-Oresbia* clade) to 105 800 (*Jacobaea*) individuals. This is extremely improbable considering the present day population sizes of Senecioneae species and the likely occurrence of bottlenecks relative to speciation events throughout the evolutionary history of these lineages. For these four lineages, ancient hybridization is a much more likely explanation, although additional morphological, karyological, and molecular studies will need to be performed to identify direct evidence for hybrid origins.

Conclusions—This study reveals new insights into the evolutionary history of Senecioneae by demonstrating strongly supported incongruence between plastid and ITS/ETS phylogenies. This incongruence is found at various taxonomic levels and affects the phylogenetic positions of six *Senecio* species, nine genera (*Caputia*, *Cineraria*, *Steirodiscus*, *Phaneroglossa*, *Lordhowea*, *Jacobaea*, *Packera*, *Senecio otites*, and *Lomanthus*), and three generic assemblages (the *Lamprocephalus-Oresbia* clade, the gynuroids, and the New World 2 group). Although the emphasis of this study has been on understanding the incongruence between plastid and ITS/ETS trees, it is important to note that our analyses also support many phylogenetic patterns that are congruent or that are only weakly incongruent in the Senecioneae. In fact, the plastid data confirms many of the taxonomic and phylogenetic conclusions that were drawn from the ITS/ETS trees presented in this study and the ITS trees in Pelser et al. (2007). For example, except for *Caputia*, the subtribal delimitation of Senecioneae identified with ITS sequences (Pelser et al., 2007) remains largely unaffected by the incongruence, well-supported generic-level incongruence was not found within subtribes Brachyglottidinae, Tussilagininae s.s., and Othonninae, and plastid data provided additional support for the new delimitation of *Senecio* proposed by Pelser et al. (2007). Therefore, the congruent patterns found in this comprehensive study of the large tribe Senecioneae provide a framework (Fig. 3) for future research focused on the incongruent phylogenetic positions of its lineages.

In addition to a better understanding of the patterns of phylogenetic incongruence and the taxa involved, our studies also provided information about the potential causes of topological incongruence in the Senecioneae. The dense generic-level sampling used in our analyses, the lack of evidence for ITS/ETS paralogy issues, and the paucity of long-branch attraction associated with the incongruent taxa suggest that ILS and/or hybridization are the most likely causes of the plastid vs. ITS/ETS incongruence. Using a novel approach based on coalescent-theory, we were able to show that ILS is unlikely to be responsible for four of the 10 strongly incongruent patterns that were observed. Our study therefore indicates that this approach may be a valuable tool for testing whether ILS can be invoked as an explanation of phylogenetic incongruence.

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