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ORIGINAL ARTICLE

Molecular phylogeny of *Nassauvia* (Asteraceae, Mutisieae) based on nrDNA ITS sequences

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Abstract The phylogeny of the genus *Nassauvia* and closely related genera was reconstructed using sequences from the internal transcribed spacer regions (ITS) of nuclear ribosomal DNA. The genus *Triptilion* is nested within *Nassauvia*, making the latter genus paraphyletic. Neither of the two subgenera *Nassauvia* and *Strongyloma* is resolved as monophyletic, and none of the sections of subgenus *Nassauvia* is recovered as monophyletic. The evolution of the compound secondary inflorescences has been complex in *Nassauvia*, with the highly aggregated forms representing the original condition in the genus. However, the ancestral condition is equivocal in several clades, and there are alternative reconstructions for the gains–losses of the variously aggregated conditions. There has been at least one gain of solitary capitula in *Nassauvia*. The evolution of flavonoid chemistry has been complex in *Nassauvia*, and flavonoids are of limited phylogenetic-taxonomic utility in the genus. Gains–losses of flavonols occur only on terminals whereas changes in flavones and C-glycosyl flavones occur at various levels in the tree. Gains–losses of methylation of flavones and flavonols occur only on terminals.

Keywords Asteraceae · Mutisieae · *Nassauvia* · Flavonoid · Phylogeny · Pseudocephalia evolution

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Introduction

Anyone studying the flora of southern South America can hardly overlook the morphologically striking genus *Nassauvia* Comm. ex Jussieu (Asteraceae, Mutisieae, Nassauviinae) with its unusual clusters of flowering heads. *Nassauvia* consists of 38 species (Cabrera 1982; Arroyo and Marticorena 1988) distributed in southern South America, especially along the Andes, in Patagonia, and on the Malvinas (Falkland Islands). It is the type genus of subtribe Nassauviinae, which contains about 24 genera (Cabrera 1971), and it has been previously suggested as being closely related to *Calopappus*, *Moscharia*, *Polyachyrus*, and *Triptilion* (Crisci 1974; Bremer 1993). Although studies on the morphology (Lessing 1832; de Candolle 1838; Bentham 1873; Cabrera 1982), phenetics (Crisci 1974), and morphological cladistics (Freire et al. 1993) of *Nassauvia* have already been published, comprehensive molecular phylogenetic insights are lacking. Katinas et al. (2008a, b) provide a broad overview of relationships within Nassauviinae using nuclear and plastid sequences, but given the objective of their study, only four species of *Nassauvia* were included.

Previous attempts have been made to estimate phylogenetic relationships within *Nassauvia* from morphology. On the basis of intuitive assessment of morphological characters and states, Cabrera (1982), in his revision of the

genus, described evolutionary relationships among infrageneric taxa. He differentiated the primitive section *Panargyrum* from the more derived sections *Mastigophorus*, *Nassauvia*, and *Caloptilium*. He also suggested that subgenus *Strongyloma* was sister to subgenus *Nassauvia*. Hunziker et al. (1991), focusing on morphology and chromosome numbers ($x = 11$ in *Nassauvia*), suggested that the genus evolved from *Calopappus*, and that section *Mastigophorus* might be the most primitive. *Triptilion* was suggested as having evolved from section *Panargyrum*.

The most comprehensive morphological phylogenetic analysis of *Nassauvia* was conducted by Freire et al. (1993), who sampled all species of *Nassauvia* and *Triptilion*, *Caloppapus*, *Moscharia*, and *Polyachyrus* for 35 morphological characters. The more distant *Cephalopappus* of subtribe Nassauviinae was used as outgroup. The results supported in large measure the classification of Cabrera (1982), except that section *Panargyrum* was recovered as paraphyletic. Furthermore, *Calopappus* was nested within *Nassauvia* rendering the latter genus paraphyletic. Subgenus *Strongyloma* was resolved as the sister group of subgenus *Nassauvia.*

Because of the striking variation of the types of synflorescence in *Nassauvia*, ranging from single heads to large clusters, some previous discussions have been directed to understanding the evolution of these types. Stuessy et al. (1988; unpublished work) suggested that single capitula, for example in the closely related genus *Calopappus*, may have evolved the small heads typical of section *Mastigophorus* followed by smaller aggregations of individual heads as in sections *Panargyrum* and *Strongyloma*, culminating in the dense aggregations of sections *Caloptilium* and *Nassauvia*.

A putative environmental correlation was observed in the morphological trend from more open secondary inflorescences (synflorescences) to tighter and more conspicuous aggregations in going from the lower and warmer regions to the higher and colder Andes. Plotting synflorescence types on the morphologically-based cladogram of Freire et al. (1993) showed tendencies for more dense synflorescence aggregations to occur in more highly derived clades, but with several notable exceptions (e.g., *N. juniperus*, *N. pentachaeta* and *N. aculeata*; Fig. 6 in Freire et al. 1993). Tortosa et al. (2004) and Katinas et al. (2008a, b) argued that the compact synflorescence is the ancestral condition in *Nassauvia* and that more lax inflorescences are the derived condition. Katinas et al. (2008a, b) suggested that the general trend has been from complex secondary head type in more arid conditions to loss of complexity associated with the ecological transition to more mesic conditions.

Flavonoid compounds have been shown to be of taxonomic utility at different levels throughout the Asteraceae (Bohm and Stuessy 2001), but there are relatively few studies of tribe Mutisieae (Bohm and Stuessy 2001, Chap. 14). A survey of the distribution of flavonoid compounds in the leaves of species of *Nassauvia* was carried out to ascertain whether specific classes of compounds characterize species, sections, or subgenera, and to trace the distribution of compounds on the molecular phylogeny to interpret evolution of the compounds within the genus.

Therefore, the purposes of this paper are:

1 to estimate a molecular phylogeny of *Nassauvia* on the basis of sequences of nuclear ribosomal ITS, including a test of the monophyly of the genus;
2 to compare the new phylogenetic inferences with morphological hypotheses;
3 to analyze the implications of all data for infrageneric and intergeneric relationships;
4 to comment on the evolution of synflorescences within the genus; and
5 to interpret the pattern of evolution of leaf flavonoid chemistry.

Materials and methods

Taxon sampling To test the monophyly of *Nassauvia*, we constructed a data matrix of 54 total species, including 23 species (24 accessions) of *Nassauvia*, 29 species from 8 other genera of Mutiseae, and *Schlechtendalia luzulifolia* (Barnadesiodeae) as an outgroup (Table 1). We included in this sample several genera (i.e., *Calopappus*, *Polyachyrus* and *Triptilion*) considered closely related to *Nassauvia* on the basis of analyses of morphological and molecular characters (Crisci 1974; Bremer 1993; Katinas et al. 2008a, b).

Molecular methods

Amplification and DNA sequencing Total DNA was extracted from herbarium specimens in accordance with either the CTAB procedure (Doyle and Doyle 1990) or by use of the DNeasy Plant Mini Kit from Qiagen (Valencia, CA, USA). The nrDNA ITS regions were amplified by use of primers 4 and 5 from White et al. (1990). The double-stranded PCR products were produced via 35 cycles of 95°C for 1 min, 48°C for 1 min, and 72°C for 1 min, with a 10-min final extension cycle at 72°C.

PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen) and sequenced using the same primers in 10 µl reactions using 2 µl BigDye, 1 µl primer (20 pmolar), and template DNA and purified water to reach the reaction volume. Cycle sequencing used 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min.

Table 1 Collections of *Nassauvia* and related Mutisieae genera analyzed in this study

Taxon	Authority	Collection no.	GenBank accession no.
Actinoseris stenophylla	Cabrera	Roque, 482	AF546085
Actinoseris sp.		Roque, 493	AF546086
Calopappus acerosus	Meyen	Stuessy, 12744	EU239276
Chaetanthera	Ruiz & Pav.		
C. acerosa	(J. Rémy) Benth. & Hook. F.	Arroyo et al., 25077	DQ355905
C. apiculata	(J. Rémy) F. Meigen	Arroyo et al., 25244	DQ355910
C. ciliata	Ruiz & Pav.	Arroyo et al., 25002	DQ355887
C. glabrata	(DC) F. Meigen	Arroyo et al., 25021	DQ355859
C. aff. *gnaphalioides*	(J. Rémy) I. M. Johnst.	Hershkovitz 02-154	DQ355906
C. limbata	(D. Don) Less.	Hershkovitz 04-632	DQ355855
C. linearis var. *linearis*	Poepp. ex Less.	Arroyo et al., 25028	DQ355874
C. lycopodioides	(J. Rémy) Cabrera	Arroyo et al., 25169	DQ355920
C. minuta	(Phil.) Cabrera	Arroyo et al., 25127	DQ355907
C. moenchioides	Less.	Hershkovitz 04-630B	DQ355844
C. pentacaenoides	(Phil.) Hauman	Arroyo et al., 25168	DQ355904
C. pulvinata var. *pulvinata*-1	(Phil.) Hauman	Arroyo et al., 25083	DQ355903
C. pulvinata var. *pulvinata*-2	(Phil.) Hauman	Arroyo et al., 25240	DQ355918
C. pusilla	(D. Don) Hook. & Arn.	Arroyo et al., 25180	DQ355916
C. revoluta	(Phil.) Cabrera	Arroyo et al., 25126	DQ355899
C. stuebelii var. indet.	Hieron.	Arroyo et al., 25201	DQ355912
Chaptalia nutans	(L.) Polak	Kew 161-83.02018	AF546087
Gerbera crocea	Kuntze	Koekemoer and Funk 1924	AY504687
Gerbera sp.		Liu, 2282	AY914823
Gochnatia amplexifolia	(Gardner) Cabrera	Roque, 476	AF546090
Nassauvia	Comm. ex Juss.		
N. aculeata	Poepp. & Endl.	De Vore, 1267	EU239282
N. argentea	Phil.	Stuessy et al., 10020	EU239263
N. argyrophylla	Speg. Ex Hosseus	Stuessy et al., 68539	EU239264
N. axillaris	D. Don		EU239273
N. chubutensis	Speg.	Stuessy et al., 6946	EU239268
N. cumingii	Hook. & Arn.	B. Sparre, 10607	EU239265
N. darwinii	O. Hoffm. & P. Dusen ex P. Dusen	Stuessy et al., 6894	EU239283
N. dentata	Griseb.	Stuessy et al., 10023	EU239269
N. digitata	Wedd.	Ricadi, 5587	EU239287
N. dusenii	O. Hoffm. ex Macloskie		EU239266
N. fuegiana	(Speg.) Cabrera	Stuessy et al., 6950	EU239272
N. glomerulosa	D. Don	Stuessy et al., 6903	EU239280
N. glomerata	Wedd.	Stuessy et al., 10339	EU239270
N. juniperina	Skottsb.	Stuessy et al., 6820	EU239271
N. lagascae	Meigen	Ricardi and Marticorena, 5089	EU239288
N. looseri	Cabrera	Stuessy, 12737	EU239275
N. pinnigera	D. Don	Schlegel, 1088	EU239284
N. planifolia	Wedd.	Stuessy et al., 10235	EU239281
N. pulcherrima	Cabrera	Stuessy et al., 10135	EU239285
N. pygmaea	Hook. F.	Stuessy et al., 6855	EU239267
N. pyramidalis	Meyen	M. L. De Vore, 1350	EU239274
N. revoluta-1	D. Don	De Vore 1268	EU239277
N. revoluta-2	D. Don	Stuessy et al., 6857	EU239278

Table 1 continued

Taxon	Authority	Collection no.	GenBank accession no.
N. ulicina	Macloskie	Stuessy et al., 6942	EU239279
Pachylaena atriplicifolia	Don		EF530250
Perezia			
P. prenanthoides	Less.		FJ979670
P. nutans	Less.		FJ979671
P. poeppigii	Less.		
Polyachyrus carduoides	Phil.		EU239289
Schlechtendalia luzulaefolia	Less.		AF412835
Triptilion spinosum	Ruiz & Pav.	Stuessy and Crawford, 1173	EU239286

Sequencing products were visualized on an ABI Prism 377 automated DNA sequencer.

Phylogeny reconstruction Sequences were edited and assembled using AutoAssembler (PE-ABI, Foster City, CA, USA). Alignment of the large ITS data set for Mutiseae was easily accomplished by eye using Se-Al (Rambaut 1996). Data were exported as a nexus file and subsequently analyzed using parsimony (MP) in PAUP* (Swofford 2000) and maximum likelihood (ML) using Garli (available at http://www.bio.utexas.edu/faculty/antisense/garli/Garli.html). For MP analyses, characters were equally weighted and gaps were treated as missing data and not included in subsequent analyses. Initial MP searches used NNI branch swapping with 1,000 random addition replicates saving 5 trees per replicate. This generated a pool of starting trees that were subsequently used for more rigorous searches using TBR branch swapping. Relative support for the clades recovered was assessed by use of 1,000 jackknife replicates with 37% character deletion, enforcing the emulate "jac" option, TBR branch swapping, and two random additions per jackknife replicate and holding two trees per replicate. Before ML analyses ModelTest (Posada and Crandall 1998) was used to select the appropriate molecular model employing the AIC criterion. Heuristic ML analyses were conducted using best-fit model with Garli (Zwickl 2006). Relative support for the ML topology was assessed by use of 500 bootstrap replicates (Felsenstein 1985) as implemented in Garli.

Flavonoid chemistry Vegetative material was collected from natural populations (Table 2), and standard paper (2D) and thin-layer chromatographic methods were used to isolate and purify compounds (Mabry et al. 1970; Markham 1982). Purified compounds were identified by ultraviolet spectroscopy, by use of standard diagnostic reagents (Mabry et al. 1970; Markham 1982). Flavonoid glycosides were hydrolyzed according to the method of Wilkens and Bohm (1976), and sugars were identified by use of the circular chromatography method of Becker et al. (1977). Glycosides resistant to acid hydrolysis were assumed to be C-glycosyl flavonoids (sugars attached by carbon–carbon rather than oxygen bonds). Chromatographic and spectral data were also useful in identifying these carbon glycosides. Lack of sufficient amounts of plant material precluded exhaustive analyses of all compounds. Some identifications of flavonoid glycosides were based on co-chromatography on thin-layer plates with several solvent systems. In rare instances when there was insufficient yield for spectral analyses, the class of compound was inferred from R_F values (relative mobilities) in several solvent systems using paper and thin-layer chromatography. Color change of compounds on chromatograms when fumed with ammonia over ultraviolet light was also used to infer the structure of flavonoids (Mabry et al. 1970; Markham 1982).

Character evolution Patterns of flavonoid evolution and inflorescence architecture were explored using MacClade (Maddison and Maddison 2005) and the ML topology. Five categories of flavonoids were variously present within *Calopappus acerosus*, species of *Nassauvia*, and *Triptilion spinosum*: flavonols, flavones, C-glycosyl flavones, methylated C-glycosyl flavones, and methylated flavonols (Table 2). These were each considered a separate character with two states (present or absent), and were traced on to the ML estimate of phylogeny. The coding of inflorescence type followed that of Freire et al. (1993), with three character states (synflorescence, pseudocephalium, and solitary capitula) present among the ingroup taxa.

Results

ITS sequence data

The aligned nrDNA matrix for 53 members of Mutiseae, including 23 species from *Nassauvia*, comprised 708 characters. Maximum parsimony (MP) and maximum likelihood (ML) analyses recover highly similar topologies, thus only the ML topology is discussed here (Fig. 1). Our analyses provide strong support (95% ML bootstrap) for a

Table 2 Distribution of flavonoid compounds in *Calopappus*, *Nassauvia*, and *Triptilion*

Taxon	Collection no.	Flavonols	Flavones	C-Glycosyl flavones	Methylated C-glycosyl flavones	Methylated flavonols
Nassauvia						
N. aculeata	Stuessy et al. 6842, 6862	+	+	+		
N. argentea	Stuessy et al. 1020	+	+			
N. argyrophylla	Stuessy et al. 68539	+	+			
N. axillaris	J. Hunziker et al. 10242, 11292	+	+			+
	Stuessy et al. 6902, 6918					
N. chubutensis	Stuessy et al. 6946	+	+			
N. cumingii	J. Hunziker et al. 10384	+	+	+		
N. darwinii	Stuessy et al. 1069					+
N. dentata	Gentilli 1072	+		+		+
N. dusenii	Stuessy et al. 6859	+		+		
N. fuegiana	Stuessy et al. 6901	+		+		+
N. glomerulosa	Stuessy et al. 6810, 6903, 6917	+	+			
N. glomerata	J. Hunziker et al. 10331, 10339	+		+		
N. juniperina	Stuessy et al. 6821	+		+		+
N. lagascae	Stuessy et al. 11330	+				
N. planifolia	J. Hunziker et al. 10314	+				
N. pulcherrima	Stuessy et al. 10135	+		+		
N. pygmaea	Stuessy et al. 6855			+		+
N. pyramidalis						
N. revoluta	Stuessy et al. 6851	+		+		
N. ulicina	Stuessy et al. 6942	+	+			
Calopappus acerosus	Stuessy 12744	+	+			
Triptilion spinosum	Stuessy and Crawford 1173	+	+	+		

clade comprising 16 species of *Chaetanthera*, and place this clade as sister to a large clade comprising 10 additional genera of Mutiseae. Within the latter clade, analyses of ITS sequence variation resolves, with moderate support (67% ML bootstrap), a subclade representing the three members of *Perezia* sampled here. Sister to the *Perezia* clade is a subclade that includes 24 members of *Nassauvia* and *Triptilion spinosum* (Fig. 1, *Nassauvia* Clade). Although only weakly supported as assessed via ML bootstrap analyses (53%), this same clade is recovered in the strict MP topology with moderate support (63%).

Phylogeny of *Nassauvia*

Within the *Nassauvia* clade, there is strong support (100% ML bootstrap) for a clade comprising three of the four members of section *Strongyloma* (i.e., *N. fuegiana*, *N. ulicina*, and *N. glomerulosa*) sampled for this study. Sister to this clade is a lineage comprising *N. lagascae* (section *Caloptilium*) and *Triptilion spinosum* (71% ML bootstrap). Together, these subclades form a weakly supported clade (61% ML bootstrap) that is sister to the remainder of *Nassauvia*. The four accessions from the three members of section *Panargyrum* (i.e., *N. looseri*, *N. darwinii*, and *N. aculeata*) presently sampled are all placed within a single, weakly supported clade (59% ML bootstrap); however, these taxa are not resolved as monophyletic (Fig. 1). Our analyses place two members of section *Nassauvia* (i.e., *N. chubutensis* and *N. pulcherrima*) within this same clade. Weakly recovered as sister to this clade is a well supported clade (97% ML bootstrap) comprising the remaining 11 taxa from section *Nassauvia* sampled for this study. First branching in this clade is one of two accessions of *N. revoluta*; the second accession of this species is placed as sister to *N. pinnigera* (80% ML bootstrap, but <50% MP jackknife), with *N. argentea* resolved as sister to this clade with less than 50% ML bootstrap (Fig. 1). Although this clade is only weakly supported, the moderate support for the sister relationship between *N. pinnigera* and *N. revoluta*-1 to the exclusion of *N. revoluta*-2 suggests that this taxon is not monophyletic. The remaining seven species from section *Nassauvia* (i.e., *N. argyrophylla*–*N. dusenii*) are resolved in a weakly supported clade (<50% ML bootstrap) that is sister to a moderately supported (75% ML bootstrap) subclade comprising the remaining individual sampled from subgenus *Strongyloma* (i.e., *N. axillaris*) and

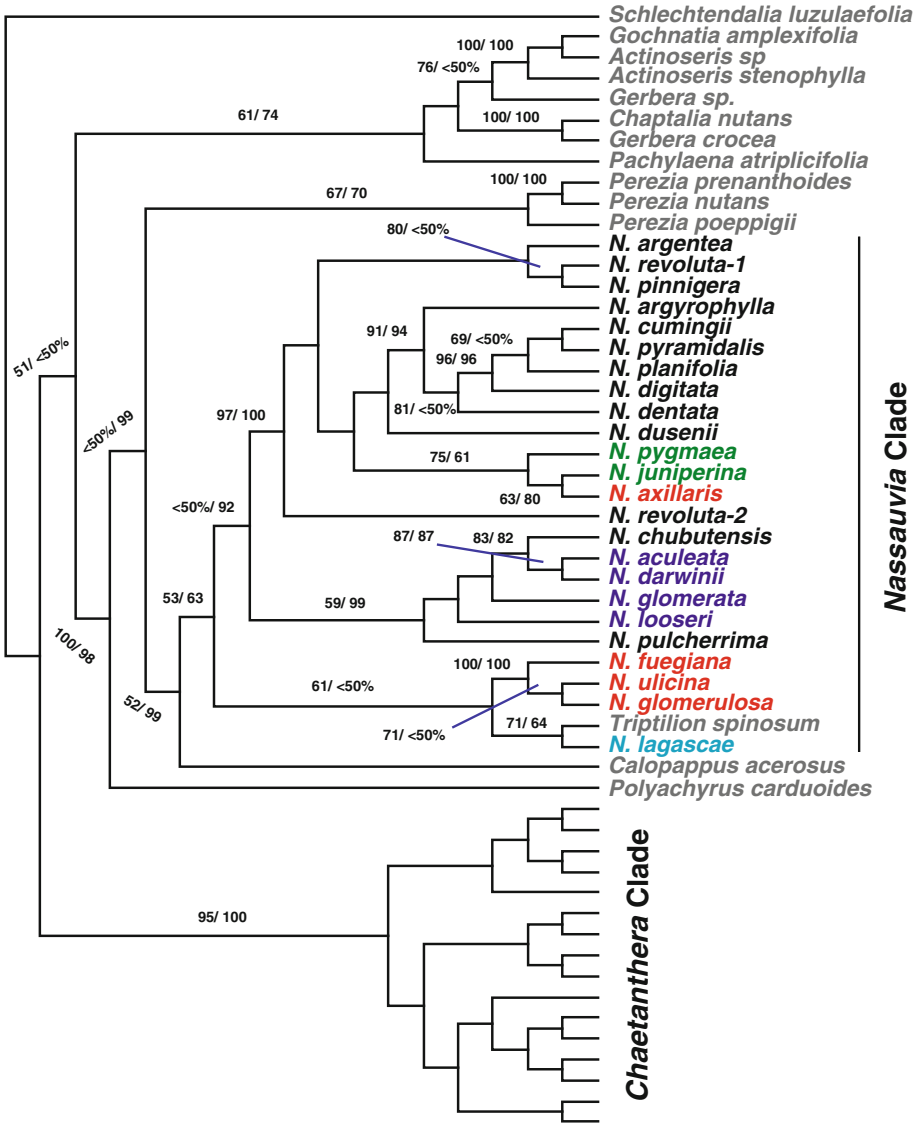

Fig. 1 Maximum likelihood topology inferred from nrDNA ITS data. *Numbers* above branches indicate bootstrap support from MP and ML analyses. Designations of subgenera and sections of *Nassauvia* are given by the color of the branches. Subgenus *Nassauvia:* section *Caloptilium, light blue*; section *Mastigophorus*, *green*; section *Nassauvia*, *black*; section *Panargyrum*, *dark blue*; subgenus *Strongyloma*, *red*

two taxa from section *Mastigophorus* (*N. pygmaea* and *N. juniperina*).

Synflorescence evolution

The molecular phylogeny suggests that the evolution of inflorescences has been complex. The original condition within *Nassauvia* is the highly aggregated pseudocephalium; the first split within the clade includes a smaller clade consisting of subgenus *Strongyloma*, *N. lagascae* (section *Caloptilium* of subgenus *Nassauvia* and *Triptilion*) in which the ancestral condition is equivocal (Fig. 2). There could have been two losses of the pseudocephalium one in the ancestor of subgenus *Strongyloma* and one in *Triptilion*; alternatively, there could have been one loss of the pseudocephalium followed by one gain in *N. lagascae* (Fig. 2). The other major, and much larger, clade consists of all members of section *Nassauvia* together with sections *Mastigophorus* and *Panargyrum* (Fig. 2). Section *Mastigophorus* includes species that often, but not always, have solitary capitula. Although *N. axillaris* is placed in section *Mastigophorus* by analyses of ITS data, it groups with other representatives of subgenus *Strongyloma* in a plastid phylogeny (Maraner and Stuessy unpubl.). Indeed, the preponderance of evidence, including inflorescence type, places *N. axillaris* in subgenus *Strongyloma* (Freire et al. 1993). Two species of section *Mastigophorus* (i.e., *N. juniperina* and *N. pygmaea*) were included in the current analysis and, as discussed earlier, the latter species is unique in this section by having a pseudocephalium. Although additional sampling within the section is needed to determine the evolution of inflorescence types, it is clear that solitary capitula have been gained at least once whereas the pseudocephalium has been retained in

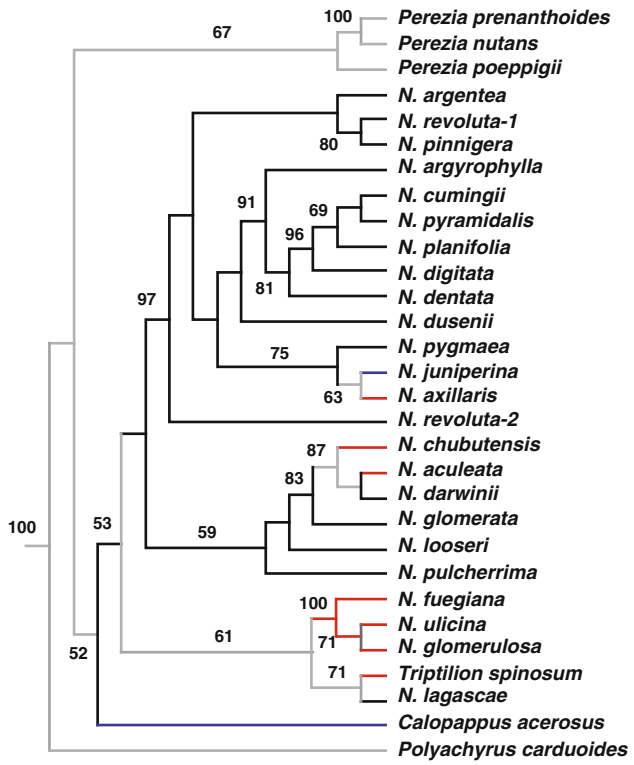

Fig. 2 Maximum likelihood topology inferred from nrDNA ITS data with inferred character state changes in inflorescence. *Black* shows pseudocephalia, *blue* shows synflorescence, and *red* shows solitary capitula

N. pygmaea (Fig. 2). Within the clade that includes section *Parnargyrum*, together with *N. chubutensis*, and *N. pulcherrima* from section *Nassauvia*, there has been either one loss of the pseudocephalium with a subsequent gain in *N. darwinii*, or two losses of the pseudocephalium (Fig. 2).

Flavonoid components and evolution

A total of 32 flavonoid compounds was detected in *Calopappus*, *Nassauvia*, and *Triptilion* (Table 2). The classes of compounds included simple flavonols and their oxygen (O) glycosides, flavones, and their O glycosides, and C-glycosyl flavones in which the sugars are attached to the flavonoid nuclei by carbon rather than oxygen bonds. Two other classes of flavonoids were flavonols and C-glycosyl flavones in which there is methylation of a hydroxyl group. Two of the components were not identified to class because of the small amount of plant material and small concentrations of compounds in the plants. Four compounds were classified only to class, with two flavones and two flavonols inferred from chromatographic properties.

Tracing the flavonoid classes on to the ML ITS tree suggests a complex pattern of secondary chemistry evolution, with each class showing several gains–losses. Optimizing flavonols on to the ML topology indicates that the ancestral condition for the *Nassauvia* clade is presence of flavonols. Within *Nassauvia*, parsimony reconstructs either four separate losses of flavanols or two losses followed by two independent gains of flavanols (Fig. 3a).

The patterns of evolution are complex for flavones and their presence is inferred as ancestral (Fig. 3b). Employing equivocal cycling in MacClade indicates that there are ten possible reconstructions of this feature. The most common pattern recovered involves three losses of flavones, with one each noted for *N. lagascae* and *N. fuegiana* (Fig. 1 and clade A, Fig. 3b) and a loss for the clade comprising *N. argentea* to *N. pulcherrima*, with eight independent gains within this large clade (clade F, Fig. 2b).

The absence of C-glycosyl flavones is interpreted as ancestral in the *Nassauvia* clade. The parsimony reconstruction reveals unequivocally that the presence of C-glycosyl flavones has arisen five times within this clade. There are two independent gains in each of the first two branching lineages and a single gain with four apparent reversals noted in the large clade comprising *N. revoluta-2* to *N. argentea* (clade A, Fig. 3c). The evolution of methylated C-glycosyl flavones is less complicated. Our analyses indicate that this class of secondary compounds has arisen three times within the *Nassauvia* clade (Fig. 3d). The absence of methylated flavonols is constructed as ancestral, and there have been either five gains or four gains and with a subsequent loss in the *N. chubutensis–N. darwinii* subclade (clade B, Fig. 3e).

Discussion

Relationships among genera and the major clades of *Nassauvia*

The placement of the genera *Calopappus* and *Triptillion* in the ITS phylogeny may be considered in the context of previous taxonomic-phylogenetic treatments. The former genus is monotypic, and has been treated as either an independent genus (Crisci 1974; Cabrera 1982; Katinas et al. 2008a, b) or as a member of *Nassauvia (*Bremer 1993). The primary features distinguishing *Calopappus* from *Nassauvia* are the larger capitula and differences in pollen, whereby the former has an ectosexine that is thicker than the endosexine, and with both these layers columellate and divided by a zig-zag internal tectum (Crisci 1974a; Tellería et al. 2003). The morphological cladistic analysis of Freire et al. (1993) showed *Calopappus* nested within *Nassauvia.* The results of this study are in agreement with Crisci and Freire (1986), who viewed *Calopappus* as sister to a *Nassauvia–Triptilion* group, and the molecular phylogenetic study of Simpson et al. (2009) that also resolved *Calopappus* as sister to a *Nassauvia–Triptilion* clade. In contrast to *Calopappus*, the genus *Triptilion*, which

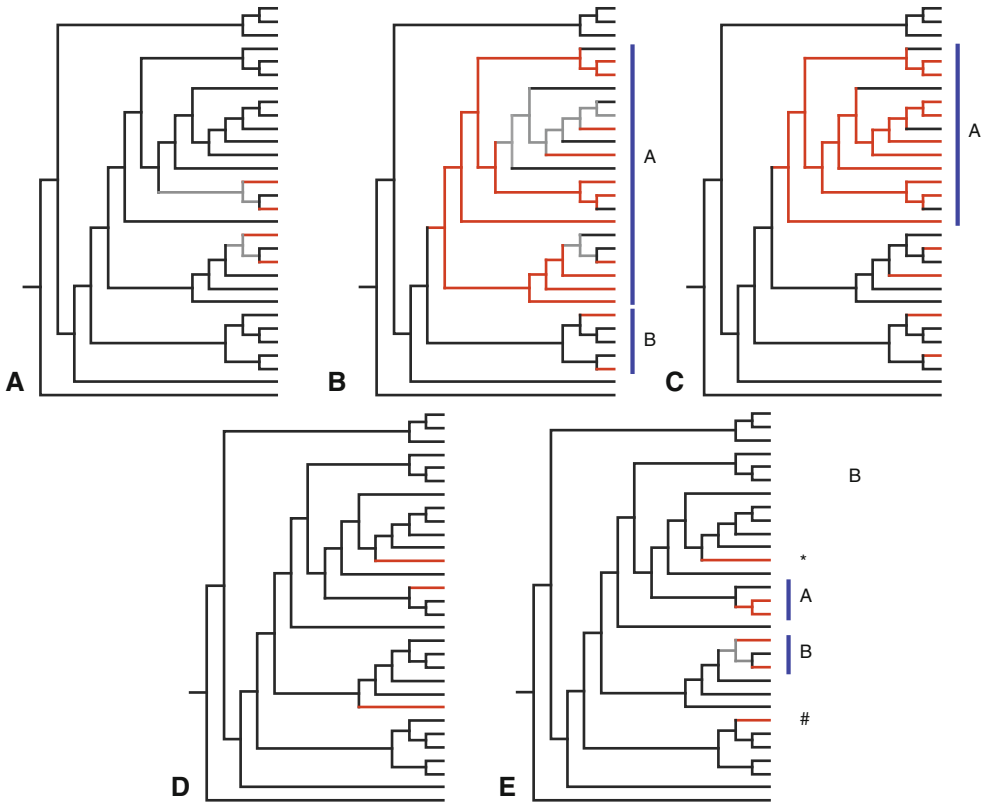

Fig. 3 MacClade reconstructions of changes in classes of flavonoids on the ML nrDNA ITS tree for the following classes of secondary compounds: **a** flavonols (*red* loss); **b** flavones (*red* loss); **c** C-glycosyl flavones (*red* gain); **d** methylated C-glycosyl flavones (*red* gain); and **e** methylated flavonols (*red* gain). Subclades discussed in text are indicated by *blue brackets*; for **e**, **N. dentata*; #*N. fuegiana*

consists of 12 species of annual or perennial herbs, has been treated by most workers as a distinct genus (Cabrera 1982; Bremer 1993; Katinas et al. 2008a, b). The genus is morphologically similar to *Nassauvia*, with the nature of the pappus being the only consistent distinguishing character (Cabrera 1982; Bremer 1993). In a cladistic analysis of morphological characters (Freire et al. 1993), *Triptilion* was resolved as sister to a clade that includes *Calopappus* and *Nassauvia.* The ITS phylogeny places *Triptilion* within *Nassauvia* (Fig. 1) and is in agreement with the results of Simpson et al. (2009) that place *Triptilion* within *Nassauvia*. Cabrera (1982) considered *Triptilion* as most closely related to basal elements within *Nassauvia* section *Panargyrum*, but the molecular phylogeny places it in a subclade with *N. lagascae* (section *Caloptilium*) and that subclade is sister to species of subgenus *Strongyloma* in a weakly supported clade (61% ML bootstrap; Fig. 1).

There is general support for the sister group relationship between the two subgenera of *Nassauvia* (Cabrera 1982; Freire et al. 1993). Cabrera (1982) considered section *Caloptilium* (*N. lagascae*) to be "derived" and it is embedded in section *Panargyrum* on the basis of morphological analyses (Freire et al. 1993). The molecular phylogeny suggests different placement of this monotypic section, with sections *Caloptilium*, *Triptilion* and subgenus *Strongyloma* allied in one clade (Fig. 1). The second major clade includes two subclades, one comprising primarily section *Panargyrum* and the other including members of sections *Mastigophorus* and *Nassauvia*. Section *Mastigophorus* seems to be derived from within section *Nassauvia* (Fig. 1). By contrast, the morphological phylogeny of Freire et al. (1993) places section *Mastigophorus* in a large clade that is sister to section *Nassauvia* rather than nested within it.

Relationships among sections of *Nassauvia*

The most recent monograph of *Nassauvia* (Cabrera 1982) recognized five sections within the genus. Included in this study is *N. lagascae* of the monotypic section *Caloptilium*, and two members from section *Mastigophorus*, four members of section *Panargyrum*, and 13 accessions (12 species) of section *Nassauvia*. The results of our MP and ML analyses indicate that the sections of the genus, as currently defined, are not monophyletic. There is strong support (100% ML and 100% MP bootstrap) for a subclade comprising three of the four members of section *Strongyloma* (Fig. 1), but strongly excluded from this subclade is *N. axillaris*. Similarly, the four members of section *Panagyrum* are all placed within a single clade, but nested within this subclade is *N. chubutensis* with *N. pulcherrima* supported as sister to this clade with moderate to strong support (59% ML bootstrap, but 99% MP bootstrap). Most taxa sampled from section *Nassauvia* are placed within a

large, well supported clade (97% ML and 100% MP bootstrap); however, nested within this and rendering these taxa paraphyletic is a moderately supported clade (75% ML and 61% MP bootstrap) including two members of the putatively non-monophyletic section *Mastigophorus* and *N. axillaris* (section *Strongyloma*).

Evolution of inflorescences, geographic distribution, and ecology

One of the characteristic features of *Nassauvia* and related genera is the reduction of the number of florets within capitula and the secondary aggregation of capitula, with the largest and most dense aggregations forming pseudocephalia. Secondary inflorescences (synflorescences) are rare but widely distributed in Asteraceae (Kunze 1969; Crisci 1974). Because they are rather peculiar morphologically and rare, synflorescences have been viewed as evolutionarily advanced (Stuessy 1978). However, within *Nassauvia* and closely related genera such as *Calopappus* and *Triptilion* there have been various phylogenetic interpretations of the different levels of aggregations, which vary from solitary capitula to highly condensed pseudocephalia. The pseudocephalia of sections *Nassauvia* and *Caloptilium* have generally been viewed as derived. However, in the morphological cladistic analysis of Freire et al. (1993), all other inflorescence types were shown as having originated from the synflorescence type found in *Triptilion.* Freire et al. (1993) thus viewed the solitary capitula of section *Mastigophorus* as derived. In contrast, Stuessy (1978) suggested that the solitary capitula of section *Mastigophorus* represent the ancestral condition in *Nassauvia*, with all other inflorescence types evolving from solitary heads. The results of Tortosa et al. (2004) and Katinas et al. (2008a, b) indicate that the more complex synflorescence type is ancestral in *Nassauvia*, in agreement with the hypothesis of Freire.

Inflorescence type may be examined relative to the geographic distribution and ecology of the elements in *Nassauvia* within the framework of the general hypothesis that the highly aggregated capitula would be at an advantage at higher altitudes where pollinators are at a premium (Bingham 1998). Presumably, the larger aggregations would be more efficient in attracting pollinators and pollination would be more efficient (Stuessy unpubl.). The members of section *Nassauvia* occur primarily in the Andes, and are characterized by the ancestral condition of highly aggregated pseudocephalia. This suggests that *Nassauvia* originated in the mountains, and there were subsequent migrations to other areas. An ancient divergence within *Nassauvia* produced the ancestor of subgenus *Strongyloma*, which subsequently migrated to lower elevations in Patagonia, with the evolution of synflorescences consisting of a dichasial inflorescence on short stalks originating from the axils of the upper leaves.

Evolution of flavonoid compounds

The evolution of flavonoid classes in the *Nassauvia–Triptilion* group (hereafter referred to as *Nassauvia* unless specifically considering *Triptilion*) has been rather complex, and as such flavonoids are not useful for delimiting groups within the genus. The presence of flavonols is the ancestral condition; four of the species of *Nassauvia* sampled for this study have lost these compounds. These taxa are placed in two separate subclades, each with a single taxon that has flavonols (Fig. 3a). This pattern can be explained by four separate losses; two losses with a single reversal in each subclade; or some combination of those scenarios. A comparison of the taxonomic distribution of flavonol loss with gain of methylated flavonols (Fig. 3a, e) shows that in several instances the loss of flavonols occurs in the same taxa showing gain of methylated flavonols. This pattern could be explained because the flavonols serve as a substrate for methylation and thus the methylated derivatives are accumulated at the expense of sequestering simple flavonols (Bohm and Stuessy 2001, Chap. 5).

Interpreting the evolution of flavones is more complex. MacClade provides 10 equally parsimonious reconstructions of these compounds. The presence of flavones is the ancestral condition within *Nassauvia*. The most common pattern recovered involves three losses of flavones. Two losses are inferred within the *N. lagascae–N. fuegiana* subclade (clade B; Fig 3b). A third loss can be inferred for the remaining ingroup taxa (clade A; Fig. 3b), with eight reversals to flavone presence; however, other combinations of gains and losses within this subclade are possible.

The parsimony reconstruction of the evolution of C-glycosyl flavones indicates that these compounds were absent in the ancestor of *Nassauvia*. Five separate gains of C-glycosyl flavones are inferred; two gains are inferred within the first two branching subclades of *Nassauvia*. A fifth gain of C-glycosyl flavones is ancestral for the *N. argentea–N. revoluta* subclade (clade A; Fig. 3c), with four independent losses in this same subclade. Methylated C-glycosyl flavones occur in a small subset of the taxa where C-glycosyl flavones are found (Fig. 3c, d). We infer three independent gains of methylated C-glycosyl flavones (Fig. 3d). Because methylation is a late step in the biosynthetic pathway to C-glycosyl flavones (Bohm and Stuessy 2001, Chap. 5), it follows that methylated C-glycosyl flavones would occur only in those taxa that synthesize simple C-glycosyl flavones.

The absence of methylated flavonols is constructed as ancestral in *Nassauvia* (Fig. 3e). There have been either

three or four gains of methylated flavones; three unequivocal gains are inferred for *N. fuegiana*, *N. dentata*, and the subclade including *N. juniperina* and *N. axillaris* (clade A; Fig. 3e). The ancestral state for the *N. chubutensis–N darwinii* subclade is equivocal (clade B; Fig. 3e). Thus it is equally parsimonious that this represents two separate gains, or a single gain with a subsequent reversal.

The evolution of flavonoid classes during the diversification of *Nassauvia* has been complex. The gains or losses have occurred throughout the clade, in some cases only in terminals and in others at deeper branches in the phylogeny. However, looking more closely it is evident that gains and losses of flavonols (including methylated derivatives) occur only in terminal taxa whereas changes in flavones and C-glycosyl flavones occur both early in the diversification of the clade and in numerous terminals (Fig. 3a, b, c). The gain of methylation occurs only in terminals (Fig. 3d, e). These collective patterns of gains and losses, although interesting and comprehensible within a biosystematic context, make the use of flavonoids per se of very limited taxonomic–phylogenetic utility in *Nassauvia*.

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