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Molecular phylogeny and biogeography of the Qinghai-Tibet Plateau endemic *Nannoglottis* (Asteraceae)

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Abstract

All taxa endemic to the Qinghai-Tibet Plateau are hypothesized to have originated in situ or from immediately adjacent areas because of the relatively recent formation of the plateau since the Pliocene, followed by the large-scaled biota extinction and recession caused by the Quaternary ice sheet. However, identification of specific progenitors remains difficult for some endemics, especially some endemic genera. *Nannoglottis*, with about eight species endemic to this region, is one such genus. Past taxonomic treatments have suggested its relationships with four different tribes of Asteraceae. We intend to identify the closest relatives of *Nannoglottis* by evaluating the level of monophyly, tribal delimitation, and systematic position of the genus by using molecular data from *ndh*F gene, *trn*L-F, and ITS region sequences. We find that all sampled species of *Nannoglottis* are a well-defined monophyly. This supports all recent taxonomic treatments of *Nannoglottis*, in which all sampled species were placed in one broadly re-circumscribed genus. *Nannoglottis* is most closely related to the Astereae, but stands as an isolated genus as the first diverging lineage of the tribe, without close relatives. A tentative relationship was suggested for *Nannoglottis* and the next lineage of the tribe was based on the ITS topology, the ''basal group,'' which consists of seven genera from the Southern Hemisphere. Such a relationship is supported by some commonly shared plesiomorphic morphological characters. Despite the very early divergence of *Nannoglottis* in the Astereae, the tribe must be regarded to have its origin in Southern Hemisphere rather than in Asia, because based on all morphological, molecular, biogeographical, and fossil data, the Asteraceae and its major lineages (tribes) are supposed to have originated in the former area. Long-distance dispersal using Southeast Asia as a steppingstone from Southern Hemisphere to the Qinghai-Tibet Plateau is the most likely explanation for this unusual biogeographic link of *Nannoglottis*. The 23–32-million-year divergence time between *Nannoglottis* and the other Astereae estimated by DNA sequences predated the formation of the plateau. This estimation is further favored by the fossil record of the Asteraceae and the possible time of origin of the Astereae. *Nannoglottis* seems to have reached the Qinghai-Tibet area in the Oligocene–Eocene and then re-diversified with the uplift of the plateau. The molecular infragenetic phylogeny of the genus identifies two distinct clades, which reject the earlier infrageneric classification based on the arrangement of the involucral bracts and the length of the ligules, but agree well with the habits and ecological preferences of its current species. The ''alpine shrub'' vs. ''coniferous forest'' divergence within *Nannoglottis* was estimated at about 3.4 million years ago when the plateau began its first large-scale uplifting and the coniferous vegetation began to appear. Most of the current species at the ''coniferous forest'' clade of the genus are estimated to have originated from 1.02 to 1.94 million years ago, when the second and third uprisings of the plateau occurred, the climate oscillated and the habitats were strongly changed. The assumed evolution, speciation diversity, and radiation of *Nannoglottis* based on molecular phylogeny and divergence times agree well with the known geological and paleobotanical histories of the Qinghai-Tibet Plateau.

Keywords: *Nannoglottis*; Astereae; Asteraceae; *trn*L-F; *ndh*F; ITS; The Qinghai-Tibet Plateau; Endemics; Phylogeny; Historical biogeography

1. Introduction

The Qinghai-Tibet Plateau is the highest plateau over the world. Its short period of formation, since the Pliocene, has considerably influenced the structure and evolution of its component flora (Shi et al., 1998).

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Furthermore, the ice sheet that occurred in the Quaternary period covered almost the entire plateau, which must have resulted in the large-scale recession and extinction of the biota (Li et al., 1995). Despite this, the plateau has an exceptionally diverse flora with about 4385 species of 1174 genera in 189 families (Wu, 1980). It is estimated that more than 25% of the total number of species are endemic to the plateau (Wu, 1987). However, only about 20 genera have considered endemic (Wu, 1980, 1987; Wu et al., 1995), amounting to less than 2% of the total number of genera. The estimates of endemics partly supported the hypothesis suggested by Wulff (1944) that the Quaternary ice sheet had wiped out the flora of the plateau and the current flora migrated or originated from adjacent areas. Because of the short time since then, endemic species, rather than endemic genera, were formed under the arid environments (Ward, 1927, 1935; Wulff, 1944; Wolfe, 1975; Wu, 1987). Most genera occurred in this area can be found in eastern Asia and belong to the temperate Northern Hemisphere groups (Wu, 1980, 1987; Wu et al., 1995). Only a few genera were found to occur in Central Asia and Mediterranean region around the plateau (Wu, 1987). The endemic plateau genera were hypothesized to be closely related to and have originated from local or adjacently distributed genera (Wu, 1980, 1987; Wu et al., 1995). If this assumption is correct, all endemics should find their specific progenitors sympatrically or immediately around the Plateau. But morphological identifications of close relatives are still difficult for some endemics, especially some endemic genera.

Nannoglottis of Asteraceae is such a Qinghai-Tibet endemic genus with unidentified relatives. The genus is distributed primarily on the east and south of the Plateau with about eight species (Gao et al., unpublished data). It is distinguished from other Asteraceae in this area by its trimorphous flowers: pistillate (filiform) which are 2–3 seriate, the outer ligulate, and the inner, staminate shortly tubular disc flowers with a truncate apex. The ligules are short and pale-red or long and yellow while the filiform and staminate disc florets are yellow. All florets are sparsely hairy. *Nannoglottis* was established by Maximowicz (1881) for *Nannoglottis carpesioides*. Two species described under *Stereosanthus* by Franchet (1896) and one under *Vierhapperia* by Handel-Mazzetti (1937) were transferred into *Nannoglottis* by Ling and Chen (1965). More recently, two additional species, *Nannoglottis hookeri* and *Nannoglottis ravida*, originally placed in *Doronicum* and *Senecio* of the Senecioneae, were transferred into *Nannoglottis* (Kitamura and Gould, 1982; Jeffrey and Chen, 1984). *N. ravida* is a subshrub growing among dry alpine shrubs while the remaining species of *Nannoglottis* are all perennials occurring under the damp coniferous forests. The species in *Nannglottis* are mainly diagnosed by layers of involucral bracts, length and color of ligules, and shape and hair of leaves. The capitula are solitary or a few arranged in a corymb. Ling and Chen (1965) presented an infragenetic system of *Nannoglottis* based on disposition of involucral bracts and length of ligules. The morphological range, habit, and ecological preference of *Nannoglottis* are summarized in Table 1.

The central flowers of *Nannoglottis* are functionally staminate and features of the style branches (the stigmatic positions) critical in the interpretation of its tribal position are absent. Thus the tribal position and generic affinities of *Nannoglottis* have long been in dispute. Hoffmann (1894) placed *Nannoglottis* in the Senencioneae while Franchet (1896) suggested a relationship with Inuleae when he established *Stereosanthus*. Handel-Mazzetti (1937) placed *Vierhapperia,* however, in the Astereae, emphasizing the similarities in the eligulate pistillate florets to those of *Erigeron* and *Conyza*. Ling and Chen (1965) followed Hoffmann (1894) by maintaining the genus within the Senecioneae, but pointed out its relationship with *Homogyne* and *Liabum*. The latter is now treated as a separate tribe, Liabeae by Bremer (1994). Grau (1977) reiterated the observation of the filiform floret similarity between *Nannoglottis* and *Erigeron* ; and on the basis of achene anatomy, Jeffrey and Chen (1984) suggested again that *Nannoglottis* is Astereaean rather than a member of the Senecioneae. Zhang and Bremer (1993) accepted *Nannoglottis* in the Astereae as an isolated genus. However, *Nannoglottis* was viewed by Nesom (1994) as a basal member of the subtribe Solidagininae of the Astereae and closely related to *Solidago* and *Oreochrysum*. Ling (1997) argued

Table 1
Morphological range, habit, and ecological preference of *Nannoglottis*

Species	Capitulescence	Involucres	Ligules	Habit	Ecological preference
N. carpesioides Maxim.	Corymb	2–3-layered	Short	Perennial	Coniferous forest
N. delavayi (Franch.) Ling and Y.L. Chen	Corymb	2–3-layered	Long	Perennial	Coniferous forest
N. gynura (C. Winkl.) Ling and Y.L. Chen	Corymb	3–4-layered	Long	Perennial	Coniferous forest
N. hieraciphylla (Hand.-Mazz.) Ling and Y.L. Chen	Corymb	2–3-layered	Short	Perennial	Coniferous forest
N. latisquama Ling and Y.L. Chen	Corymb	2–3-layered	Long	Perennial	Coniferous forest
N. macrocarpa Ling and Y.L. Chen	Corymb	2–3-layered	Long	Perennial	Coniferous forest
N. ravida (C. Winkl.) Y.L. Chen	Solitary	2–3-layered	Long	Subwood	Alpine shrub
N. yuennanensis (Hand.-Mazz.) Hand.-Mazz.	Corymb	2–3-layered	Short	Perennial	Coniferous forest

that *Nannoglottis* should be classified in the Senecioneae, in a subtribe of its own, related to *Petasites* and *Tussilago* of the subtribe Tussilagininae based on their similar trimorphous florets. Ho et al. (1997) suggested that *Nannoglottis* might have evolved from *Senecio* of the Senecioneae on the basis of the yellow florets of both genera whereas most genera of the Astereae in Asia have white to bluish or pinkish flowers. Thus previous suggestions for the tribal affinity of *Nannoglottis* have involved four distinct tribes of Asteraceae: Inuleae, Liabeae, Senecioneae, and Astereae. The tribal placement of *Nannoglottis* is thus unresolved. Furthermore, the monophyly of *Nannoglottis* needs confirmation because the current species of the genus were originally described under five genera of different tribes and merged together only recently.

DNA data, particularly DNA sequences, have greatly contributed to the understanding of the phylogeny, evolution, and taxonomy of Asteraceae (Jansen and Kim, 1996). This is particularly true for the problematic genera of the family, where morphological data are lacking or ambiguous (Francisco-Ortega et al., 2001, 1997; Karis et al., 2001; Kim et al., 1998; Panero et al., 1999; Park et al., 2001). In the Asteraceae, the commonly used DNA sequence data for phylogenetic analyses are those from ITS, *ndh*F, and *trn*L-F regions. The *trn*L-F region sequence usually comprises two non-coding chloroplast DNA sequences, *trn*L intron, and *trn*L/*trn*F intergenic spacers (Taberlet et al., 1991). Although relatively short with about 700 bps, the *trn*L-F region sequence has proven to be phylogenetically informative from infrageneric phylogeny to tribal delimitation of Asteraceae (Bayer and Starr, 1998; Bayer et al., 2000; Fernández et al., 2001). Therefore, the *trn*L-F region data are ideally suited to examine the monophyly, tribal placement, and infrageneric phylogeny of a problematic genus in one parsimonious tree. The *ndh*F gene sequence is the most useful data set for inferring the phylogeny at and below family level of Asteraceae (Kim and Jansen, 1995; Olmstead et al., 2000). With a longer length and more phylogenetic information than other chloroplast sequences, such as *rbc*L and *trn*L-F data, *ndh*F provided enough informative characters to infer a finer position for a problematic genus in the Asteraceae (Eldenás et al., 1999; Watson et al., 2000). For Asteraceae, which must have undergone recent and rapid evolution, ITS, the nuclear sequence of the internal transcribed spacers, a rapidly evolving region, has been proven to be more suitable for investigating phylogeny within a genus or closely related genera than chloroplast sequences (Bain and Golden, 2000; Baldwin et al., 1995; Noyes, 2000; Noyes and Rieseberg, 1999). Furthermore, the phylogeny inferred from ITS can be evaluated against that from chroloplast genome. Therefore, we performed phylogenetic analyses of *Nannoglottis* based on three data sets: *trn*L-F regions and *ndh*F and ITS sequences at the different taxonomic levels. A combination of three data set analyses will provide a good resolution to the phylogenetic and taxonomic problems of *Nannoglottis* as detailed above.

Despite abundant research on the origin of island endemics or identification of specific progenitors of the narrowly distributed species by using molecular data (Baldwin et al., 1991; Francisco-Ortega et al., 2001, 1997; Kim et al., 1998; Lia et al., 2001; Panero et al., 1999), fewer works have focused on the origin of the island-like Qinghai-Tibet Plateau endemic flora. The endemic distribution of *Nannoglottis* is ideally suited to examine hypotheses for the origin of the Qinghai-Tibet Plateau endemic flora based on the molecular phylogeny. In addition, one major goal of modern biogeography is to reconstruct the phylogeny of endemic genera and evaluate their origin and evolution against the geologic and paleoclimatic histories of their distribution (Avise, 2000).

The three major objectives in this study were to (1) evaluate the monophyly, tribal delimitation, and systematic position of *Nannoglottis* and identify its close relatives if possible, (2) examine the infrageneric relationship of the genus as inferred from DNA sequence data against the existing supraspecific classification on the basis of the morphological characteristics proposed by Ling and Chen (1965), and (3) assess the biogeographical origin and evolutionary history of *Nannoglottis* in a phylogenetic context.

2. Materials and methods

2.1. Data sets, sample strategy, and plant materials

Three data sets were designed to detect monophyly, the systematic position, and infrageneric relationships of *Nannoglottis*. The first data set was constructed for *trn*L-F (two non-coding chloroplast regions flanking the *trn*L and *trn*F genes, the *tr*nL, intron, and *trn*L/*trn*F intergenic spacer) data of eight species of *Nannoglottis* and 26 genera belonging to 17 tribes of Asteraceae to give a preliminary assessment of monophyly, tribal placement, and infrageneric phylogeny of *Nannoglottis*. This data set was designed with a further emphasis to test the possible relationship of *Nannoglottis* with *Senecio* and *Tussilago* of the Senecioneae suggested recently by Ho et al. (1997) and Ling (1997). According to the molecular and morphological works (Bremer, 1994; Kim and Jansen, 1995), Asteraceae comprises three subfamilies: Barnadesieae, Cichiorioideae, and Asteroideae, with the Barnadesieae at the basal position for the family. The four tribes (Inuleae, Liabeae, Senecioneae, and Astereae) which were supposed to be possibly related to *Nannoglottis* fall in Cichiorioideae and Asteroideae. Therefore, *Doniophyton* in Barnadesieae was selected as

the outgroup to conduct a parsimony analysis of other taxa. The *trn*L-F data recovered a preliminary result of a tribal position of *Nannoglottis* in the Astereae and an infrageneric phylogeny with two major clades within the genus.

To further test the tribal position of *Nannoglottis* and possible position within Astereae, one species from each of the two major clades inferred from the initial *trn*L-F analysis was selected for inclusion in *ndh*F analysis. The second *ndh*F (the chloroplast gene *ndh*F, the ND5 protein of chloroplast NADH dehydrogenase) data set was constructed for *Nannoglottis* and 36 genera collectively representing all of the currently recognized tribes of three subfamilies in Asteraceae of Bremer (1994) and Kim and Jansen (1995). Seven genera (*Erigeron*, *Conyza*, *Baccharis*, *Bellis*, *Aster*, *Pyrrocoma*, and *Felicia*) of Astereae were included in this data set. They represent five subtribes of Nesom (1994) and major evolutionary lines of Zhang and Bremer (1993) based on morphological analyses. Among them, *Felicia* is recently inferred to be one genus of the basal lineage on the basis of molecular data (Noyes and Rieseberg, 1999). *Erigeron* and *Conyza* with eligulate pistillate flowers in this data set were supposed to be related to *Nannoglottis* by those authors, who placed this genus in Astereae (Grierson, 1964; Grau, 1977). *Barnadesia* in Barnadesieae was again selected as the outgroup.

The third data set was based on nuclear genome, independent from the chloroplast sequence data set. This data set was designed to test the systematic position of *Nannoglottis* within Astereae recovered by the *ndh*F analysis and obtain more detailed infrageneric phylogeny of the genus against the *trn*L-F region analysis. We used ITS (ITS1, the 5.8S gene, and ITS2) sequences from a different inheritance pathway. This data set includes eight species of *Nannoglottis* and 42 genera of Astereae. Four genera from the Anthemideae, Calenduleae, and Gnaphalieae were selected as outgroups because these three tribes were found to be closely related to Astereae based on the *ndh*F parsimony analysis. The sampled genera in Astereae include *Erigeron*, *Conyza*, and *Solidago*, which have been suggested to be related to *Nannoglottis* by Grau (1977), Grierson (1964), and Nesom (1994). The Astereae is the second largest tribe in the Asteraceae with over 170 genera and 3000 species worldwide (Bremer, 1994; Nesom, 1994). The Asterinae is the only subtribe of Nesom (1994) that is primarily Asian. Most genera in this subtribe in China (Ling and Chen, 1985) and the Himalayan area are closely related to *Aster* and *Kalimeris*, which have been included in the data matrix. The sampled genera cover 12 subtribes of Nesom (1994) and major evolutionary lines of Astereae (Zhang and Bremer, 1993) based on morphological analyses. They represent major lineages and include "basal" genera of the Astereae based on the molecular evidence over the world, but mainly from the Northern Hemisphere (Noyes and Rieseberg, 1999). Another reason why we had not sampled more genera for the ITS matrix is that the *ndh*F analysis placed *Nannoglottis* at the base of Astereae. In addition, the Qinghai-Tibet flora has a close relationship with North Hemisphere flora with temperate nature (Wu, 1987); therefore, we mainly tested the possible link of *Nannoglottis* with genera of Astereae distributed in this area.

The taxonomic revision of *Nannoglottis* is badly needed. From our specimen examination of the materials, the more recently described species of Ling and Chen (1965), *Nannoglottis macrocarpa*, might represent an extreme type of *N. hookeri.* Both species have a major overlap in distribution in southeast Tibet and Nepal and are difficult to discriminate morphologically in the field and the herbarium. The great variation of *N. hookeri* in the field had been detailed by Kitamura and Gould (1982). We temporally named our sequencing material from southeast Tibet as *N. macrocarpa* before a formal taxonomic revision is made for them. The type specimen of *N. souliei* belongs to *Nannoglottis gynura* while the specimens cited under *N. souliei* by Ling and Chen (1965) should be ascribed to *Nannoglottis yuennanensis*. All these and other unmentioned taxonomic problems made it difficult to name the collected materials. The preliminary names of materials studied were tentatively given by the second author, who is conducting a taxonomic revision of *Nannoglottis*. These sampled materials covered all morphological ranges of *Nannoglottis* especially in terms of characters used for the infrageneric classification, such as the layers of involucral bracts and length of ligules. The morphological characters of the sampled species are listed in Table 1.

All sequences of *Nannoglottis* and *trn*L-F sequence of *Tussilago* are first reported in the present study. Sequences of other taxa were downloaded from GenBank, mainly referred to Bayer and Starr (1998) for *trn*L-F, Kim and Jansen (1995) for *ndh*F, and Noyes and Rieseberg (1999) for ITS sequences. All GenBank accession numbers used in the analyses and their authorization are listed in Table 2.

2.2. DNA extraction, amplification, and sequencing

Total genomic DNA was isolated from silica-dried leaves following the CTAB method of Doyle and Doyle (1987). A sample of about 50 mg of leaf material from each of the specimens was used.

The *trn*L-F regions of cpDNA were amplified with primers e and f (Taberlet et al., 1991). The PCR was performed in a 25 μL volume, containing about 10–40 ng plant DNA, 50 mM Tris–HCI, 1.5 mM $MgCl_2$, 250 μg/mL BSA, 0.5 mM dNTPs, 2 μM of each primer, and 0.75 unit *Taq* polymerase. Initial template denaturation was programmed at 94 °C for 3 min, followed by 32 cycles of

Table 2
Accessions used in this study

Species	Tribe and subtribe	Source	GenBank Accession Nos.
*Trn*L-F region sequence			
Ageratum houstonianum Mill	Eupatorieae	GH-95011[a]	U82012, U82013
Antennaria luzuloides Torr. and A. Grays	Gnaphalieae	OR-91002[a]	U82014, U82015
Artemisia tribdentata Nutt.	Anthemideae	CO-90072[a]	U82016, U82017
Aster novae-angliae L.	Astereae	AB-95003[a]	U82018, U82019
Calendula officinalis L.	Calenduleae	GH-95009[a]	U82020, U82021
Cirsium subniveum Rydb.	Cardueae	WY-90044A[a]	U82024, U82025
Crepis tectorum L.	Lactuceae	AB-95002[a]	U82026, U82027
Doniophyton anomalum (D. Don) Wedd.	Barnadesieae	Stuessy et al. 12857[a]	U82028, U82029
Echinops exaltatus Schrad.	Cardueae	AB-95005[a]	U82030, U82031
Gaillardia aristata Pursh	Helenieae	GH-95006[a]	U82032, U82033
Gazania rigens R. Br.	Arctoteae	GH-95012[a]	U82034, U82035
Gerbera jamesonii Bolus ex Hook.	Mutisieae	GH-95004[a]	U82036, U82037
Helianthus annuus L.	Heliantheae	GH-95007[a]	U82038, U82039
Inula helenium L.	Inuleae	GH-95013[a]	U82040, U82041
Lactuca satira L.	Lactuceae	AB-95007[a]	U82042, U82043
Liabum solidagineum (Kunth) Less.	Liabeae	Dillon and Sánches 6253[a]	U82044, U82045
Matricaria matricarioides (Less.) Port.	Anthemideae	AB-95005[a]	U82046, U82047
Nannoglottis carpesioides Maxim.	Unknown	Liu Jianquan 538	**AY017153**
N. delavayi (Franch.) Ling and Y.L. Chen	Unknown	Gao Tiangang 99505	**AY017159**
N. gynura (C. Winkl.) Ling and Y.L. Chen	Unknown	H.B.G. 1942	**AY017154**
N. hieraciphylla (Hand.-Mazz.) Ling and Y.L. Chen	Unknown	Gao Tiangang 708	**AY017157**
N. latisquama Ling and Y.L. Chen	Unknown	Gao Tiangang 99605	**AY017156**
N. macrocarpa Ling and Y.L. Chen	Unknown	Gao Tiangang 99806	**AY017158**
N. ravida (C. Winkl.) Y.L. Chen	Unknown	Liu Jianquan 645	**AY017152**
N. yuennanensis (Hand.-Mazz.) Hand.-Mazz.	Unknown	Gao Tiangang 99407	**AY017155**
Osteospermum clandestinum (Less.) Norl.	Calenduleae	WA-94070[a]	U82048, U82049
Petasites frigidus (L.) Fr.	Senecioneae	Starr 96001[a]	U82050, U82051
Senecio vulgaris L.	Senecioneae	AB-95006[a]	U82052, U82053
Stokesia laevis Greene	Vernonieae	GH-95014[a]	U82054, U82055
Streptoglossa cylindriceps (J.M. Black) Dunlop	Inuleae	WA-94049[a]	U82056, U82057
Stuatrina muelleri Sond.	Gnaphalieae	Bayer s. n.[a]	U82058, U82059
Townsendia exscapa (Richard-son) Porter	Astereae	Co-93037[a]	U82062, U82063
Tussilago farfara L.	Senecioneae	Liu Jianquan 354	**AYAF468166**
*ndh*F gene sequence			
Achillea millefolium L.	Anthemideae	Kim and Jansen s. n.[b]	L39442
Anisothrix integra (Compton) A. Anderb.	Gnaphalieae	Kim and Jansen s. n.[b]	L39437
Antennaria neodioica Greene	Gnaphalieae	Kim and Jansen s. n.[b]	L39436
Arctotis stoechadifolia Berg	Arctoteae	Kim and Jansen s. n.[b]	L39425
Aster cordifolius L.	Astereae: Asterinae	Kim and Jansen s. n.[b]	L39449
Atractylodes japonica Koidz.	Cardueae	Kim and Jansen s. n.[b]	L39413
Baccharis neglecta Britton and A.Br.	Astereae: Bacchardinae	Kim and Jansen s. n.[b]	L39448
Barnadesia caryophylla S.F. Blake	Barnadesieae	Kim and Jansen s. n.[b]	L39394
Bellis perennis L.	Astereae: Bellidinae	Kim and Jansen s. n.[b]	L39446
Blennosperma nanum (Hook.) S.F. Blake	Senecioneae	Kim and Jansen s. n.[b]	L39433
Calendula officinalis L.	Calenduleae	Kim and Jansen s. n.[b]	L39439
Carlina vulgaris L.	Cardueae	Kim and Jansen s. n.[b]	L39412
Cichorium intybus L.	Lactuceae	Kim and Jansen s. n.[b]	L39390
Conyza sp.	Astereae: Conyzinae	Kim and Jansen s. n.[b]	L39451
Coreopsis tinctoria Nutt.	Heliantheae	Kim and Jansen s. n.[b]	L39461
Delairea odorata Lem.	Senecioneae	Kim and Jansen s. n.[b]	L39435
Eremothamnus marlothianus O. Hoffm.	Arctoteae	Kim and Jansen s. n.[b]	L39424
Erigeron hybridus Hieron.	Asterea: Conyzinae	Kim and Jansen s. n.[b]	L39450
Eupatorium atrorubens G. Nicholson	Eupatorieae	Kim and Jansen s. n.[b]	L39376
Felicia aethiopica (Burm.) Bolus and Wolley Dod ex Adams. and Salter	Astereae: Feliciinae	Kim and Jansen s. n.[b]	L39445
Flaveria ramosissima Klatt	Helenieae	Kim and Jansen s. n.[b]	L39465
Inula sericea Kit. Ex Kanitz	Inuleae	Kim and Jansen s. n.[b]	L39453
Liabum glabrum Hemsl.	Liabeae	Kim and Jansen s. n.[b]	L39421
Munnozia gigantea Rusby	Mutisieae	Kim and Jansen s. n.[b]	L39400

Table 2 (*continued*)

Species	Tribe and subtribe	Source	GenBank Accession Nos.
Nannoglottis gynura (C. Winkl.) Ling and Y.L. Chen	Unknown	H.B.G. 1942	**AY017151**
N. ravida (C. Winkl.) Y.L. Chen	Unknown	Liu Jianquan 645	**AY017150**
Nassauvia gaudichaudii Cass.	Mutisieae	Kim and Jansen s. n.[b]	L39405
Osteospermum muricatum E. Mey.	Calenduleae	Kim and Jansen s. n.[b]	L39440
Piptocarpha axillaris Baker	Vernonieae	Kim and Jansen s. n.[b]	L39431
Pyrrocoma sp.	Astereae: Machaerantherinae	Kim and Jansen s. n.[b]	L39447
Pluchea sericea Coville	Plucheeae	Kim and Jansen s. n.[b]	L39452
Santolina chamaecyparissus L.	Anthemideae	Kim and Jansen s. n.[b]	L39444
Sinclairia pringlei (B.L. Rob. and Greenm) H. Rob. and Brettell	Liabeae	Kim and Jansen s. n.[b]	L39422
Stokesia laevis Greene	Vernonieae	Kim and Jansen s. n.[b]	L39430
Syneileisis parmata L.	Senecioneae	Kim and Jansen s. n.[b]	L39432
Tragopogon porrifolius L.	Lactuceae	Kim and Jansen s. n.[b]	L39391
ITS sequence			
Achillea millefolium L.	Anthemideae	R.D. Noyes 1185[c]	GBANAF046939
Amellus strigosus (Thunb.) Less.	Astereae: Feliciinae	G. Germishuizen 4204[c]	GBANAF046942
Anaphalis margaritacea (L.) Benth. ex C.B. Cl.	Gnaphalieae	B. Boyle 368[c]	GBANAF046937
Aphanostephus ramosissimus DC.	Astereae: Brachycominae	E. Ventura 794[c]	GBANAF046990
Aster amellus L.	Astereae: Asterinae	A.K. Skvortsov s. n.[c]	GBANAF046961
Astranthium integrifolium (Michx.) Nutt.	Astereae: Brachycominae	D.E. Boufford 25607[c]	GBANAF046984
Baccharis dracunculifolia DC.	Astereae: Bacchardinae	M. Lewis 35355[c]	GBANAF046958
Bellis perennis L.	Astereae: Bellidinae	J.C. Solomon 8238[c]	GBANAF046950
Boltonia asteroides (L.) L'Her	Astereae: Asterinae	N.C. Henderson 94-1072[c]	GBANAF046975
Calendula officinalis L.	Calendulae	R.D. Noyes 1233[c]	GBANAF046938
Calotis dentex R. Br.	Astereae: Brachycominae	P.I. Forester 5075[c]	GBANAF046956
Chaetopappa bellioides (A. Gray) Shinners	Astereae: Feliciinae	R.D. Noyes 872[c]	GBANAF046980
Chiliotrichum diffusum (Forst.) O. Kuntze	Astereae: Hinterhuberinae	G.T. Prance 28630[c]	GBANAF046945
Chrysothamnus viscidiflorus (Hook.) Nutt.	Astereae: Solidagininae	J.D. Morefield 4008[c]	GBANAF046967
Commidendron robustum DC.	Astereae: Bacchardinae	H.H. Schimidt 664[c]	GBANAF046943
Conyza canadensis (L.) Cronq.	Astereae: Conyzinae	C. Ochs 248[c]	GBANAF046987
Crinitaria linosyris (L.) Less.	Astereae: Asterinae	A.K. Skvortsov s. n.[c]	GBANAF046949
Dichaetophora campestris A. Gray	Astereae: Brachycominae	G. Nesom 7552[c]	GBANAF046983
Diplostephium rupestre (H.B.K.) Wedd.	Astereae: Hinterhuberinae	L. Holm-Nielsen 28233[c]	GBANAF046962
Doellingeria umbellata (Miler) Nees	Astereae: Symphyotrichinae	H.H. Schimidt 1060[c]	GBANAF046966
Erigeron byei Sundberg and Nesom	Astereae: Conyzinae	R. Scott 477[c]	GBANAF046974
Euthamia graminifolia (L.) Nutt.	Astereae: Solidagininae	R.D. Noyes 1183[c]	GBANAF046982
Felicia aethiopica (Burm.) Bolus and Wolley Dod ex Adams. and Salter	Astereae: Feliciinae	J.P. Rourke 1918[c]	GBANAF046941
Geissolepis suaedifolia B.L. Robinson	Astereae: Brachycominae	G. Nesom 6634[c]	GBANAF046995
Grindelia lanceolata Nutt.	Astereae: Machaerantherinae	R. Rudman CO875[c]	GBANAF046976
Heterotheca villosa (Pursh) Shinners	Astereae: Chrysopsidinae	B. Stein 1823[c]	GBANAF046994
Hysterionica jasionoides Willd.	Astereae: Conyzinae	J. Conrad 2402[c]	GBANAF046986
Kalimeris integrifolia Turcz. ex DC.	Astereae: Asterinae	W. Wei 6003a[c]	GBANAF046960
Laennecia sophiifolia (Kunth) Nesom	Asterinae: Podocominae	L.L. Lopez 346[c]	GBANAF046964
Macharanthera pinnatifida (Hook.) Shinners	Astereae: Machearantherinae	C. Sherman 98[c]	GBANAF046977
Minuria integerrima (DC.) Benth.	Astereae: Podocominae	E.M. Canning 6313[c]	GBANAF046957
Monoptilon bellioides (A. Gray) H.M. Hall	Astereae: Feliciinae	G. Yatskievych 93-06[c]	GBANAF046981
Myriactis humilis Merr.	Astereae: Lageniferinae	T. Chiang 141[c]	GBANAF046959
Nannoglottis carpesioides Maxim.	Unknown	Liu Jianquan 538[c]	**AY017161**
N. delavayi (Franch.) Ling and Y.L. Chen	Unknown	Gao Tiangang 99505[c]	**AY017167**
N. gynura (C. Winkl.) Ling and Y.L. Chen	Unknown	H.B.G. 1942	**AY017162**
N. hieraciphylla (Hand.-Mazz.) Ling and Y.L. Chen	Unknown	Gao Tiangang 708	**AY017165**
N. latisquama Ling and Y.L. Chen	Unknown	Gao Tiangang 99605	**AY017164**
N. macrocarpa Ling and Y.L. Chen	Unknown	Gao Tiangang 99806	**AY017166**
N. ravida (C. Winkl.) Y.L. Chen	Unknown	Liu Jianquan 645	**AY017160**
N. yuennanensis (Hand.-Mazz.) Hand.-Mazz.	Unknown	Gao Tiangang 99407	**AY017163**
Olearia argophylla (Labill.) E. Muell. ex Benth.	Astereae: Hinterhuberinae	B.J. Conn 3071[c]	GBANAF046944
Oreostemma alpigenum (Torrey and A. Gray) E. Greence	Astereae: Symphyotrichinae	M. Merello 819[c]	GBANAF046978
Oritrophium hieracioides (Wedd.) Cuatr.	Astereae: Hinterhuberinae	J.C. Solomon 16570[c]	GBANAF046946

Table 2 (*continued*)

Species	Tribe and subtribe	Source	GenBank Accession Nos.
Pentachaeta aurea Nutt.	Astereae: Feliciinae	D. Bramlet 2367[c]	GBANAF046972
Podocoma notobellidiastrum (Griseb.) Nesom	Astereae: Podocominae	E. Zardini 3009[c]	GBANAF046963
Psiadia punctulata (DC.) Vatke	Astereae: Bacchardinae	E. Brusse 5607[c]	GBANAF046954
Pteronia incana (Burm.) DC.	Astereae: Hinterhuberinae	H. Joffe 850[c]	GBANAF046947
Sericocarpus tortifolius (Michx.) Nees	Astereae: Symphyotrichinae	M. Merello 429	GBANAF046969
Solidago petiolaris Ait.	Astereae: Solidagininae	N.C. Henderson 92-361[c]	GBANAF046968
Symphyotrichum oblongifolium (Nutt.) Nesom	Astereae: Symphyotrichinae	A.E. Brant 2704[c]	GBANAF046979
Townsendia florifer (Hook.) A. Gray	Astereae: Brachycominae	M. Merello 773[c]	GBANAF046985
Tracyina rostrata S.E. Blake	Astereae: Feliciinae	J. Strother 1363[c]	GBANAF046970
Ursinia nana DC.	Anthemideae	M. Bourell 2554[c]	GBANAF046940

Tribal classfication of Asteraceae follows Bremer (1994) and subtribal treatment of Astereae follows Nesom (1994).
Boldface sequences are first reported in the present study.
[a] Detailed by Bayer and Starr (1998).
[b] By Kim and Jansen (1995).
[c] By Noyes and Rieseberg (1999).

94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1.75 min plus a final extension of 72 °C for 7 min.

The internal forward primer (5′-TTGGGAATTG GTGGGAATGTG) and reverse primer (5′-TTCCT ATGGACCCAACGAAC) located near the two ends of the *ndh*F gene were used to amplify *ndh*F sequences by following Olmstead and Reeves (1995). Amplifications were performed in 25 μL with 25 ng plant DNA, 50 mM Tris–HCI, 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 2 μM of each primer ,and 0.75 unit *Taq* polymerase. The PCR was programmed for the initial template denaturation at 95 °C for 5 min, followed by 25 thermal cycles of 95 °C for 1 min, 45 °C for 1 min, and 72 °C for 2 min plus a final extension of 72 °C for 4 min.

The primers ITS4 and ITS5 (White et al., 1990) were used to amplify the ITS region sequences. The PCR was performed in a 25 μL volume, containing about 10–40 ng plant DNA, 50 mM Tris–HCI, 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 2 μM of each primer, and 0.75 unit *Taq* polymerase. The standard PCR program began with 2 min at 94 °C, 40 s at 92 °C, 40 s at 52 °C, 1 min at 72 °C, and followed by 30 cycles of 92 °C for 40 s, 55 °C for 40 s, and 72 °C 1.5 min plus a final extension of 72 °C for 5 min.

All double-stranded PCR products were purified using 1% agarose gel following the protocol of the Wizard Kit. The sequencing primers used for amplifying ITS and *trn*L-F were the same as those above. Except for the forward and reverse primers as used in the amplifying whole sequence, other three pairs of primers at the site 480, 972, and 1600 designed according to the published sequences in Asteraceae were further used to sequence the *ndh*F gene. The sequencing reactions were carried out in a Perkin–Elmer GenAmp model 9600 thermocycler using the Applied Biosystems (ABI) Tag DyeDeoxy Terminator Cycle Sequencing Kit following the suggested protocol. Sequencing products were separated and analyzed on an ABI 377 Automated DNA Sequencer. Both strands of DNA were sequenced through the use of forward and reverse primers. All sequences were recorded in both strands with an overlap of at least 70%.

2.3. Sequence alignment, boundary determination, and data analysis

The *trn*L-F and ITS sequence alignments were made with CLUSTAL W (Thompson et al., 1994) and refined manually. The *ndh*F sequences were aligned manually. All boundaries of the sequences were made by comparison to the published sequences of other genera of Asteraceae downloaded from GenBank. For the *trn*L-F region, we recovered a continuous sequence including the *trn*L intron, the *trn*L 3′ and 5′ exons (UAA), and the *trn*L/F intergenic spacer with the forward and reverse primers e and f (Taberlet et al., 1991) for all newly sequenced species. The sequences from GenBank registered by Bayer and Starr (1998) only comprise the *trn*L intron and the *trn*L/F intergenic spacer. In the final *trn*L-F matrix, the *trn*L 3′ and 5′ exons (UAA) with 61 bp were trimmed off. This short sequence showed no variation within *Nannoglottis*. The downloaded *ndh*F gene sequences from GenBank comprise the entire sequence of the gene through using the external forward and reverse primers to the gene by Kim and Jansen (1995). However, in the present study, the reverse primer was located at the internal end of the gene, so that 108 bp at the 3′ end of the gene was not obtained. This small part of the gene has shown little variation in the Asteraceae especially within Astereae and its absence in the data matrix had no impact on our assessing the systematic position of *Nannoglottis*.

Phylogenetic analyses were performed for all the three data sets by using PAUP 4.0 b (Swofford, 2000) with all characters unweighted. Heuristic parsimony searches were conducted with 100 replicates of random addition of sequences, in combination with ACCTRAN character optimization and MULPARS + TBR branch-swapping and STEEPEST DESCENT options on to search for

multiple islands of most parsimonious trees (Maddison, 1991). Bootstrap analyses (Felsenstein, 1985) were performed to assess the relative support for monophyletic groups. Bootstrap values were calculated from 1000 replicates by using a heuristic search with 10 subreplicate random addition with TBR and MULPARS options on. When gaps were treated as "NEW STATE," the topology of the strict concensus trees of three data sets had not changed, but bootstraps for some clades were greatly elevated. Because some gaps with more than 1 bp, which result from once insertion/deletion, will be weighted as one mutation site in the parsimony analysis, we treated all gaps as missing and rechecked the gap phylogeny information on the most parsimonious tree.

2.4. Divergence time

Comparisons of *ndh*F sequence divergence and the relative rate tests involved nine taxa of the Astereae and a reference taxon *Anisothrix* of Gnaphalieae. But only eight *Nannoglottis* species and a functional reference taxon *Olearia* were used to conduct divergence and the relative rate tests of ITS sequences. The nucleotide sequence divergences of *ndh*F were calculated for synonymous differences under Jukes and Cantor's one-parameter model, while those of ITS were made under Kimura's two-parameter model by following Kim et al. (1998). The relative rate tests were used to assess rate homogeneity between different lineages against the same reference taxon. Standard errors for estimated relative rate difference were used for significance tests to assess whether a molecular clock could be rejected. Time of divergence was calculated as the value of DNA sequence divergence divided by twice the sequence rate (Li, 1997). All these analyses were performed by MEGA 2.0 (Kumar et al., 2001).

3. Results

3.1. TrnL-F region data set

Length variation for *trn*L intron ranged from 424 to 453 bp and for the *trn*L/F intergenic spacer from 255 to 345 bp, corresponding to the reports by Bayer and Starr (1998). The ranges of nucleotide divergences for two fragments within the total family are also the same as in their reports. Within *Nannoglottis*, no variation was detected between *N. latisquama* and *Nannoglottis hieraciphylla* and between *Nannoglottis delavayi* and *N. macrocarpa*. The largest nucleotide sequence divergence, 2.26%, was found between *N. ravida* and *N. gynura* (Table 3). Most of the insertion and deletion patterns are the same as those described by Bayer and Starr (1998). Half of the indels are informative to support the clades in the following phylogenetic analyses. Five insertion/deletions (ranging from 1 to 5 bps) were identified within *Nannoglottis*. One deletion is found in all *Nannoglottis* species, which supports the monophyly of the genus. Three for *N. gynura* and one for *N. carpesioides* bear no phylogenetic information.

The aligned *trn*L-F region data set included 262 variable characters and 129 informative characters when gaps are excluded among the 839 aligned sites. Parsimony analysis produced 56 trees in one island with 421 steps, a consistency index of 0.770, and a retention index of 0.774. One of them is depicted as Fig. 1. Monophyly of *Nannoglottis* was well supported with a bootstrap of 98%. Some clades in the Asteraceae collapsed in the bootstrap test, but in all trees, *Nannoglottis* always grouped with *Aster* and *Townsendia* of the Astereae with a bootstrap support of 85%. The 50% consensus tree topology of all parsimonious trees corresponded well with that by Bayer and Starr (1998).

Two major clades were recovered within *Nannoglottis*: *N. ravida* and the other species. In the second clade, *N. carpesioides* and *N. yuennanensis* were identified as sister group with high bootstrap support of 98%. These groups, however, did not correspond with the existing infrageneric classifications of *Nannoglottis*. *N. gynura*, the only species with a 3–4-layered involucre, recognized as a separate section in the genus (Ling and Chen, 1965), shows a relationship with *N. carpesioides–N. yuennanensis* subclade of the second clade with a low bootstrap support of 59%.

Table 3
Nucleotide sequence divergence comparisons of eight species in *Nannoglottis*

Species	1	2	3	4	5	6	7	8
1 *N. ravida*	–	0.01191	0.01731	0.01583	0.00528	0.00528	0.00396	0.00396
2 *N. carpesioides*	0.09072	–	0.01864	0.00661	0.00926	0.00926	0.00795	0.00795
3 *N. gynura*	0.09601	0.01260	–	0.02264	0.01462	0.01462	0.01331	0.01331
4 *N. yuennanensis*	0.08354	0.00627	0.01259	–	0.01055	0.01055	0.01187	0.01187
5 *N. latisquamala*	0.08753	0.05064	0.05738	0.05059	–	0.00000	0.00132	0.00132
6 *N. hieraciphylla*	0.09857	0.05407	0.06434	0.05748	0.01593	–	0.00132	0.00132
7 *N. macrocarpa*	0.09285	0.06086	0.06768	0.06429	0.05748	0.05575	–	0.00000
8 *N. delavayi*	0.09504	0.03891	0.04557	0.03888	0.04412	0.05275	0.06110	–

Divergence values of ITS data based on Kimura's two-parameter are below the diagonal and divergence values of *trn*L-F region data are above the diagonal.

3.2. ndhF data set

The added sequences of two *Nannoglottis* species are similar to those from Astereae. Therefore, the characteristics of this data set correspond well with those reported by Kim and Jansen (1995). The *ndh*F sequence used for phylogenetic analysis furnished a total of 2100 sites. Of these characters, 627 variable sites were uninformative and 317 were informative (gaps excluded). Parsimony analysis identified 36 trees in one island with 1265 steps, a consistency index of 0.640, and a retention index of 0.633. The topology of the inferred tree agrees well with the subfamilial division and tribal relationship previously recovered from this gene data set by Kim and Jansen (1995). The monophyly of the groupings corresponding to the subfamily Asteroideae is supported by a bootstrap value of 99%. Within the subfamily, intertribal relationships are not well resolved and some subclades cannot be supported by the bootstrap test. In the strict consensus tree, Senecioneae comprises a separate clade and Astereae is placed in a clade with Gnaphalieae, Calenduleae, and Anthemideae. Liabeae is

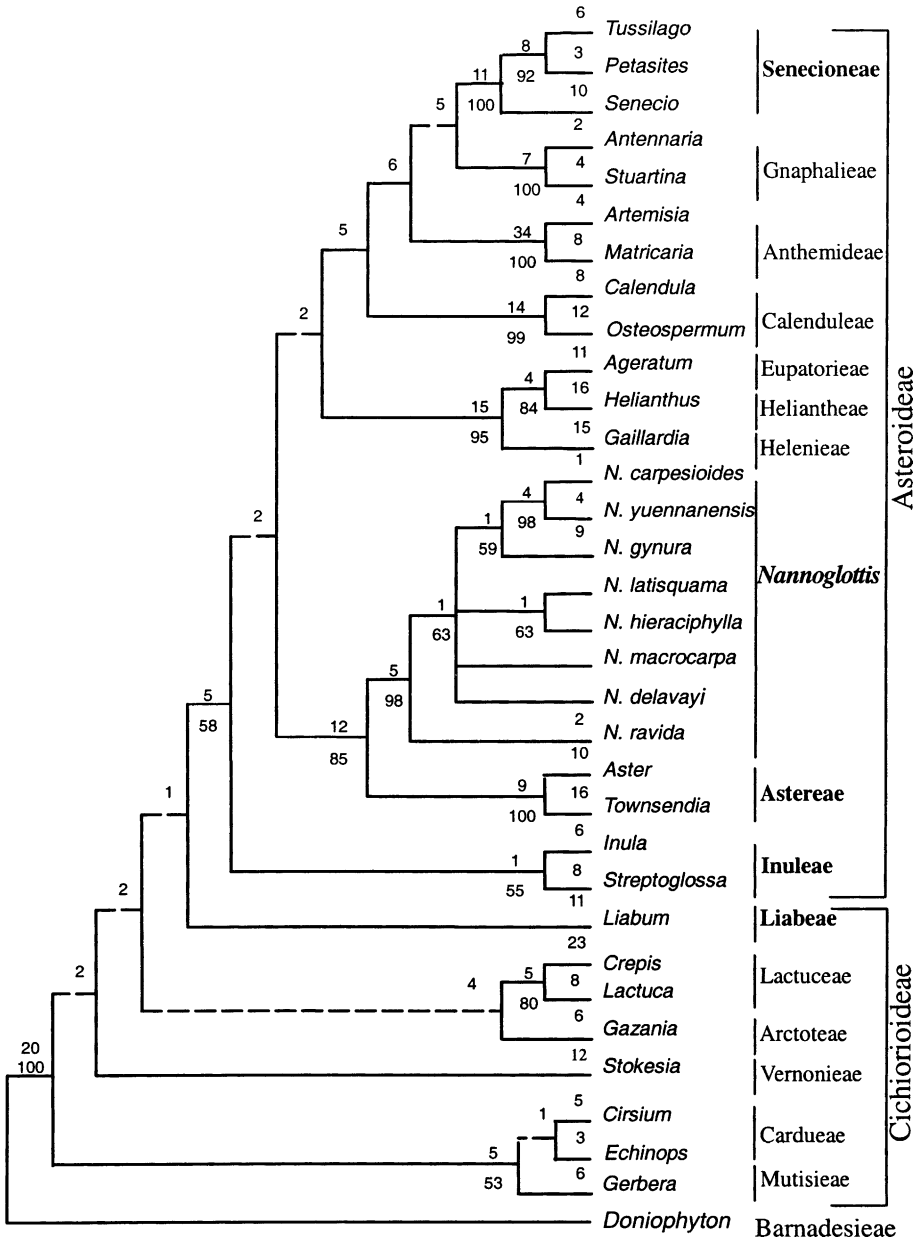

Fig. 1. One of the 56 equally parsimonious trees (length = 421 steps; RC = 0.770; RI = 0.774) from analysis of *trn*L-F sequence data of Asteraceae, showing the monophyly and position of *Nannoglottis*. Numerals above and below the branches indicate character and bootstrap support, respectively. Dashed lines denote branches that collapse in the strict consensus tree. Vertical bars indicate tribes and subfamilies following Bremer (1994). Boldface taxa are *Nannoglottis* and tribes previously hypothesized to be related to it.

sister group of Vernonieae with a bootstrap support of 78% in the subfamily Cichioriodeae.

Nannoglottis did not fall within clades representing Inuleae, Liabeae, and Senecioneae, but rather was placed at the first diverging lingeage of the Astereae (Fig. 2). The clade grouping *Nannoglottis* with Astereae was strongly supported by the bootstrap test (100%).

3.3. ITS data set

The aligned ITS encompassed a total of 677 sites. *Nannoglottis* showed a pairwise distance variation of 30–34% with four non-Astereae outgroups, 18–30% with Northern Hemisphere (Asia + North America) genera, and 14–22% with Southern Hemisphere genera. Within *Nannoglottis*, the small pairwise distance, detected between *N. carpesioides* and *N. yuennanensis*, is 0.63%. The largest nucleotide sequence divergence, 9.86%, was found between *N. ravida* and *N. hieraciphylla* (Table 3). Most of the insertion and deletion patterns in this data set are the same as those described by Noyes and Rieseberg (1999). More than half of the indels are informative and they increased the bootstrap value greatly when included in the phylogenetic analyses. When gaps were excluded, the range of ITS sequences used for phylogenetic analyses contained 407 variable sites among the total aligned, 336 of which were phylogenetically informative changes. Parsimony analysis produced 58 trees in two islands (48 in one island and 10 in the other) with 1796 steps, a consistency index of 0.40, and a retention index of 0.593. In all trees, *Nannoglottis* always comprised a distinct clade as the first diverging lineage of Astereae, showing no relationship with Eurasian or Northern Hemisphere genera (Fig. 3). The re-

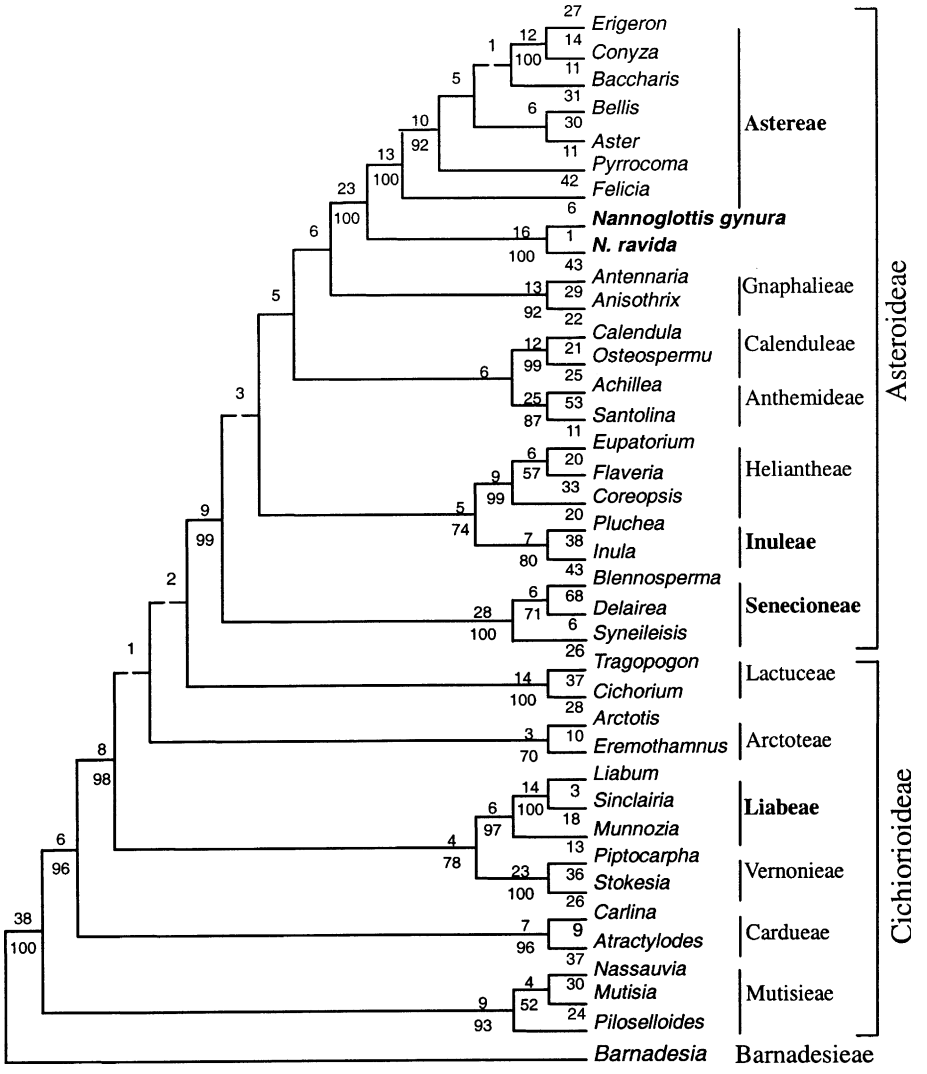

Fig. 2. One of the 36 equally parsimonious trees (length = 1265 steps; RC = 0.640; RI = 0.633) from analysis of *ndh*F sequence data of Asteraceae, showing the position of *Nannoglottis*. Numerals above and below the branches indicate character and bootstrap support, respectively. Dashed lines denote branches that collapse in the strict consensus tree. Vertical bars on the right indicate tribes and subfamilies following Bremer (1994). *Nannoglottis* and tribes previously hypothesized to be related to it are marked in boldface.

maining genera clustered into the other clade (bootstrap support = 74%). Three lineages of this clade recovered by Noyes and Rieseberg (1999) were identified in the present analysis: the "basal group," "Southern Hemisphere grade," and "North America clade." The "basal group" comprises seven genera from Australia, South America, and Africa, of which four genera have only woody species (Fig. 3) whilst the other three include woody, subwoody, and herbaceous species (Nesom, 1994; Noyes and Rieseberg, 1999). The terminal "North American clade" includes genera from North America and other areas of Northern Hemisphere. The "Southern Hemisphere grade" consists of the genera from both Southern Hemisphere and Northern Hemisphere, but

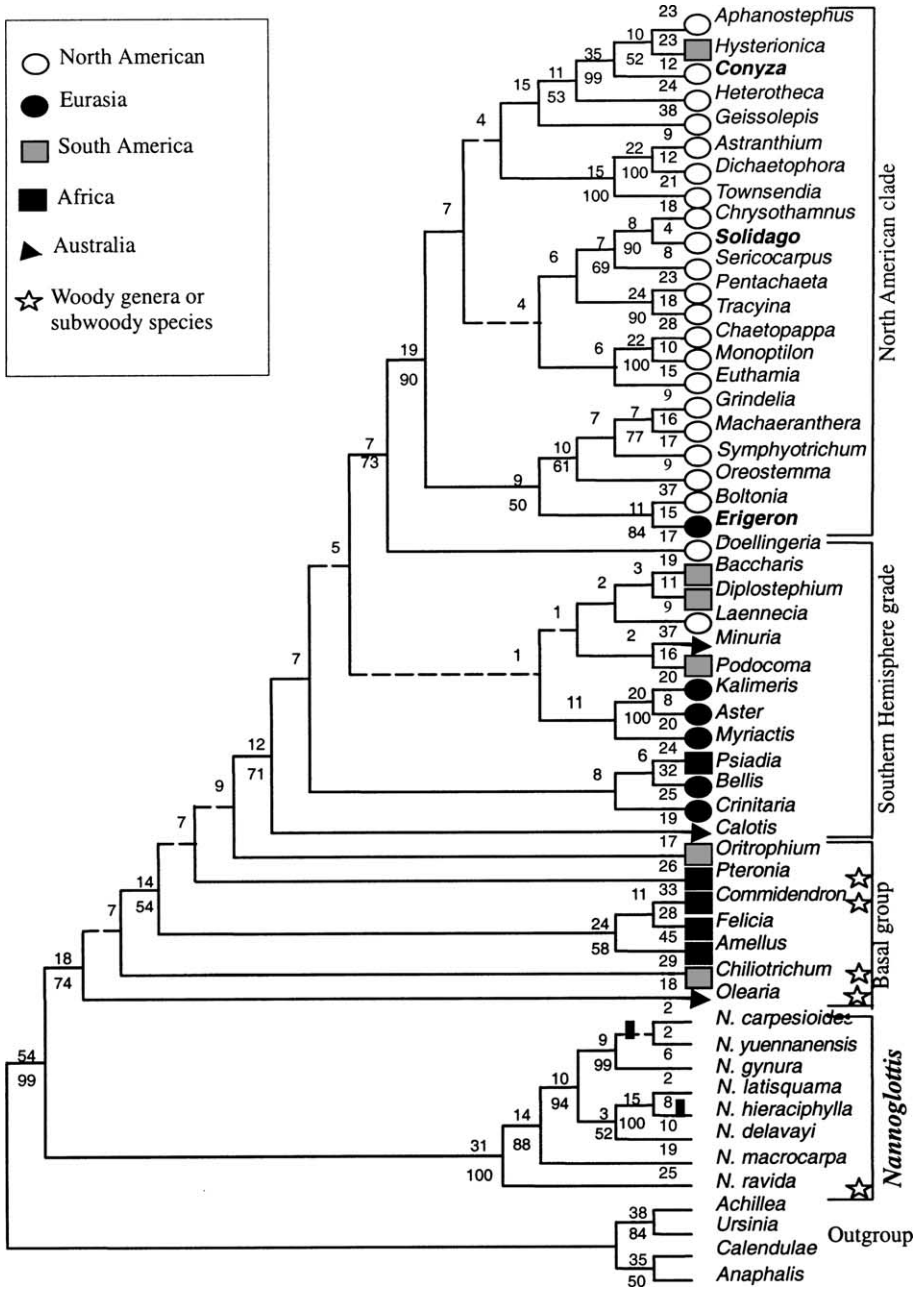

Fig. 3. One of the 58 equally parsimonious trees (length = 1796 steps; RC = 0.400; RI = 0.593) from analysis of ITS sequence data of Astereae, showing the position of *Nannoglottis*. Numerals above and below the branches indicate character and bootstrap support, respectively. Dashed lines denote branches that collapse in the strict consensus tree. Brackets on the right indicate *Nannoglottis* and three lineages recovered by Noyes and Rieseberg (1999): the "basal group," "Southern Hemisphere grade" and "North America clade." Solid bars (■) indicate paralleling evolution of the short ligules within *Nannoglottis*. The woody genera of the "basal group" or subwoody species in *Nannoglottis* and distributions of sampled genera are marked by symbols as indicated on the upper part of the figure. *Nannoglottis* and genera previously hypothesized to be related to it in Astereae are marked in boldface.

mainly from the former area. Our analyses agreed well with the results by Noyes and Rieseberg (1999). However, there are several minor topological differences between our tree and the tree recovered by them. These differences mainly result from different outgroup selections. In their phylogenetic analyses, an initial analysis was performed on seven non-Astereae outgroup taxa and a 16-taxa sample of Astereae to identify basal taxa within Astereae. For the latter analysis, they used the basal taxa as outgroups to root an expanded analysis of 55 taxa. We used four non-Astereae outgroup taxa to root 43 taxa of Astereae. However, these differences are unimportant given that the positions of most genera in the supported clades of two trees have not been changed and the aim of the present analysis was to identify the position of *Nannoglottis*.

Within *Nannoglottis* (Fig. 3), two distinct clades were recovered: one comprising a single species, *N. ravida*, and the other including the remaining species (bootstrap support = 88%), consistent with the *trn*L-F topology but with a high bootstrap support. These two clades correspond well with ecological habitats of *Nannoglottis*. *N. ravida* is a typical alpine shrub habitat species and the remaining species are distributed within coniferous forests. ITS data analysis further recovered three subgroups in the second "coniferous forest" clade. *N. macrocarpa*, comprising a distinct lineage, is sister to the other species. The remaining six species then fall into two subgroups: a subgroup of *N. carpesioides*, *N. yuennanensis*, and *N. gynura* and the other subgroup of *N. delavayi*, *N. hieraciphylla* and *Nannoglottis latisquamala*. The phylogeny inferred from ITS data rejected the traditional supraspecific classification of *Nannoglottis*. *N. gynura*, the only member of Sect. Stenolepis with 3–4 layers of involucral bracts grouped with *N. carpesioides* and *N. yuennanensis* with high bootstrap support. *N. yuennanensis*, *N. carpesoides*, and *N. hieraciphylla*, all with short ligules, could not be identified as a monophylogenetic group. These conclusive results are mainly in accord with the analyses from chloroplast *trn*L-F sequence data.

3.4. Divergence time

Most relative rate tests based on pairwise comparisons of Jukes and Cantor's one-parameter for *ndh*F sequence divergence did not reject the hypothesis of equal evolutionary rate among nine taxa of the Astereae in comparison with a reference taxon *Anisothrix*. The comparisons with *Erigeron* showed significant rate heterogeneity at the $P = 0.05$ level. Exclusion of *Erigeron* had little impact on the estimated divergence times between *Nannoglottis* and the remaining genera of Astereae. Therefore, the data set for estimating divergence times still included *Erigeron*. The clocklike evolution of *ndh*F sequences allowed us to estimate the divergence time between *Nannoglottis* and the other Astereae. An overall divergence rate of approximately 0.05 or 0.07% per Myr (million year) for *ndh*F was suggested by Selanen et al. (1997) and followed by Kim et al. (1998) in estimating the divergence times of Asteraceae. Based on these two rates, the divergence time of *Nannoglottis* and the remaining genera of Astereae was estimated as 22.86–32.00 Myr. Between two species within *Nannoglottis*, which represent "alpine shrub" and "coniferous forest" clades recovered by *trn*L-F and ITS data, the divergence time was 2.41–3.37 Myr.

Relative rate tests for ITS sequences within *Nannoglottis* based on Kimura's two-parameter did not reject the hypothesis of equal evolutionary rate in comparison with the reference taxon *Olearia* at a significance level of 5%. Given the apparent clocklike evolution of the ITS sequences within *Nannoglottis*, we approximated their divergence times by using a calibration rate of 1.57% per Myr. There has been considerable debate on rates of ITS sequences, ranging from 0.86 to 1.57% per Myr (Baldwin et al., 1995; Sang et al., 1994, 1995; Wendel et al., 1995). We chose a relatively fast rate of 1.57% sequence divergence per Myr in *Robinsonia* suggested by Sang et al. (1995) to estimate the divergence times within *Nannoglottis* for three seasons. First, *Robinsonia* and *Nannoglottis* are in the same family Asteraceae. Second, both are endemic to oceanic islands or island-like plateau. Third, species in both genera are herbs or subwood (*N. ravida*), thus, minimizing the effect of generation time on substitution rates. The time of the first divergence clade for *N. ravida* and the remaining species, "alpine shrub" vs. "coniferous forest," was estimated as 3.34 Myr, almost the same as 3.37 Myr estimated by *ndh*F sequence based on a rate of 0.05% per Myr. The smallest divergence time is 0.2 Myr between *N. carpesioides* and *N. yuennanensis*. The divergence times for remaining species range from 1.02 to 1.94 Myr according to the average K2P sequence pairwise differences in Table 3.

4. Discussion

4.1. Monophyly, tribal delimitation, systematic position, and close relatives of Nannoglottis

Both *trn*L-F region and ITS parsimonious tree (Figs. 1 and 3) data sets indicated that all sampled species of *Nannoglottis* form a well-supported monophyly. The genetic distances, 0–1.866% for *trn*L-F region data and 0.63–9.86% for ITS data (Table 3), fall within the infrageneric variation reported for Asteraceae (Bayer et al., 2000; Fernández et al., 2001; Noyes, 2000). Therefore, these molecular phylogenetic analyses fully support recent taxonomic treatments of *Nannoglottis* (Jeffrey and Chen, 1984; Kitamura and Gould, 1982; Ling and Chen,

1965), in which all the sampled species were placed in one re-circumscribed genus. In fact, the trimorphous flowers of *Nannoglottis*, a combination of ligulate florets, eligulate pistillate florets, and staminate disc florets within a capitula, rarely reported for the Asteraceae, are an unambiguous synapomorphy. Further morphological evidence for monophyly includes (1) stipitate-white tomentum vestiture on the stems, leaves, and phyllaries; (2) close, grayish-white tomentum particularly on the lower leaf surfaces; and (3) solitary capitula or few, relatively larger heads in a loose, terminal corymb. The undergoing chromosome comparison also supports the monophyly of the genus. All species have $2n = 18$ with one pair of stable st chromosomes, which can serve as the chromosomal markers of *Nannoglottis* (Gao et al., unpublished data; Liu et al., 2001).

Nannoglottis was grouped with Astereae with a high bootstrap support in both *trn*L-F and *ndh*F analyses (Figs. 1 and 2). These results rejected the relationships of *Nannoglottis* with Liabeae, Inuleae, and Senecioneae as suggested by various authors. Both *Nannoglottis* and *Liabum* have long and slender style and more than two layers of involucral bracts (Ling and Chen, 1965). These traits, however, have also occurred in other tribes of Asteraceae (Bremer, 1994). A relationship of *Nannoglottis* with Inuleae was suggested by Franchet (1896) based on habit and the inuloid leaf shape. However, these two features are extremely variable within a tribe and seem to be of little taxonomic value for assessing tribal delimitation (Bremer, 1994; Karis, 1993). The eligulate pistillate florets in *Homogyne* of Senecioneae are similar to the filiform florets of *Nannoglottis* (Ling and Chen, 1965). The staminate disc flowers of *Nannoglottis* are also found in *Tussilago* of Senecioneae (Ling, 1997). These two traits have also been used to suggest a morphological connection between *Nannoglottis* and Senecioneae. But the trimorphous flowers of *Nannoglottis*, which consist of ligulate, staminate disc, and filiform floret, are distinctively different from the "trimorphous flower" combination in *Homogyne* (ligulate, fertile disc, and eligulate pistillate florets), and in *Petasites* and *Tussilago* (ligulate, staminate, and fertile disc florets), and thus indicate their different origins.

Important morphological characters used to circumscribe Astereae and other tribes of Asteraceae are those features related to style, pollen, and achene (Bremer, 1987). Although staminate, the disc flowers of *Nannoglottis* show the Asteroid-type slender style with obtuse sweeping hairs extending from top to bifurcation (Liu, 2001). A combination of shape and distribution of stylar sweeping hairs of *Nannoglottis* is different from those of Senecioneae and Inuleae, but similar to that of Astereae. Senecioneae has obtuse stylar sweeping hairs at the truncated apex whilst the Inuleae is characterized by acute sweeping hairs covering the large part of the stylar branches. The "helianthoid" pollen type with internal foramina of *Nannoglottis* is one of the diagnostic features for Astereae (Grau, 1977; Nesom, 1994), which is different from the "senecioid" type in Inuleae and most genera of Senecioneae (Liu, 2000). *Doronicum* of Senecioneae also has the "helianthoid" pollen type. This feature seems to suggest a relationship between *Nannoglottis* and *Doronicum* of Senecioneae. But in the Asteroideae, the "helianthoid" pollen must have undergone convergent evolution while the "senecioid" type represents a plesiomorphic character state (Karis, 1993). Inclusion of *Doronicum* within the Senecioneae was further supported by the molecular evidence (Fernández et al., 2001). *Nannoglottis* has Asteroid achenes with a single-layered epidermis of cells thickened on three sides ("u"-cells) or less commonly all round (Jeffrey and Chen, 1984), but Grau (1980) found such a feature to also occur in other tribes. In addition, $x = 9$ of *Nannoglottis* is a common chromosome base number for Astereae, but different from $x = 10$ of Inuleae and Senecioneae (Liu et al., 2000). As pointed out by Nesom (1994), none of the listed diagnostic features of Grau (1977), e.g., Asteroid-type style and sweeping hairs "helianthoid" pollen, Asteroid achenes and $x = 9$, is restricted to Astereae and all can be found in other tribes. Astereae, in fact, is delimitated by a combination of these morphological characters. In general, morphological data available now for *Nannoglottis* agree well with such a combination. Therefore, tribal delimitation of *Nannoglottis* within Astereae inferred from the present molecular analyses parallels morphological evidence.

Unexpectedly, we could not identify the close relatives of *Nannoglottis* in Astereae. On both *ndh*F and ITS phylogenetic trees, *Nannoglottis* comprises a well-supported monophyly, but with an isolated position as the first diverging lineage of the tribe. Whether the genus consists the sister group to all remaining genera of Astereae cannot be answered because many genera in the tribe have not yet been sampled. The Astereae includes approximately 170 genera and more than 3000 species worldwide (Bremer, 1994), but most genera and species are centered in North America, Africa, South America, and Australia (Nesom, 1994; Noyes and Rieseberg, 1999). Although Grau (1977) and Nesom (1994) proposed that *Nannoglottis* should be placed in Astereae, the molecular data did not support their suggestions for its close relationship with *Erigeron*, *Conyza*, and *Solidago* (Fig. 3). A suggestion for the close relationship of *Nannoglottis* and *Erigeron* and *Conyza* was based on the trimorphous flowers (Grau, 1977). The fertile disc flowers in *Erigeron* and *Conyza* are distinctively different from the staminate disc florets of *Nannoglottis*, although they shared similar filiform florets and ligules out of the central disc flowers. A relationship of *Nannoglottis* and *Solidago* was guessed by Nesom (1994) based on leafy habit and shape, color and shape of ray flowers, and the

size of capitula, and achenes. The paralleling evolutions of these characters at higher taxonomic levels are well recorded in Asteraceae (Bremer, 1994), considerably weakening their indicative value. The isolated position of *Nannoglottis* within Astereae is further supported by the floral micromorphology (Liu, 2001). *Nannoglottis* has the polarized endothecial pattern while the other Astereae were found to be of the radial type. The polarized pattern must be regarded as a plesiomorphic character state in Asteroideae and the radial type must have undergone paralleling evolution among different tribes (Karis, 1993).

Despite the Northern Hemisphere links of most of the Qinghai-Tibet flora (Wu, 1987), we have not found a close relationship of *Nannoglottis* with any genus of Astereae from this area. Next to the *Nannoglottis* lineage on the ITS tree is the "basal group" of Astereae recovered by Noyes and Rieseberg (1999), which consists of seven genera from Southern Hemisphere, *Olearia*, *Chiliotrichum*, *Amellus*, *Felicia*, *Commidendron*, *Pteronia*, and *Oritrophium* (Fig. 3). *Olearia*, *Chiliotrichum*, *Commidendron*, and *Pteronia* are shrubs to small trees. *Felicia* and *Amellus* both comprise annuals to small shrubs. These genera are distributed on three continents of the Southern Hemisphere. Although as indirect relatives of *Nannoglottis*, they do show a relationship with genus if viewed from the ITS topological lineages and *Nannoglottis* can be further treated as one of the expanded "basal groups." This relationship is further enforced by the following evidence. First, although *Nannoglottis* is morphologically distinguished from the Asian Astereae, both *ndh*F and ITS phylogenies robustly recovered its position in Astereae. Second, *N. ravida*, sister to all other species, is a subshrub (see below), corresponding with the woody characteristics of the "basal group" (Fig. 3). Third, both *Nannoglottis* and *Pteronia* of the "basal group" have yellow and relatively large capitula. Fourth, the similar sterile disc flowers of *Nannoglottis* are found in some genera (e.g., *Oritrophium*) of the "basal group." Undoubtedly, some features, such as woody habits and yellow flowers, which link *Nannoglottis* and the "basal group" of Astereae, represent plesiomorphic states in Astereae.

4.2. Infrageneric phylogeny of Nannoglottis

The morphological features for the infragenetic phylogeny of *Nannoglottis* are listed in Table 1. *N. gynura* has 3–4 layers of involucral bracts while the other species of the genus have only 2–3 layers with the inner indistinct. According to the length of ligules, all species of *Nannoglottis* can be classified into two types, the short ligules in *N. carpesioides*, *N. yuennanensis*, and *N. hieraciphylla* and the long ligules in the remaining species. *N. ravida* is different from the remaining species with the solitary capitula and subwoody habit growing among the alpine shrubs. Ling and Chen (1965) delimited *N. gynura* as a separate section, Sect. Stenolepis Ling and Y.L. Chen, and the remaining species as the other section, Sect. *Nannoglottis*, based on disposition of involucral bract layers in their infrageneric classification of *Nannoglottis*. The latter section was further subdivided into two series: Ser. Delavayanae Ling and Y.L. Chen and Ser. Carpesioides (correctly Ser. *Nannoglottis*) according to the length of the ligules. The molecular analysis does not support this treatment. In the *trn*L-F and ITS phylogenetic trees (Figs. 1 and 3), *N. gynura* nested within the species with 2–3 layers of involucral bracts. *N. yuennanensis*, *N. carpesioides*, and *N. hieraciphylla* with short ligules grouped, respectively, with *N. gynura* and *N. latisquama* with the long ligules on both inheritance pathway trees. This strongly suggests that the infrageneric classification of *Nannoglottis* needs revision. On the other hand, the molecular phylogeny of *Nannoglottis* broadly agrees with the habits, the number of heads, and ecological preferences of its species (see Table 1). *N. ravida*, a subshrub with single head, comprises the sister clade to the other species. This species is an extremely endangered species with no more than 1000 individuals narrowly distributed under dry alpine shrubs among four sites in the Chengduo and Qumalai countries of Qinghai Province along the Long River. The remaining species, with several capitula arranged in a corymb, are more widely distributed perennials growing in the damp coniferous woods (such as *Picea* and *Pinus*) of the plateau. Three groups were further recovered for the latter clade on ITS tree (Fig. 3), but we failed to find morphological features to support such subdivision. The long ligule, the single head, 2–3-layered involucres, and the subwoody habit, must represent plesiomorphic states within *Nannoglottis* if assessed from the molecular infrageneric phylogeny. The short ligules must have undergone a paralleling evolution (Fig. 3). These implications correspond well with the comparison with the "basal group" or outgroup of Astereae.

4.3. Biogeography and evolution of Nannoglottis

Both *ndh*F and ITS tree recovered the "basal" position of *Nannoglottis* in Astereae. It is tempting to hypothesize that the Astereae arose in eastern Asia, rather than in Southern Hemisphere as has been suggested by most previous authors (Bremer, 1994; Nesom, 1994; Noyes and Rieseberg, 1999; Zhang and Bremer, 1993). We refuted this hypothesis based on two reasons. First, only *Nannoglottis* among the "basal group" of Astereae on the molecular tree is distributed in Asia whereas the other genera are in Southern Hemisphere. The morphological and geographical studies of Astereae further recovered most genera and species related to the "basal group" occurring in the latter area (Nesom, 1994). Such

a distribution pattern suggests that it is impossible that Astereae arose in Asia, but retained most taxa in a faraway Southern Hemisphere area. Second, the Asteraceae and its major lineages (tribes) have their origin in the ancient Gondwanaland area (Africa, South America, and Australia) based on all morphological, molecular, biogeographical, and fossil data (Bremer, 1987, 1994; Bremer and Gustafsson, 1997). Thus it seems unlikely that only the Asterae arose in eastern Asia while most tribes of Asteraceae closely related to it originated in Southern Hemisphere.

Following the rationale that Astereae arose from Southern Hemisphere, how did *Nannoglottis* reach the Qinghai-Tibet Plateau given the fact that these two areas are currently separated by such a long distance? The first hypothesis involves a vicariant origin of *Nannoglottis*. Because the India plate was a part of Southern Hemisphere, the simplest hypothesis in this regard is that *Nannoglottis* was rafted to the Qinghai-Tibet Plateau by the floating plate. This Asia-Southern Hemisphere biogeographical model has been applied to the origin of the Asian arowanas (Yoshinori and Mutsumi, 2000). Asian arowanas vicariantly diverged from Australasian arowanas in the eastern margin of Gondwanaland at the early Cretaceous and migrated into Eurasia on the Indian subcontinent or on smaller continental blocks. Under such a hypothesis, the divergence time of *Nannoglottis* should date back to the early Cretaceous. The India Plate separated from Gondwanaland at this time, then commenced its northward movement at about 100 Myr at the middle to late Cretaceous, and collided with Asia by the middle Eocene (Fang et al., 1995; Li et al., 1995; Shi et al., 1998). Nesom (1994) retained the similar opinion that the origin of the Astereae should be ancient, perhaps dating back to at least the middle to late Cretaceous. We believe that *Nannoglottis* could not have originated at Cretaceous and its current distribution was not associated with rafting of the India Plate for two reasons. First, the earliest angiosperm fossil record is from the early Cretaceous (Dilcher, 2000) and the derived position of Asteraceae in the angiosperm phylogeny suggests it could not have an origin older than the less derived taxa. Second, no species of *Nannoglottis* is currently distributed in India.

Instead of a vicariant model, an alternative assumption involves a dispersal origin of *Nannoglottis*. The long-distance dispersal origins, directly or through steppingstones, were recorded in disjunct endemics and their progenitors in Asteraceae (Baldwin et al., 1991; Francisco-Ortega et al., 1997, 2001; Kim et al., 1998; Panero et al., 1999). The direct long-distance origin without steppingstone was assumed in the endemic Hawaiian silversword alliance and its close relatives, which are disjunctly distributed in North American, separated by a 3900-km Pacific ocean (Baldwin et al., 1991). Another Hawaiian endemic genus, *Hesperomannia*, was identified to be closely related to African species of *Vernonia*, the long-distance dispersal of the genus assumed to use southeast Asia and southeast Pacific islands as steppingstones for the transportation of propagules by birds (Kim et al., 1998).

We cannot exclude a direct long-distance dispersal origin of *Nannoglottis* from the Southern Hemisphere without any steppingstone. The bracts of all *Nannoglottis* species are sticky with glandular hairs. The sticky bracts, and more important than that, the fruit appendages, are excellent dispersal adaptations, for migratory birds, which have been flourishing in the Qinghai-Tibet areas since the uplifting of the plateau (Shi et al., 1998). The best explanation for origin of *Nannoglottis* from Southern Hemisphere, we suggest, is a long dispersal using Southeast Asia as a steppingstone. This assumption is favored by two facts: (1) the flora of east Qinghai-Tibet Plateau is mainly derived from Southeast Asia and has a tropical link (Wu, 1980, 1987); (2) a part of Southeast Asia, the Burma-Malaya Plate, had been a part of the ancient Gondwana land (Audley-Charles, 1987). Such a hypothesis does not reject the possibility that *Nannoglottis* has a first vicariant origin on the Burma–Malaya Plate with Southern Hemisphere, but then a long-distance dispersal to the Qinghai-Plateau. Also it is possible that the dispersal is a gradual progress from Southern Hemisphere, to Southeast Asia and then to the plateau, but all the ancestors along the way died out because of changes in climate and habitats.

Because of the common occurrence of long-distance dispersal, ancient and recent origins are possible for endemics of Asteraceae. The time of origin has to be estimated using molecular calibration and guesses from the phylogenetic implication because of the lack of fossil record for most Asteraceae endemics. The Hawaiian Islands endemic *Hesperomannia* was estimated to be a 17–26 Myr ancient origin based on DNA sequence divergence (Kim et al., 1998). In contrast, the estimated time of divergence for the Macaronesian endemic *Argyranthemum* and its Mediterranean sister groups has always been less than 3 Myr based on all molecular ITS, isozyme, and cp DNA data (Francisco-Ortega et al., 1997). The recent origin was further guessed for Macaronesian *Pericallis* (Panero et al., 1999) based on its derived position in Senecioneae. Because the Qinghai-Tibet Plateau began to arise until the late Miocene (Shi et al., 1998), *Nannoglottis* seems to have a more recent origin than that. Despite this, we believe that *Nannoglottis* has an ancient origin, at least 23–32 Myr ago, as estimated from the *ndh*F sequence divergence for three reasons. First, unambiguous pollen fossils of Asteraceae were found at Paleocene–Eocene from South Africa (Zavada and Villiers, 2000). Most lineages of the family could have been in existence at low levels at this time, although without having diversified extensively. Second, in spite of the derived position of the tribe on the

morphological and molecular analyses (Bremer, 1987; Kim and Jansen, 1995), Astereae could not have originated after the Oligocene–Eocene border. Oligocene pollens of Asteraceae are specialized type, similar to current Astereae pollens, and were well recorded in several continents (Bremer, 1994; Bremer and Gustafsson, 1997). Third, the "basal" position of *Nannoglottis* recovered in all topologies indicates the fact that the genus should have its origin almost at the same time with the tribe Astereae.

The geological and paleobotanical studies on the Qinghai-Tibet Plateau area provide a good framework to develop a scenario regarding evolution and radiation of *Nannoglottis*. The Burma–Malaya Plate, a part of ancient Gondwana land, separated from the Australia Plate at the Eocene and joined the Eurasia Plate in South Asia in the Oligocene (Audley-Charles, 1987). The separation time of the Burma–Malaya Plate predates the origin time (23–32 Myr) of *Nannoglottis* estimated from DNA sequences. Its joining time with the Eurasia Plate was before the intensive uplift of the Qinghai-Tibet Plateau. Therefore, it is possible that *Nannoglottis* dispersed to Southeast Asia by the Burma-Malaya Plate and then to the Qinghai-Tibet area before the uplifting of the Qinghai-Tibet Plateau. From the Oligocene to Late-Miocene, the Qinghai-Tibet Plateau area was still covered by tropical or subtropical forests and the average altitude had not reached 1000 meters (Shi et al., 1998; Wu et al., 1995). The ancestors of *Nannoglottis* should have grown in such a habitat when they arrived at the Qinghai-Tibet area. The first large-scale uplifting of the Qinghai-Tibet Plateau occurred at about 3.4 Myr ago, which was accompanied by the largest glaciers in the North Hemisphere (Li et al., 1995). The divergence time, 3.34 or 3.37 Myr, estimated by both *ndh*F and ITS DNA sequences for two major clades, "alpine shrub" vs. "coniferous forest," in *Nannoglottis*, is in agreement with the extensive change of the climate resulted from this uplifting (Shi et al., 1998). The paleobotanical data of the Qinghai-Tibet Plateau indicate that the "coniferous forest" vegetation appeared at this stage because of the colder habitats than before (Wu et al., 1995). After that, the plateau strongly uplifted again about 2.5 Myr ago. At this stage, the largest ice age occurred through the Northern Hemisphere and glaciers developed in the major mountain chains of the Qinghai-Tibet area, which continued from late Pliocene of the Tertiary to the Quaternary (Fang et al., 1995). The third phase of uplifting occurred at about 1.6 Myr ago. This event led to a colder, drier climate and the formation of the modern river systems. The latest uplift was a turning point for climate change and vegetation replacements by alpine shrub, alpine meadow, and the expanded coniferous forests (Shi et al., 1998).

Geological studies demonstrated that a unified ice sheet did not cover the whole Qinghai-Tibet Plateau during the Quaternary Ice Age (Li et al., 1995; Shi et al., 1998). So, an entire biotic wipeout as suggested by Wulff (1944) was unlikely. The fossil faunas and biographical data also support this viewpoint (Yu et al., 2000). Two clades of *Nannoglottis* must both have survived the Pleistocene glaciation. But most ancestral species of *Nannoglottis* in the "alpine shrub" clade might have become extinct at the glacial period or when they at last entered the alpine habitats and the currently survived ancestor-like species *N. ravida* became extremely endangered and narrowly distributed. In contrast, the "coniferous forest" clade of *Nannoglottis* developed more species and wider distribution in the subsequent radiation. A major reason is that the coniferous forest rapidly expanded its distribution during interglacial periods or after the retreat of the ice in the Quaternary (Liu, 1988; Shi et al., 1998). Such a rapid speciation resulted also from the fast habitat isolation due to the climatic oscillation in combination with the second and third large-scaled uprising of the plateau. The divergence times of most current species at this clade, estimated from ITS sequences, from 1.02 and 1.94 Myr, correspond well with this hypothesis.

4.4. Origins of the Qinghai-Tibet Plateau endemics

The historical origin and evolution of *Nannoglottis* reject the general view that the Qinghai-Tibet Plateau flora had receded from or had been extinguished by the Quaternary ice sheet (Wulff, 1944) and all endemics in the plateau, originated in situ or from adjacently distributed genera with the uplift of the plateau (Wu, 1987). We must point out that these two assumptions are true for some endemic taxa of the plateau. Several recent molecular researches on plateau endemic genera not only confirmed these hypotheses, but also recovered a more interesting issue. The Qinghai-Tibet endemic *Milula* (Liliaceae), a monotypic genus represented by *Milula spicata*, differs from *Allium* by having a distinctly elongated, spicate inflorescence instead of the capitate or umbellate inflorescences of the rest of the genus. Molecular data recovered its close relationship with *Allium cyathophorum* of subgenus *Rhizirideum* (Friesen et al., 2000). The highly aberrant spicate inflorescence of *Milula*, which resulted in the difficulty of identifying its progenitor, is not paralleled by additional molecular evidence. Although the specific progenitor of *M. spicata* has not been identified, it is obvious that this species should be included in *Allium* subgenus *Rhizirideum* as *A. spicatum*. *Sinadoxa*, a monotypic genus endemic to the Qinghai-Tibet Plateau, was thought to have ambiguous relationship with Adoxaceae, Valeraceae of Dipsacales or other members of Araliales because of its unique and highly complicated inflorescence like a spike with several glomerate interrupted clusters. The result from the molecular study indicated its close relationship with

Adoxa (Liu et al., 2000). In contrast to its morphological distinctness, a very low ITS sequence divergence, 3.4%, was found between *Sinadoxa* and its progenitor *Adoxa*. This might indicate that *Sinadoxa* is derived more recently than expected, without sufficient time to accumulate adequate mutational differences. The third example involves the plateau endemic *Lomatogoniopsis* in Gentianaceae (Liu et al., 2001). *Lomatogoniopsis* is distinctly from *Lomatogonium* in having protruding glands at the corolla base and non-vascularized scale at the inner lobes. Despite the distinctive difference in corolla morphology, very low genetic mutations based on ITS sequences (less than 2%) were detected between them. Undoubtedly these endemics originated in situ with the formation of the plateau since the Pliocene. We note that all the superficially very distinctive characters used for the recognition of these apparently distinct endemics are related to their flowers or inflorescences. Kadereit (1994) suggested that such characters might have arisen by single macromutation of the structural genes. Because of selective advantages or because they occurred in small and isolated populations, these mutations were brought to fixation. Up to now, no data regarding the selective advantages could be obtained for these endemics. The rich geological and ecological diversities of the Qinghai-Tibet Plateau, together with habitat isolation due to changing climatic conditions during the uplift of the plateau, might well promote rapid speciation in small, isolated populations, thus, allowing the fixation of peculiar or rare morphological, especially floral characters (Friesen et al., 2000). Recent studies indicate that dramatic shifts in organismal structure in some taxa can arise from mutations at key regulatory loci, rather than structural genes (Doebley and Lukens, 1998), and the regulatory gene was found to have an accelerated evolution in an adaptive radiation (Barrier et al., 2001). We cannot exclude the possibility that the distinctive morphology of these plateau endemics resulted from the accelerated regulatory gene evolution in their adaptive radiation. The rapid diversification in morphological features, which means discordant evolutionary tempos at molecular and morphological levels, remains to be explained for these endemics, but provides an extremely good opportunity to study the rapid morphological speciation (such as selective advantages and lineage sorting) under the fierce selection pressure.

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