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Radiation and diversification within the *Ligularia–Cremanthodium–Parasenecio* complex (Asteraceae) triggered by uplift of the Qinghai-Tibetan Plateau

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Abstract

The *Ligularia–Cremanthodium–Parasenecio* (*L–C–P*) complex of the Tussilagininae (Asteraceae: Senecioneae) contains more than 200 species that are endemic to the Qinghai-Tibetan Plateau in eastern Asia. These species are morphologically distinct; however, their relationships appear complex. A phylogenetic analysis of members of the complex and selected taxa of the tribe Senecioneae was conducted using chloroplast (*ndh*F and *trn*L-F) and nuclear (ITS) sequences. Phylogenetic trees were constructed from individual and combined datasets of the three different sequences. All analyses suggested that *Doronicum*, a genus that has been included in the Tussilagininae, should be excluded from this subtribe and placed at the base of the tribe Senecioneae. In addition, the Tussilagininae should be broadly circumscribed to include the Tephroseridinae. Within the expanded Tussilagininae containing all 13 genera occurring in eastern Asia, *Tussilago* and NS*Petasites* diverged early as a separate lineage, while the remaining 11 genera comprise an expanded *L–C–P* complex clade. We suggest that the *L–C–P* clade, which is largely unresolved, most likely originated as a consequence of an explosive radiation. The few monophyletic subclades identified in the *L–C–P* clade with robust support further suggest that some genera of Tussilagininae from eastern Asia require generic re-circumscriptions given the occurrence of subclades containing species of the same genus in different parts of the phylogentic tree due to homoplasy of important morphological characters used to delimit them. Molecular-clock analyses suggest that the explosive radiation of the *L–C–P* complex occurred mostly within the last 20 million years, which falls well within the period of recent major uplifts of the Qinghai-Tibetan Plateau between the early Miocene to the Pleistocene. It is proposed that significant increases in geological and ecological diversity that accompanied such uplifting, most likely promoted rapid and continuous allopatric speciation in small and isolated populations, and allowed fixation or acquisition of similar morphological characters within unrelated lineages. This phenomenon, possibly combined with interspecific diploid hybridization because of secondary sympatry during relatively stable stages between different uplifts, could be a major cause of high species diversity in the Qinghai-Tibetan Plateau and adjacent areas of eastern Asia.

Keywords: Radiation; Diversification; Qinghai-Tibetan Plateau; Molecular systematics; Senecioneae; Tussilagininae; Eastern Asia

1. Introduction

A central goal of the study of biological diversity is to understand why different regions with similar environments contain different numbers of species (Qian and Ricklefs, 2000; Qian et al., 2005). Determining the causes of high biodiversity in some regions is of primary importance in biology and a principal aim of biogeographic research (Willis and Niklas, 2004; Willis and Whittaker, 2002). Molecular phylogenetic reconstructions of evolutionary relationships between living organisms are increasingly used to infer

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these putative causes of diversification within an historic and geographic context (Avise, 2000). Recent studies show that high numbers of plant species within regions might be due in part to bursts of speciation that occurred during the last few million years triggered by major geophysical and/or climate change (Richardson et al., 2001a,b), and that a significant proportion of plant diversity originated during the late Tertiary, i.e., since approximately 10 million years ago (Willis and Whittaker, 2002). However, the number of studies conducted on species rich floras remains low with most centered on groups in the Southern Hemisphere (Pennington et al., 2004). Several areas recognised as biodiversity hotspots in the Northern Hemisphere (Myers et al., 2000; Wilson, 1992), have yet to be subjected to detailed investigation. Here, we report the first molecular phylogenetic investigation of the history and evolution of a component of the flora of the Qinghai-Tibetan (Q-T) Plateau.

The Q-T Plateau is the highest and largest plateau in the world, having a mean elevation of ~4.5 km and an area of 2.5×10^6 km^2 (Zheng, 1996). The eastern part of this region and the adjacent area of southeast China has been listed as one of the world's 25 or 34 biodiversity hotspots, based on species richness and greatest danger of anthropogenic extinction (Myers et al., 2000; Wilson, 1992; http://www.biodiversityhotspots.org/xp/Hotspots). The Q-T Plateau contains more than 12,000 species of plants in more than 1500 genera, and it is estimated that about 50 genera and more than 20% of the total species are endemic to this region (Wang et al., 1993; Wu and Wu, 1996). Although levels of plant diversity and endemism in this region are much less than those of the Cape flora (Linder, 2003) and tropical rainforests (Richardson et al., 2001a), the flora is more speciose than might be expected based on comparisons made at similar latitudes in the Northern Hemisphere (Wu and Wu, 1996). For example, the Q-T flora contributes to the high plant diversity in eastern Asia (Wang et al., 1993; Wu, 1988; Wu and Wu, 1996), which is roughly twice as rich as that of eastern North America, a region of similar area and climate (Qian et al., 2005). The high species richness of the flora of the Q-T Plateau and adjacent areas has been attributed to two major factors (Axelrod et al., 1996). One hypothesis is that an unbroken gradient of vegetation from tropical rain forest to boreal coniferous forests was maintained in the region and adjacent areas throughout the Quaternary when massive extinctions occurred elsewhere in the Northern Hemisphere. This therefore acted as a major refugium for organisms in the region during the period of marked climatic oscillation. The other scenario assumes that accelerated speciation occurred following the collision of the Indian subcontinent with Asia commencing about 40 Ma. Some ancient taxa, i.e., Trochodendraceae, Cecidiphyllaceae, Eucommiaceae, and several primitive genera found in the area, are monotypic or contain few species (Wang et al., 1993; Wu, 1988; Wu and Wu, 1996), indicating that the existence of Quaternary refugia might not have played an important part in generating great species richness despite having maintained some ancient groups.

The uplift of the Q-T Plateau began approximately 40 million years ago (Ma) (Chung et al., 1998) following the collision of India with Asia. Recent evidence indicates that the southern margin of the plateau reached its present elevation approximately 15 Ma (Spicer et al., 2003), if not earlier (22 Ma) (Guo et al., 2002), with the total plateau being uplifted to its present altitude by 7–8 Ma (Harrison et al., 1992) or more recently during the late Pliocene and early Pleistocene (Shi et al., 1998). These uplifts since the early Miocene have created high mountains and deep valleys within the plateau (Li et al., 1995), which could have accelerated the production of new species in allopatry, and been partly responsible for the high local and regional species richness. To investigate this possibility, we have conducted a phylogenetic analysis of the *Ligularia–Cremanthodium–Parasenecio* complex (hereafter referred to as the *L–C–P* complex) and possible allies that comprise the subtribes Tussilagininae and Tephroseridinae of the tribe Senecioneae (Asteraceae). This group exhibits high species richness in the region and in adjacent eastern Asia (Liu, 2001, 2004).

Senecioneae, the largest tribe in the Asteraceae with ~3200 species and ~120 genera (Bremer, 1994), has been the subject of much debate with regard to its phylogenetic composition. Nordenstam (1977) recognized two subtribes: Blennospermatinae and Senecioninae, while Jeffrey and Chen (1984) divided the Senecioneae of eastern Asia into three subtribes: Senecioninae, Tussilagininae, and Tephroseridinae. Bremer (1994) incorporated the Tephroseridinae into Tussilagininae, and acknowledged Blennospermatinae and Senecioninae as additional subtribes. But this treatment was rejected by Chen (1999) who maintained the Tussilagininae and the Tephroseridinae as separate subtribes. The *L–C–P* complex of the Tussilagininae is composed of ~120 species of *Ligularia*, ~70 species of *Cremanthodium*, ~60 species of *Parasenecio* plus six monotypic or small satellite genera, i.e., *Farfugium*, *Syneilesis*, *Ligulariopsis*, *Sinacalia*, *Miricacalia*, and *Dendrocacalia* (Chen, 1999; Jeffrey and Chen, 1984; Liu, 1989, 2001, 2004). Species of *Ligularia* occur in a great variety of habitats in the Q-T plateau region from forests to high alpine meadows, at elevations ranging from 1000 to 4000 m. *Cremanthodium* species occur in alpine meadow and scree areas at altitudes ranging from 2400 to 5600 m, while most species of *Parasenecio* are restricted to coniferous forests. More than 200 species in the complex are endemic to the Q-T Plateau (Liu, 2004) and comprise a typical group which exhibits great diversification in this region (Wu and Wu, 1996). Most endemics are restricted to small hills or valleys, and occur either allopatrically or occasionally sympatrically. These endemics are morphologically well defined and easily recognized in the field (Chen, 1999; Liu et al., 1994, 2002b). However, generic circumscriptions are extremely ambiguous, especially between members of *Ligularia*, *Parasenecio*, and *Cremanthodium* (Liu, 2001; Liu et al., 2001), due to a lack of diagnostic morphological traits (Liu, 2001, 2004). This may reflect possible bursts of recent speciation and random fixation of similar morphological features among

unrelated lineages. Two small satellite genera, *Ligulariopsis* and *Sinacalia*, of the three large genera also occur mainly in the Q-T Plateau (Chen, 1999; Liu, 2001, 2004). *Ligulariopsis*, a monotypic genus, is distinguished from the three speciose genera in having a morphological combination of radiate capitula and none-vaginate leaf sheathing, while the latter genus comprising four species, differs by having a morphological combination of radiate capitula, none-vaginate leaf sheathing and tuberiform rhizomes (Chen, 1999; Jeffrey and Chen, 1984; Liu, 2001, 2004). The five genera comprising the core components of the *L–C–P* complex mainly distributed in the Q-T Plateau have similar morphology and their delimitation is unclear. Of the remaining four satellite genera, *Farfugium* and *Syneilesis* occur from central China to Japan, while *Miricacalia*, and *Dendrocacalia* are endemic to Japan. The relationships of the complex to other genera of the Tussilagininae of eastern Asia, i.e., *Tussilago*, *Petasites*, and *Doronicum,* and to genera of the Tephroseridinae, i.e., *Sinosenecio*, *Tephroseris*, *Nemosenecio*, are not well established. Both floral microcharacters and chromosomal data suggest that the *L–C–P* complex is more closely related to some species of three genera of the Tephroseridinae than to the remaining genera of the Tussilagininae (Liu, 2001, 2004).

Subtribal relationships in the Senecioneae remain poorly known despite the accumulation of molecular data for the group within recent years (e.g., Bain and Golden, 2000; Comes and Abbott, 2001; Fernandez et al., 2001; Pelser et al., 2002, 2003). Blennospermatinae has been widely assumed to be the basal group of the Senecioneae (Bain and Golden, 2000; Bremer, 1994; Pelser et al., 2002); however, Swenson and Bremer (1999) found that *Abrotanella*, a genus of the Blennospermatinae, is only weakly (one step) associated with four sampled genera (*Blennosperma*, *Syneilesis*, *Senecio*, and *Lopholaena*) of the Senecioneae, casting doubt upon which genus is basal to the tribe. *Doronicum* has traditionally been placed in the Tussilagininae based on its cylindrical anther-collars and $x = 30$, suggesting a close relationship with the *L–C–P* complex (Bremer, 1994; Chen, 1999; Jeffrey and Chen, 1984). However, its "Helianthoid" pollen and small chromosomes indicate an aberrant position in this subtribe (Liu, 2001, 2004). Recently, Fernandez et al. (2001) placed it at the base of the sampled genera of the Senecioneae, sister to a clade containing *Blennosperma*, *Lopholaena*, *Senecio*, and *Syneilesis* (one genus of the *L–C–P* complex). These findings suggest that the traditionally circumscribed Asian Tussilagininae might not be monophyletic. However, except for these aberrant genera, other genera of Senecioneae that occur out of Asia have been shown to form two monophyletic clades: the Senecioninae group and the Tussilagininae group (Bain and Golden, 2000; Panero et al., 1999; Pelser et al., 2002, 2003). Although not all non-Asiatic genera of Senecioneae have been examined, the available morphological traits indicate that most un-sampled genera fit well within the Senecioninae and Tussilagininae groups (Jeffrey, 1992). Most genera of Senecioneae whose phylogenetic position is unresolved occur in eastern Asia, within two subtribes: the Tussilagininae and the Tephroseridinae (Chen, 1999; Jeffrey, 1992; Liu, 2001). Therefore, our sampling strategy focused on the *L–C–P* complex, but extended to cover the most representative genera of the Tussilagininae, the Tephroseridinae and a few of the Senecioninae in eastern Asia.

Following a survey of newly sequenced chloroplast and nuclear DNA data of representative species of the *L–C–P* complex and related genera of the Senecioneae, we aimed to (1) evaluate the relationship of the *L–C–P* complex to the Tephroseridinae, and to refine its circumscription in eastern Asia; (2) examine the generic delimitation of the complex against the previous classification based on morphological characters; and (3) determine underlying causes of the radiation and diversification within the *L–C–P* complex, which might be correlated with past geological changes in the Q-T plateau.

2. Materials and methods

2.1. Sampling strategy, plant materials, and datasets

Our sample of species within the *L–C–P* complex included 20 species representing eight of the nine sections in *Ligularia* and *Cremanthodium*, five species representing three of five sections in *Parasenecio*, and eight species representing the satellite genera: *Sinacalia*, *Ligulariopsis*, *Farfugium*, *Syneilesis*, *Miricacalia*, and *Dendrocacalia* from eastern Asia (Fig. 1). We further sampled four species representing the other three genera of Tussilagininae: *Doronicum*, *Tussilago*, and *Petasite*. Only the genus*Diceroclados* of the Tussilagininae in eastern Asia was excluded from the present analysis. This was due to the unavailability of material. Five species representing all three genera of the Tephroseridinae, i.e., *Sinosenecio*, *Tephroseris*, and *Nemosenecio*, and 12 species representing *Senecio* and *Synotis* of the Senecioninae were also included in the analysis. Except for *Dendrocacalia*, *Miricacalia*, and *Nemosenecio*, whose leaves were collected from herbaria specimens, leaves of species were collected directly in the field and dried with silica gel. The origins of material are listed in the Table 1. Voucher specimens have been deposited in the Northwest Plateau Institute of Biology, Chinese Academy of Sciences, China.

Molecular datasets were produced for *ndh*F, *trn*L-F and ITS DNA sequences, although not all of these sequences were available for all species examined due to failure of amplification in certain species. Accession numbers for new sequences are also listed in Table 1. Additional sequences reported previously (Kim and Jansen, 1995; Liu et al., 2002a; Sang et al., 1995) were downloaded from GenBank.

New sequences of *ndh*F were obtained for 40 species of 12 genera of the Tussilagininae and the Tephroseridinae from eastern Asia and 12 species representing the other two genera of the Senecioninae. The *ndh*F gene sequence is considered particularly useful for inferring phylogeny at and below family level within the Asteraceae (Kim and Jansen, 1995; Olmstead et al., 2000). This sequence is longer and

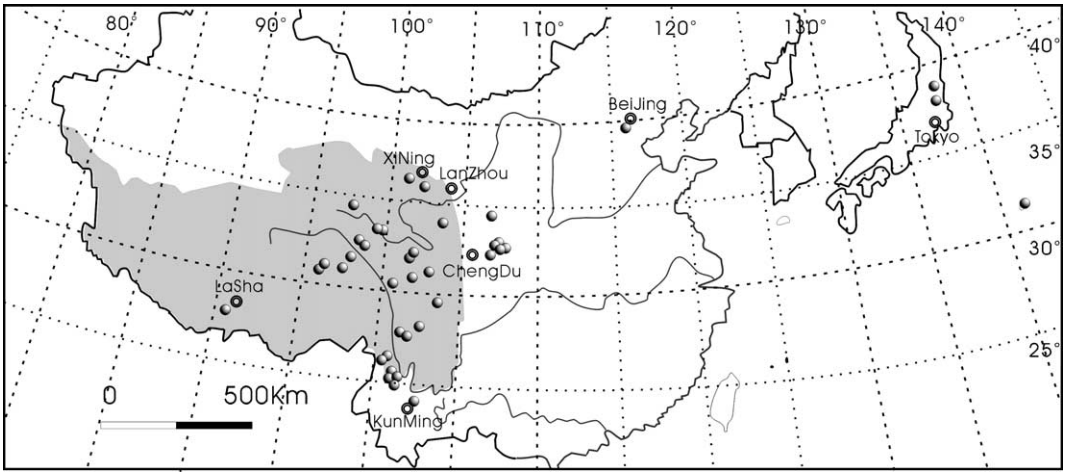

Fig. 1. Locations (indicated as circles) from which taxa of the *Ligularia–Cremanthodium–Parasenecio* complex and related genera of the Tussilagininae and Tephroseridinae were sampled. The shaded area indicates the Qinghai-Tibetan Plateau.

potentially contains more phylogenetic information than other chloroplast sequences, such as *rbc*L and *trn*L-F. Thus, *ndh*F has been found to provide sufficient informative characters to circumscribe tribes and refine the position of problematic genera in the Asteraceae (e.g., Panero et al., 1999; Francisco-Ortega et al., 1997, 2001). Based on the established intertribal frame in the family (Kim and Jansen, 1995), additional *ndh*F sequences for nine other genera, representing eight additional tribes of Asteraceae, were downloaded from GenBank and added to the data matrix. *Barnadesia* (sub-family Barnadesieae) is assumed to be the basal group of Asteraceae according to all analyses based on both morphological and molecular evidence (e.g., Bremer, 1994; Kim and Jansen, 1995) and therefore was selected as outgroup in the analysis.

The *ndh*F dataset was so formed to establish a phylogenetic frame of the Senecioneae and provide a preliminary assessment of the circumscription and monophyly of both the Tussilagininae and the *L–C–P* complex from eastern Asia. A *trn*L-F dataset was also generated to evaluate tribal circumscription and intertribal relationship in the Asteraceae (e.g., Bayer and Starr, 1998; Bayer et al., 2000), but failed to yield a discernible phylogeny for either intertribal relationships of Asteraceae or circumscription of the Senecioneae.

A subsequent chloroplast *trn*L-F sequence dataset limited to Senecioneae species was constructed for analysis. This dataset included 38 species of the *L–C–P* complex and related genera of the Tussilagininae, Tephroseridinae, and Senecioninae. Finally, a dataset was generated comprising rDNA ITS sequences of 25 species of the *L–C–P* complex, 13 species of other Asian Tussilagininae and Tephroseridinae genera, and 6 species of the Senecioninae. Sequences covered both ITS1 and ITS2, but excluded the 5.8S subunit which had been found to be invariant. Analyses were conducted separately on all three datasets, and then on combinations of the *trn*L-F and *ndh*F datasets, and the ITS, *trn* L-F and *ndh*F datasets for those species for which sequences were available. We initially obtained *trn*L-F and ITS sequences for three or more accessions in each of 14 species, i.e., *Cremanthodium humile*, *C. ellisii*, *C. lineare*, *C. discoideum*, *Ligularia. przewalskii*, *L. tsangchanensis*, *L. purdomii*, *L. dentata*, *L. sagitta*, *L. virgaurea*, *L. rumicifolia*, *Sinacalia tangutica*, *Tephroseris rufa*, and *Farfugium japonicum*. Either no or low variation (i.e., 1–2 bp differences) was found among accessions within species except *L. virgaurea*. Consequently, accessions grouped together as monophylogenetic species clades in an initial analysis. Thereafter, we selected one accession of each species for subsequent analyses. In *L. virgaurea*, four different *trn*L-F sequences and five different ITS sequences were recorded among 102 individuals from 11 populations. This species was excluded from further analysis.

2.2. DNA extraction, amplification, and sequencing

Total genomic DNA was isolated using the CTAB method of Doyle and Doyle (1987). Amplification of the *ndh*F gene was carried out using primers and procedures described by Liu et al. (2002a). PCRs of 25 μl contained 25 ng plant DNA, 50 mM Tris–HCl, 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 2 μM of each primer and 0.75 U of *Taq* polymerase. The reaction profile used was: 1 cycle at 95 °C for 5 min; 25 cycles at 95 °C for 1 min; 45 °C for 1 min; and 72 °C for 2 min; 1 cycle at 72 °C for 4 min. The *trn*L-F region of cpDNA was amplified with primers e and f (Taberlet et al., 1991). PCRs were as for *ndh*F except that plant DNA varied between 10 and 40 ng and 250 μg/mL BSA was included. The reaction profile was: 1 cycle at 94 °C for 3 min; 32 cycles at 94 °C for 1 min; 50 °C for 1 min; and 72 °C for 1.75 min; 1 cycle at 72 °C for 7 min. The primers ITS2, ITS3, ITS4, and ITS5 (White et al., 1990) were used to amplify the total ITS sequence. PCRs were as for *trn*L-F with the exclusion of BSA. The reaction profile was: 1 cycle at 94 °C for 2 min at, 40 s at 92 °C, 40 s at 52 °C, 1 min at 72 °C; 30 cycles at 92 °C for 40 s, 55 °C for 40 s, 72 °C 1.5 min; 1 cycle at 72 °C for 5 min.

PCR products were purified using a CASpure PCR Purification Kit following the protocol recommended by the

Table 1
List of taxa and sources of plant material analyzed for the first time plus accessions with sequences available from GenBank as well as the capitula characters and chromosome numbers (CN) (2n) of those investigated species (Liu, 2004)

Species	Origins	*ndh*F	ITS	*trn*L-F	Capitula	CN
Subtribe Tussilagininae Dum						
Cremanthodium Benth						
Sect.*Cremanthodium*						
C. decaisnei C. B. Clarke	Xiangcheng, Sichuan	AY723232	AY723269	AY723194	Radiate	58
C. stenoglossum Ling et S. W. Liu	Yushu, Qinghai	AY723233	AY176136	AF468175	Radiate	58
Sect. Parallelinervus Ling et S. W. Liu						
C. lineare Maxim.	Banma, Qinghai	AY723237	AY723273	AY723198	Radiate	58
C. microglossum S. W. Liu and J. Q. Liu	Chindu, Qinghai	AY723238	AY176135	AF468183	Radiate	58
Sect. Pinnatinervus Ling et S. W. Liu						
C. discoideum Maxim.	Chindu, Qinghai	AY723235	AY723271	AY723196	Discoid	58
C. ellisii (Hook. f) Kitam.	Madoi, Qinghai	AY723236	AY723272	AY723197	Radiate	58
C. humile Maxim.	Maq n, Qinghai	AY723234	AY723270	AY723195	Radiate	60
Dendrocacalia crepidifolia (Nakai) Nakai	Ogasawara, Japan	—	AY723280	—	Discoid	52
Doronicum stenoglossum Maxim.	Yushu, Qinghai	AY723253	AY176138	AY723179	Radiate	60
D. pardalianches L.		AY723254	—	—	Radiate	60
Farfugium japonicum (Bain and Golden) Kitam.	Nanchuan, Chongqin	AY723242	AY176166	AF468163	Radiate	60
Ligularia Cass.						
Sect. *Corymbosae* (Franch.) Hand.-Mazz						
L. cymbulifera (W.W. Smith.) Hand.-Mazz.	Zhongdian, Yunnan	AY723222	AY723259	AY723184	Discoid	58
L. dentata (A. Gray) Hara	Kunming, Yunnan	AY723219	AY723256	AY723181	Radiate	58
L. purdomii (Turrill) Chittenden	Banma, Qinghai	AY723220	AY723257	AY723182	Discoid	58
L. yunnanensis (Franch.) Chang	Dali, Yunnan	AY723221	AY723258	AY723183	Discoid	58
Sect. *Ligularia*						
L. lamarum (Diels) Chang	Dali, Yunnan	AY723225	AY723262	AY723187	Radiate	58
L. przewalskii (Maxim.) Diels	Songpan, Sichuan	AY723226	AY723263	AY723188	Radiate	58
L. tsangchanensis (Franch.) Hand.-Mazz.	Dali, Yunnan	AY723227	AY723264	AY723189	Radiate	58
L. sagitta (Maxim.) Mattf.	Daohu, Sichuan	AY723228	AY723265	AY723190	Radiate	58
Sect. Senecillis (Gaertn.) Maxim.						
L. brassicoides Hand.-Mazz.	Xiangcheng, Sichuan	AY723229	AY723266	AY723191	Radiate	58
L. pleurocaulis (Franch.) Hand.-Mazz.	Daocheng, Sichuan	AY723230	AY723267	AY723192	Radiate	58
L. liatroides (C. Winkl.) Hand.-Mazz.	Nangq n, Qinghai	AY723231	AY723268	AY723193	Radiate	58
Sect. *Scapiculis* S. W. Liu						
L. vellera (Franch.) Hand.-Mazz.	Lijiang, Yunnan	AY723224	AY723261	AY723186	Radiate	58
Sect. *Stenostegia* Pojark						
L. rumicifolia (Drumm.) S. W. Liu	Qushui, Tibet	AY723223	AY723260	AY723185	Radiate	58
Ligulariopsis shichuana Y. L. Chen	Mt. Taibai, Shannxi	AY723241	AY176148	AF468161	Discoid	58
Miricacalia makineana (Yatabe) Kitam.	Honshu, Japan	—	AY723281	—	Discoid	52
Parasenecio						
Sect. *Cacalia*						
P. cyclotus Bur. et Franch.	Zhongdian	AY723251	AY723277	AY723205	Discoid	?
P. taliensis Franch.	Dali, Yunnan	AY723250	AY723276	AY723204	Discoid	60
Sect. Palmate						
P. hastiformis Y. L. Chen	Mt. Gongga, Sichuan	AY723252	AY723278	AY723206	Discoid	?
Sect. *Parasenecio*						
P. deltophyllus (Maxim.) Y. L. Chen	Maq n, Qinghai	AY723248	AY723274	AY723202	Discoid	60
P. maowenensis Y.L. Chen	Jinchuan, Sichuan	AY723249	AY723275	AY723203	Discoid	?
Petasites japonicus (Sieb. et Zucc.) Maxim.	Nanchuan, Chongqin	AY723240	AY176152	AF468187	Radiate	60
Sinacalia tangutica (Maxim.) B. Nord.	Xunhua, Qinghai	AY723243	AY176157	AY723199	Radiate	60
Syneilesis aconitifolia (Bge.) Maxim.	Beijing	L39432	AY176163	AF468162	Discoid	52
Tussilago farfara L.	Xining, Qinghai	AY723239	AY176167	AF468166	Radiate	60
Subtribe Tephroseridinae C. Jeffrey et Y. L. Chen						
Nemosenecio nikoensis (Miq.) B. Nord	Honshu, Japan	—	AY723279	—	Radiate	48
Sinoseneci bodinieri (Vant.) B. Nord.	Nanchuan, Chongqin	AY723245	AY176158	AY723201	Radiate	48
S. globigerus (Chang) B. Nord.	Nanchuan, Chongqin	AY723247	AY176159	AF468170	Radiate	48
S. subcoriaceus C. Jeffrey et Y. L. Chen	Nanchuan, Chongqin	AY723246	AY176162	AF468173	Radiate	60
Tephroseris rufa (Hand.-Mazz.) B. Nord.	Seda, Sichuan	AY723244	AY176166	AF468180	Radiate	48

(*continued on next page*)

Table 1 (*continued*)

Species	Origins	*ndh*F	ITS	*trn*L-F	Capitula	CN
Subtribe Senecioninae						
Sencio argunensis Turcz.						
S. densiserratus Chang	Songpan, Sichuan	AY723210	—	—	Radiate	40
S. diversipinnus var. discoideus C. Jeffrey et. Y. L. Chen	Maerkang, Sichuan	AY723211	—	—	Radiate	40
S. laetus Edgew.	Dali, Yunnan	AY723213	—	—	Radiate	?
S. nemorensis L.	Ruoergai, Sichuan	AY723209	—	—	Radiate	?
S. nigrocinctus Franch.	Dali, Yunnan	AY723212	—	—	Radiate	?
S. pseudomairei Levl.	Huoqing, Yunna	AY723215	—	—	Radiate	?
S. pteridophyllus Franch.	Dali, Yunnan	AY723216	—	—	Radiate	?
S. scandens Buch.-Ham. Ex D. Don	Huoqin, Yunnan	AY723214	—	—	Radiate	?
S. thianshanicus Regel and Schmalh.	Cehngduo, Qinghai	AY723207	AY176156	AF468168	Radiate	40
Synotis alata (Wall. ex. DC.) Jeffrey et Y. L. Chen	Huoqin, Yunnan	AY723217	—	—	Radiate	?
S. lucorum (Franch.) C. Jeffery et Y. L. Chen	Dali, Yunnan	AY723218	AY723255	AY723180	Radiate	40

Subtribal and infrageneric classification follows Jeffrey and Chen (1984), Chen (1999), and Liu (1989).

manufacturer (Casarray, Shanghai, China). Sequencing primers used for amplifying *trn*L-F and ITS were the same as those mentioned above. For *ndh*F the forward and reverse primers were the same as used in amplifying the whole sequence. In addition, three other pairs of primers at sites 480, 972, and 1600 were designed according to published sequences in Asteraceae and used to sequence the *ndh*F gene (Liu et al., 2002a). Sequencing reactions were carried out in a Biometra thermocycler using a DYEnamic Dye Terminator Cycle Sequencing Kit (Amersham) following the manufacturer's protocol. Sequencing products were separated and analyzed on a MegaBACE 500 DNA Analysis System. Both strands of DNA were sequenced using forward and reverse primers. Sequences were recorded in both strands with an overlap of at least 70%.

2.3. Sequence alignment, boundary determination, and data analysis

Alignment of *trn*L-F and ITS sequences was conducted using CLUSTAL W (Thompson et al., 1997) and refined manually. Alignment of *ndh*F sequences was done manually. Sequence boundaries were determined by comparison with published sequences of other genera of Asteraceae downloaded from GenBank. Downloaded *ndh*F sequences comprised the entire gene sequence obtained using external forward and reverse primers (Kim and Jansen, 1995). However, in the present study, the reverse primer annealed internally, so that 108 bp at the 3′ end were not determined. The informative indels in both cpDNA sequences were coded as binary characters or treated as missing. Most gaps of ITS sequences comprise only a 1-bp difference, and therefore in the analyses were treated as both missing and the fifth state.

Each dataset was subjected to maximum parsimony (MP), maximum likelihood (ML), and Bayesian analyses. We used Modeltest (Posada and Crandall, 1998) to select parameters and assumptions for ML analyses in PAUP 4.0b10 (Swofford, 2000). During the ML analysis of a combination of different datasets (*ndh*F + *trn*L-F and *ndh*F + *trn*L-F + ITS), the best model for each dataset was used. Maximum likelihood heuristic search parameters were simple addition sequence of taxa with TBR branch swapping, MULTREES and COLLAPSE. For Bayesian analyses (Huelsenbeck and Ronquist, 2001), four simultaneous Monte Carlo Markov Chains (MCMC) were run for 5,000,000 generations, saving a tree every 1000 generations. Because the fittest models selected for the analyzed datasets were not implemented in MrBayes, two common models GTR + I + Γ and HKY85 + Γ were used on the different datasets in Bayesian analyses. The datasets and corresponding figures were deposited in TreeBase with accession numbers SN2447–9402, 9403, 944, 9418, and 9419.

Maximum parsimony analyses (equally weighted characters and nucleotide transformations) involved a heuristic search strategy with 100 replicates of random addition of sequences, in combination with ACCTRAN character optimization and MULPARS + TBR branch-swapping and STEEPEST DESCENT options on.

Posterior probability (shown as percentages, PP) for Bayesian analyses (Huelsenbeck and Ronquist, 2001) and bootstrap values (BP) (Felsenstein, 1985) for MP trees assessed relative support for monophyletic groups. Burn-in, the generation time for each parameter to reach the stationary state, was determined by visual inspection of the log-likelihood values. We discarded the first 499 trees and collected 4501 trees (whose log-likelihoods converged to stable values) to construct a 50% majority rule consensus tree with posterior probabilities with PAUP* v4.0b10 (Swofford, 2000). Bootstrap values were calculated from 1000 replicates using a heuristic search with simple addition with TBR and MULPARS options on. Sequence characteristics were calculated using PAUP Version 4.0b10 (Swofford, 2000). Congruence between different DNA datasets were evaluated by the incongruence-length-difference (ILD) test with 1000 replicates on parsimony-informative characters using the TBR branch swapping algorithm and number of trees retained for each replicate limited to 1000 (Farris et al., 1995).

2.4. Molecular calibration

In the absence of a fossil record, we used ITS sequences to infer the onset of diversification in the most recent

common ancestor (MRCA) of the *L–C–P* complex. Because the putative pseudogenes of ITS usually originated more recently than the functional copies (Bailey et al., 2003), their existence in the aligned dataset might confound phylogenetic reconstruction and affect the dating of the divergence among orthologous sequences (Alvarez and Wendel, 2003). We distinguished functional sequences from putative pseudogenes through examining the nucleotide substitution in a highly conserved region (5.8S gene), a relatively reliable indicator for discerning ITS orthologs (Hershkovitz et al., 1999). *Robinsonia* of Senecioneae, a genus within the Senecioninae clade, comprises two closely related subgenera distributed in the Juan Fernández Islands (Sang et al., 1995). We used the estimated earliest time that these two subgenera of *Robinsonia* split from their MRCA due to the formation of Masatierra (Sang et al., 1995) as a calibration point to date the MRCA node of the *L–C–P* complex within the Tussilagininae lineage. This calibration point may overestimate the dating time because the occupation of plants in the islands should be more recent than their geological formation. The hypothesis of rate constancy was evaluated with a likelihood ratio test that is twice the difference in log likelihood of branch lengths between a rate-constrained tree (forcing the molecular clock in PAUP) and a tree that has no constraints on branch lengths. The molecular clock was rejected because constrained and unconstrained analyses differed significantly (4861.13 vs. 4930.19, df = 44, $P < 0.005$), so Sanderson's method of nonparametric rate smoothing (NPRS) (Sanderson, 1997) based on the ML tree without molecular clock enforced was used to produce an ultrametric tree with TreeEdit version 1.0 alpha 10 (Rambaut and Charleston, 2000). We further estimated the confidence intervals for date of onset of diversification in the most recent common ancestor (MRCA) of the *L–C–P* complex by fixing the tree and bootstrapping the dataset 100 times. Error in divergence time estimates attributable to mistaken tree topology was estimated from trees generated by parsimony analysis of 100 bootstrap replicates. Estimates of 100 times were used to construct a histogram of the possible divergence range time. Because NPRS is inaccurate if sequence divergence is low, we further estimated the date without NPRS under TreeEdit directly based on ML branch lengths. The substitution rates of other taxa with similar habit or in the same sunflower family were further used to estimate the onset of diversification.

We also used the *ndh*F dataset to estimate the MRCA diversification onset. As was the case with the ITS dataset, the molecular clock was rejected because constrained and unconstrained analyses differed significantly (7763.74 vs. 7855.32, df = 60, $P < 0.005$). Therefore, the average *ndh*F nucleotide substitution from the MRCA node was calculated for synonymous mutations under the Jukes and Cantor's one-parameter model following Kim et al. (1998). Time of divergence was calculated as the value of DNA sequence mutation divided by the evolutionary rate of *ndh*F sequence suggested by Seelanen et al. (1997). All these analyses were performed using TreeEdit version 1.0 alpha 10 (Rambaut and Charleston, 2000).

2.5. Character-state optimization and biogeographic analyses

The evolution of discoid capitula and chromosome number within the *L–C–P* complex (see Chen, 1999; Liu, 2004) was inferred with Fitch parsimony optimization onto the reduced ITS consensus trees of two ML trees. Character states were scored for each OTU and characters were optimized with MacClade 4.0 (Maddison and Maddison, 2000). Ancestral states were inferred through minimizing the number of character state changes on the tree. We also used Fitch parsimony optimization (Maddison et al., 1992) to assess the historical biogeography of *L–C–P* complex with the molecular ML consensus topology. This method assumes that geographic distributions are solely the result of dispersal (as opposed to vicariance) events. Thus, polymorphic area states are restricted to terminal nodes. The data matrix was constructed by coding "area" as a single multistate character, and the analysis was performed with MacClade 4.0 (Maddison and Maddison, 2000). Three areas circumscribed for the analysis were the Q-T Plateau, central China, and Japan. The ML ITS tree was used for optimization, with all species comprising operational taxonomic units (OTUs). Dispersal-Vicariance analysis (DIVA) was further used to infer ancestral areas by using DIVA 1.1a (Ronquist, 1996, 1997) based on one of two ML ITS trees. DIVA reconstructs ancestral areas by minimizing dispersal and extinction events needed to explain the observed distribution pattern based on an inferred fully resolved phylogeny, with vicariance considered as the default mode of speciation.

3. Results

3.1. Phylogenetic analyses of ndhF dataset

The *ndh*F sequence dataset analyzed comprised 52 species of the Senecioneae, representing all recognized subtribes, and 12 species representing eight additional tribes of Asteraceae. The aligned dataset contained 2131 sites of which 276 were variable but phylogenetically uninformative, and 191 that were variable and informative (gaps excluded). Five indels (one of 3-bp, another of 9-bp, and the remainder of 6-bp) were restricted to single species, and therefore yielded no phylogenetic information. Parsimony analysis identified 4239 trees with 747 steps, a consistency index (CI) of 0.767, and a retention index (RI) of 0.796. The strict MP consensus tree and the ML tree ($-\ln L = 7763.74$, the best-fit model TVN + I + G) were mostly congruent in topology with the 50% majority rule consensus tree derived from Bayesian analysis (under the GTR + I + Γ model) (Supplementary Fig. S1). Phylogenetic analysis of *ndh*F sequences refuted traditional tribal and subtribal circumscriptions of Senecioneae. *Abrotanella* occurred in a clade with *Aster* (75% BP and 77% PP) and should be excluded from the Senecioneae. The remaining 51 species of 15 genera considered to be members of the Senecioneae

comprised a monophyletic clade with strong support (80% BP and 95% PP). *Doronicum*, a genus formerly considered to be closely related to the *L–C–P* complex and placed in the Tussilagininae, was situated at the base of the Senecioneae clade and is sister to all remaining species. Two tentative subclades—the Tussilagininae lineage (including *Blennosperma*) (<50% BP and 53% PP) and the Senecioninae lineage (75% BP and 100% PP)—were recovered. These results suggest that both the subtribe Blennospermatinae and Tephroseridinae should be reduced and the latter nested within the Tussilagininae. The phylogenetic relationships of species within the Senecioninae lineage were well resolved and four subclades were recognized and received moderate to strong supports. Within the Tussilagininae lineage, *Tussilago*, *Petasites*, and *Blennosperma* were basal, while all *L–C–P* species together with Tephroseridinae species comprised a monophyletic group, designated here as the *L–C–P*clade (57% BP and 82% PP). In the *L–C–P* clade, a polytomous radiation pattern was found with 17 parallel branches. Five of these branches contained more than one species. The clustering of species within some branches has to be considered as tentative due to weak support and collapse in the strict MP consensus tree. The low resolution within the *L–C–P* clade was due mainly to a lack of phylogenetically informative synapomorphic mutations. A total of 117 mutations were detected among species within this clade, but only 21 of these were phylogenetically informative. However, within the Senecioninae lineage, more than 70% of all mutations were informative and the phylogenetic relationship were well resolved.

The results of the analysis of the *ndh*F dataset contradicted previous assumptions regarding the subtribal delimitation of the Senecioneae and suggested that: (1) *Abrotanella* should be excluded from the Senecioneae and have a close relationship with the Astereae; (2) *Doronicum* should be excluded from the Tussilagininae and positioned at the base of the Senecioneae; and (3) the remaining sampled genera of Senecioneae are clustered into two tentative lineages: the Senecioninae lineage and the Tussilagininae lineage. The latter lineage included Tephroseridinae and possible *Blennosperma* of the Blennospermatinae. Because of its basal position in the Senecioneae, *Doronicum* was selected as outgroup in subsequent phylogenetic analyses.

3.2. Phylogenetic implication of trnL-F dataset and a combined analysis of trnL-F and ndhF sequences

Following on from the results of the analysis of the *ndh*F dataset, the *trn*L-F sequence dataset was generated to include all species of the Tussilagininae lineage except for *Blennosperma*, three species of the Senecioninae and the outgroup *Doronicum*. This *trn*L-F dataset contained 890 sites, of which 801 were constant, 62 were variable but parsimony-uninformative, and 27 were variable and informative when indels were excluded. A heuristic search identified 423 most parsimonious trees (length = 112, RI = 0.91 CI = 0.83). The strict consensus MP tree was topologically the same as the 50% majority rule consensus tree derived from Bayesian analysis (under the HKY85 + Γ model) (Supplementary Fig. S2). When four indels were coded as binary characters, the topological relationships were the same as shown in Fig. S2. The same relationships were also resolved by ML analysis (not shown, $-\ln L = 1977.45$, the best-fit model K81uf + G). Both the Senecioninae and the Tussilagininae (including Tephroseridinae) were resolved as two major lineages with low to moderate support (87% BP and 100% PP for the Senecioninae, 62% BP and 100% PP for the Tussilagininae). Within the Tussilagininae lineage, *Tussilago* and *Petasites* comprised a separate clade (80% BP and 100% PP), and all remaining species showed a polytomous radiation within a monophyletic *L–C–P* clade that had low support (51% BP and <50% PP). A total of 50 mutations were present within the *L–C–P* clade, but only 16 (32%) were parsimony-informative. In the aligned *trn*L-F dataset, four indels were parsimony-informative; two of these (9 and 35 bp) supported the grouping of *Sinosenecio bodinieri* and *S. globerus*, one (4 bp) supported the *Tussilago* and *Petasites* clade, while another (5 bp) grouped two *Senecio* species (Fig. S2).

Partition homogeneity analyses showed no significant incongruence between the *ndh*F and *trn*L-F datasets ($P = 0.75$). The combined sequences were 2991 bp long, of which 2701 sites (including gaps) were constant, 222 nucleotide sites were variable but parsimony-uninformative, and 68 were variable and phylogenetically informative. MP analysis produced 5003 most parsimonious trees (Length = 349, CI = 0.89, and RI = 0.73). The strict consensus tree (Fig. 2) was completely congruent with the 50% major consensus tree obtained from Bayesian analysis (under the GTR + I + Γ model) and had a similar topology to the ML tree (not shown, $-\ln L = 6558.4$). The major clades resolved (Senecioninae, and the *Tussilago*, *Parasenecio*, and *Sinosenecio* groups) were consistent with those identified in the *trn*L-F and *ndh*F phylogenies. But this was not so for groupings of *Ligularia* and *Cremanthodium* species.

A total of 167 mutations were detected within the *L–C–P* clade in the combined alignment of *trn*L-F and *ndh*F sequences, but only 37 of these were phylogenetically informative. Within this clade, the smallest pairwise nucleotide distance (0.05%) was detected for three pairs of species, *Ligularia pleurocaulis* vs. *L. yunnanensis*, *L. brassicoides* vs. *L. liatroides*, and *Cremanthodium stenoglossum* vs. *C. microglossum*, while the greatest distance (1.29%) occurred between *Ligulariopsis shichuana* and *Ligularia cymbulifera*. Nucleotide distance between species of the *L–C–P* clade and those of other clades was 0.33–1.33% for comparisons with the *Tussilago* group, 0.91–1.89%, for comparisons with three Senecioninae species (*Senecio argunensis*, *S. thianthanicus*, and *Synotis lucorum*), and 2.24–3.19% for comparisons with the outgroup *Doronicum*.

3.3. Phylogenetic analyses of ITS dataset

The ITS dataset was composed of 45 species and included all species examined in the *trn*L-F and *ndh*F

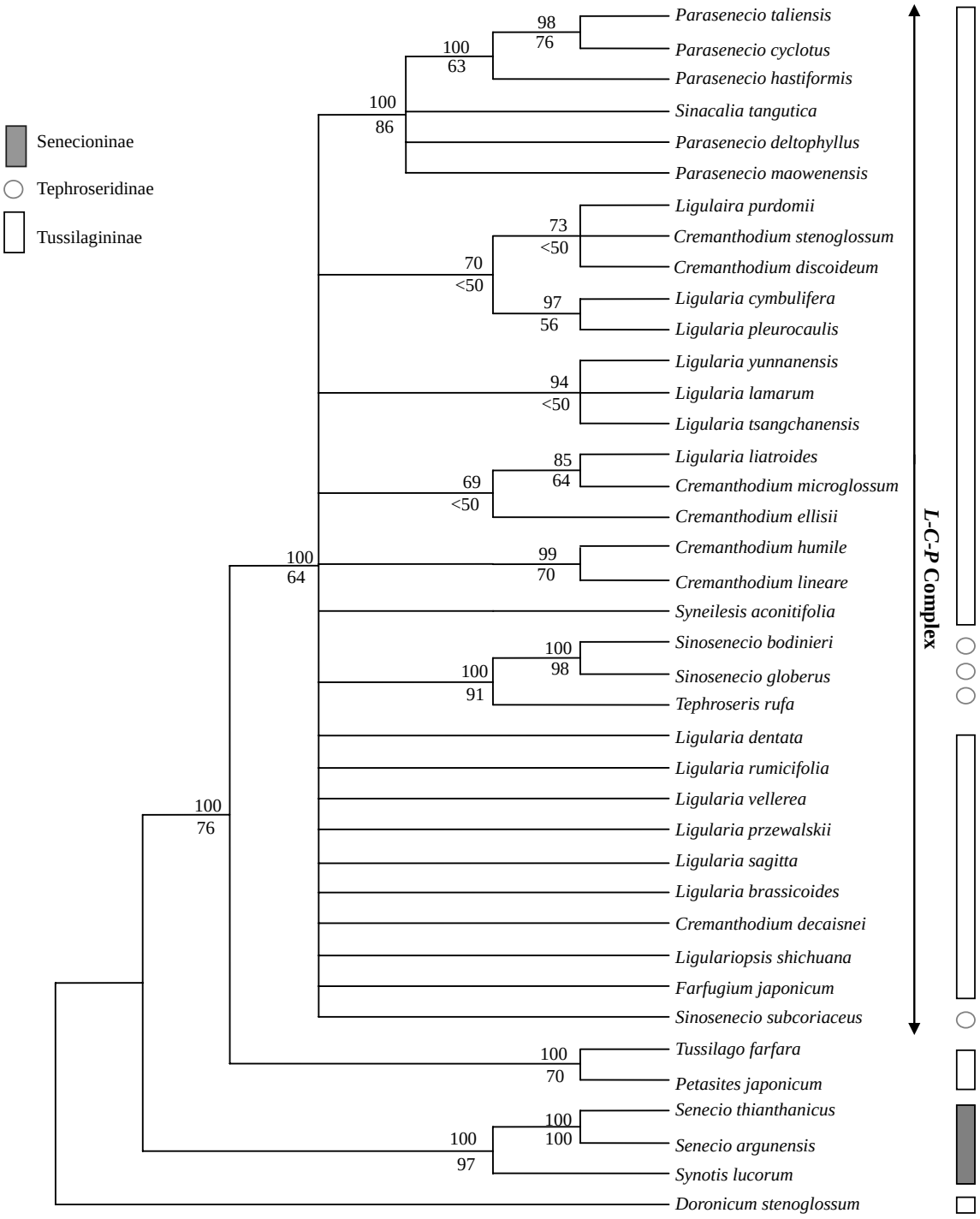

Fig. 2. The strict consensus tree of 5003 most parsimonious trees (Length = 349, CI = 0.888, and RI = 0.729) based on the analysis of *trn*L-F + *ndh*F datasets. Posterior probabilities (PP) are noted above branches and bootstrap support values (BP) are given below branches receiving >50% bootstrap support.

combined analysis, plus one species *Nemosenecio* of the Tephroseridinae, two species representing *Miricacalia* and *Dendrocacalia* of the Tussilagininae and three species representing *Robinsonia* of the Senecioninae. All newly produced sequences covered the 5.8S region (164 bp), but no variation was found within the segment. Therefore, we assumed that all of these ITS sequences are functional orthologs rather than paralogs in that they possessed the same conserved 5.8S gene (Hershkovitz et al., 1999). Due to lack of mutation in the 5.8S sequence, this fragment was omitted from further analyses. The ITS sequences were easily aligned with insertions composed of 1–3 gaps, most of which existed between ingroup taxa and outgroups. The aligned ITS1 + ITS2 sequence was 503 bp long and comprised 160 constant sites or gaps, 144 of which were variable but phylogenetically uninformative, and 199 that were variable and informative when gaps were treated as missing. MP analysis yielded 2499 MP trees (Length = 895, CI = 0.60, and

RI = 0.51) and the strict consensus of these trees was congruent with the 50% majority consensus tree obtained from Bayesian analysis (under the HKY85 + Γ model) (Fig. 3). When gaps were treated as the fifth state, MP analysis produced 1408 MP trees (Length = 939, CI = 0.62, and RI = 0.60), and all topological branches did not change, but the bootstrap support for some clades was increased. A similar tree topology was also resolved by ML analysis under the best-fit model (GTR + G) ($-\ln L = 4861.13$). The short internal distance in the phylogenetic tree (Supplementary Fig. S4) suggested that the occurrence of few synapomorphic mutations is a major factor responsible for poor differentiation within most subclades of the *L–C–P* complex. This was the case despite the long branches in some clades that indicated abundant autapomorphic mutations.

All analyses recovered two major lineages: Tussilagininae and Senecioninae. *Dendrocacalia*, a genus previously placed within the Tussilagininae, nested within the Senecioninae. This genus should therefore be excluded from the Tussilagininae of eastern Asia. Within the Tussilagininae, the *Tussilago* group (*Tussilago* and *Petasites*) was sister to the weakly supported *L–C–P*clade (<50% BP and 66% PP),

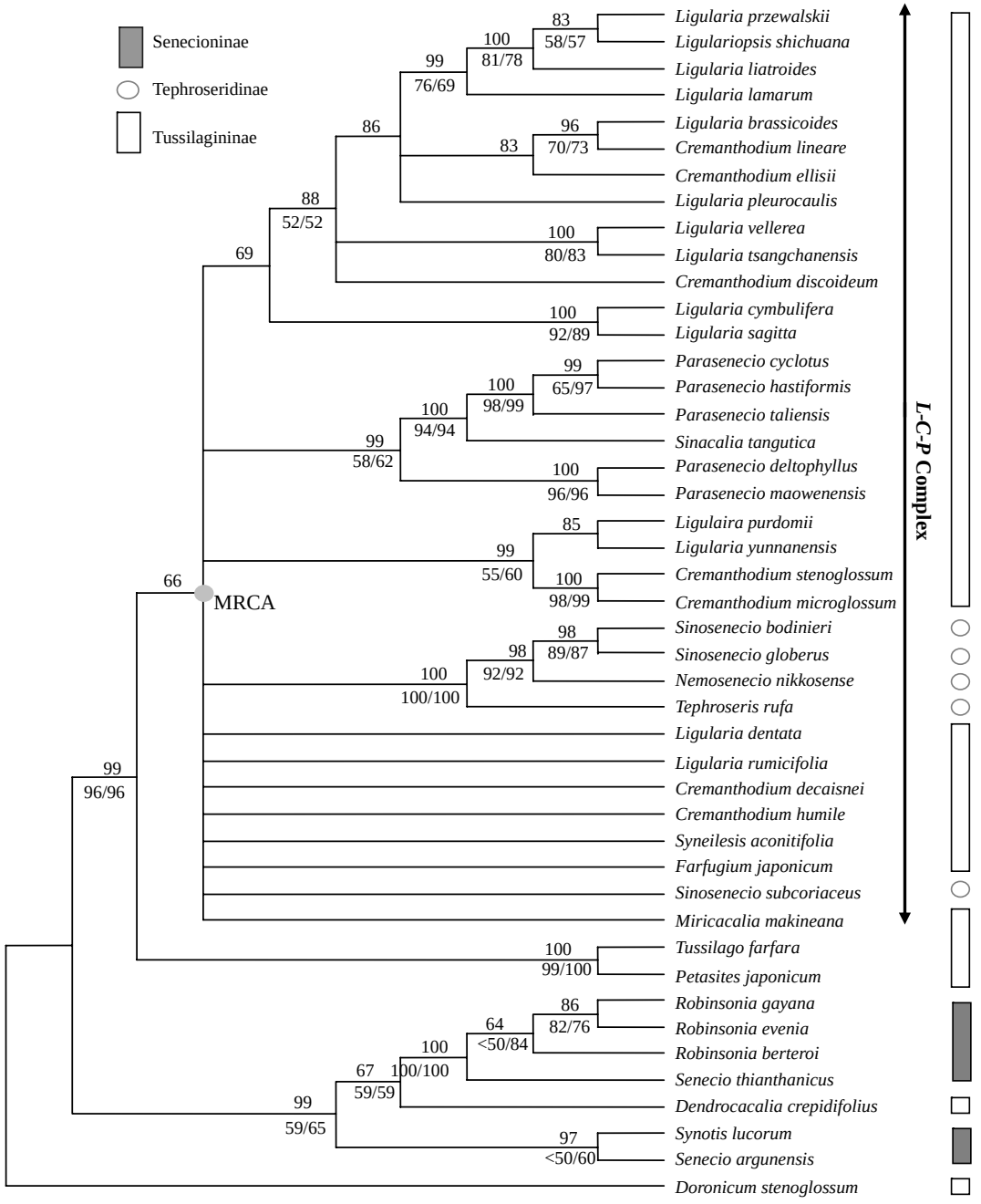

Fig. 3. The 50% majority rule consensus tree derived from Bayesian analysis of the ITS dataset. The posterior probabilities are noted above branches, while bootstrap values (gaps as missing/new state) are indicated below. MRCA denotes the most recent common ancestor.

which contained 35 species from 11 genera. This clade comprises 12 parallel polytomous branches, of which only four consist of more than one species (Fig. 3 and (Fig. S2)).

Among the 20 species of *Ligularia* and *Cremanthodium* examined, the average pairwise nucleotide distance was 4.88%, with the smallest distance (0.21%) occurring between *Cremanthodium stenoglossum* and *C. microglossum*, and the largest (9.98%) between *L. rumicifolia* and *L. dentata*. Within the entire *L–C–P* clade, average uncorrected pairwise nucleotide distance was 6.63% with the smallest distance (0.21%) detected between *C. stenoglossum* and *C. microglossum*, and the greatest distance (19.46%) between *Miricacalia makineana* and *Tephroseris rufa*. In this clade, 226 nucleotide mutations (excluding gaps) were detected, but only 103 (45.60%) are parsimony-informative, while 54.40% mutations are autapomorphic. Species within the *L–C–P* clade showed a pairwise distance variation of 9.80–18.01% with those of the *Tussilago* group (*Tussilago* and *Petasites*), 11.79–20.84% with four Senecioninae species (*Dendrocacalia crepidifolius*, *S. argunensis*, *S. thianthanicus*, *Synotis lucorum*, and three *Robinsonia* species), and 28.74–32.88% with the outgroup, *Doronicum*. The following species possessed an additive ITS sequence: *Ligularia tsangchanensis* (ITS1, site 136, C/T; ITS2, 458, A/C; ITS2, 463, A/T), *L. liatroides* (ITS1, site 47, A/T), *L. przewalskii* (ITS2, site 441, C/T), and *Cremanthodium ellisii* (ITS1, site 143, G /T; ITS2, 463, C/T) and exhibited phylogenetic relationships in the ITS tree (Fig. 3 and (Fig. S3)) that were very different from those shown in cpDNA trees (Fig. 2).

3.4. Combined analysis of ITS, trnL-F, and ndhF sequences

Partition homogeneity analysis showed that there was significant incongruence between the plastid and the nuclear ITS datasets ($P = 0.01$). The major discordances concerned relationships between *Ligularia* and *Cremanthodium* species, which might result from hybridization and introgression as indicated by some additive ITS sites. After the exclusion of nine species of these two genera from analysis, no significant incongruence was detected ($P = 0.88$). An initial phylogenetic analysis was conducted on the combined plastid and nuclear sequence data that included all species, to examine specifically the relationships between the major clades resolved previously. The combined aligned sequence was 3494 bp long, of which 2870 sites were constant when gaps were treated as missing, 379 sites were variable but uninformative, and 245 sites were variable and phylogenetically informative. The topology of the ML tree (separate models for each dataset, $-\ln L = 11248.34$) and the strict consensus tree of 1835 most parsimonious trees (Length = 1152, CI = 0.69, and RI = 0.52), was similar to that of the ITS tree (not shown). Both Bayesian (under the HKY85 + Γ model) and MP bootstrap values of the major lineages, Tussilagininae, Senecioninae, *Tussilago* group, *Parasenecio* group, and *Sinosenecio* group, were higher than those from the individual sequence analyses. The *L–C–P* clade, especially, received increased support (76% BP and 100% PP), suggesting their common origin from the MRCA. Groupings within *Ligularia* and *Cremanthodium* were the same as those indicated by the ITS phylogenetic tree.

A second analysis was conducted on 30 species that exhibited congruent datasets after excluding nine species of *Ligularia* and *Cremanthodium*. This combined sequence dataset contained 246 variable but parsimony-uninformative sites and 223 phylogenetically informative mutations. MP analysis produced 1382 most parsimonious trees (Length = 996, CI = 0.725, and RI = 0.528). The strict consensus tree was completely congruent with the 50% major consensus tree obtained from Bayesian analysis (under the GTR + I + Γ model) and had a similar topology to the ML (based on the separate best fit model for three datasets) tree ($-\ln L = 10600.87$) (Fig. 4). The major clades resolved were consistent with those identified in the initial analysis. But both Bayesian (under the HKY85 + Γ model) and MP bootstrap values of the major lineages were elevated. Bootstrap support of the *L–C–P* clade increased from 76% in this first analysis to 86% in the second analysis. However, most species or subclades within the *L–C–P* clade remained unresolved despite the greater number of variable characters available for analysis.

Taken overall, the phylogenetic analysis conducted on the *ndh*F dataset, together with the analyses of the *trn*L-F and ITS datasets, and the combination of all three datasets, tentatively suggest that: (1) *Dendrocacalia* should be excluded from the Tussilagininae and transferred to the Senecioninae; (2) the Tephroseridinae (comprising *Tephroseris*, *Sinosenecio*, and *Nemosenecio*) (Jeffrey and Chen, 1984) should be reduced and included within the broadly circumscribed Tussilagininae; (3) all 13 genera of the Tussilagininae in eastern Asia may be grouped into two tentative clades, the *Tussilago* clade (*Tusssilago* and *Petasites*), and a poorly resolved *L–C–P* complex clade containing the remaining 11 genera; (4) *Sinacalia* nests within the*Parasenecio* subclade and should be reduced into the latter genus; (5) *Sinosenecio* is paraphyletic; and (6) most species of *Ligularia* and *Cremanthodium* show no generic groupings.

3.5. Dating the onset of diversification in the L–C–P complex

Although only the enlarged lineage that included the *Tussilago* group received strong bootstrap support in MP analyses of all datasets, we assumed that the *Tussilago* group is sister to the expanded *L–C–P*complex for three reasons. First, there is a distinct genetic divergence between the *Tussilago* group and the *L–C–P*complex, which received support in the analyses of a combination of three datasets (Fig. 4) despite weak support in the separate analyses (Fig. 3 and Figs. S1, S2, and S3). Second, flowers of species in the *Tussilago* group are precocious and the inner florets of their capitula are female, which differs distinctly from those of the *L–C–P* complex. Third, although they share a chromosome number of 2n = 60 with most members of the *L–C–P*complex, their karyotypes are 2B, and therefore different from 2A, a common karyotype in the *L–C–P*complex (Liu, 2004). Fourth, monotypic *Tussilago* is

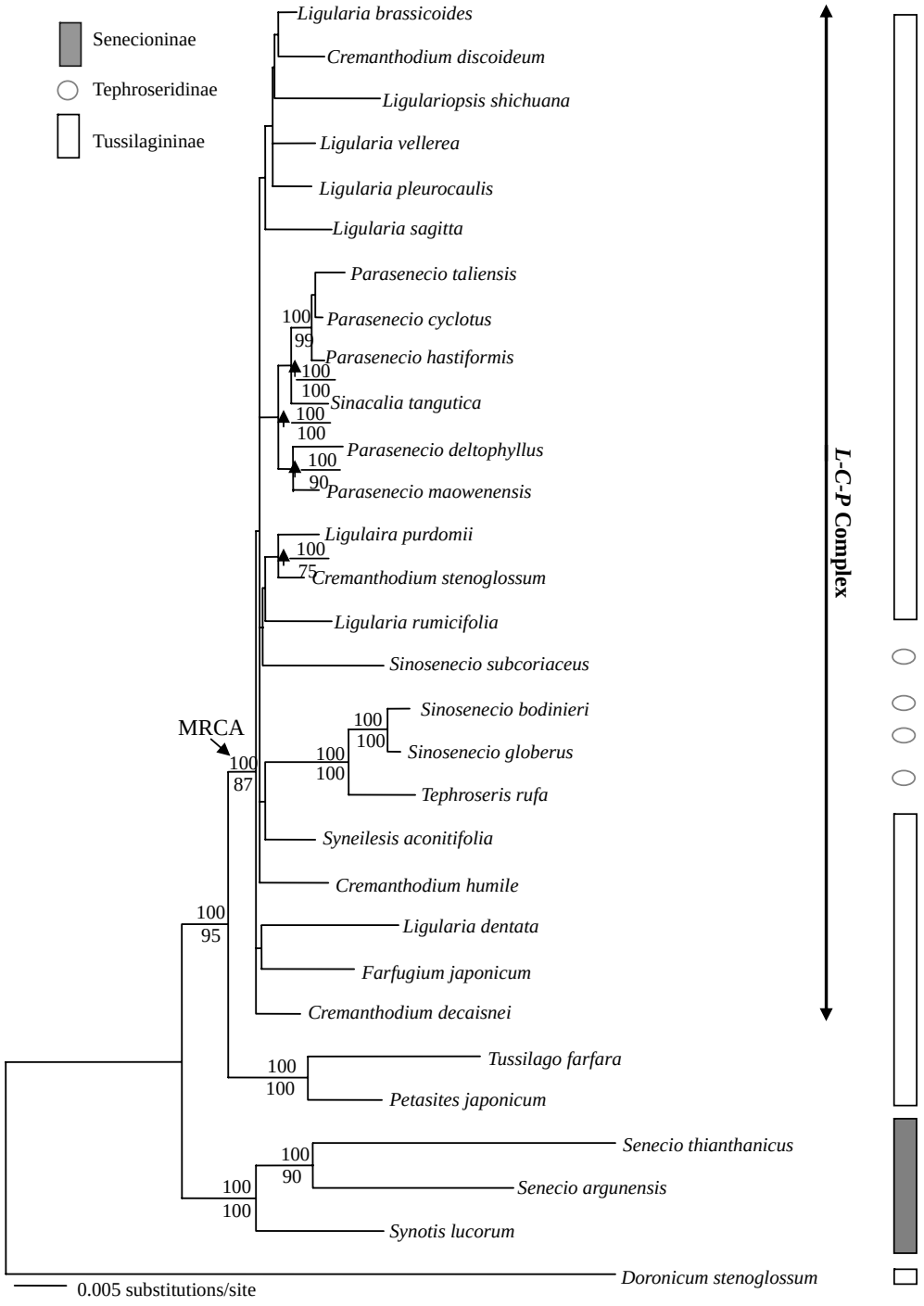

Fig. 4. The ML phylogram of an analysis of reduced taxa with congruent *trn*L-F, *ndh*F, and ITS datasets. Posterior probabilities (PP) are noted above branches and bootstrap support values (BP) are given below branches receiving >50% bootstrap support. MRCA, the most recent common ancestor.

widely distributed in the temperate North Hemisphere, and *Petasites* has its diversity centers out of Asia, in North America and Europe. Thus, we have placed the MRCA of the *L–C–P*clade at the node divergent from the *Tussilago* group. This clade includes not only most species of the three genera from the Q-T Plateau (*Ligularia*, *Cremanthodium*, and *Paransenecio*), but also those species of the remaining eight genera of the Tussilagininae from the plateau and other eastern Asian regions.

The MRCA of the *L–C–P* clade in the ITS ML tree was dated to 10.85 ± 2.7 Ma, based on NPRS and assuming the earliest possible split of two subgenera of *Robinsonia* (Sang et al., 1995) during the formation of Masatierra in the Juan Fernández Islands (approximately 4 million years ago). Bootstrapping frequencies showed that the possible age of the MRCA ranged from 6.55 to 17.03 Ma, with most ages falling within 8–13 Ma categories (Fig. 5). The date were modified to 10.96 Ma based on ML branch lengths without

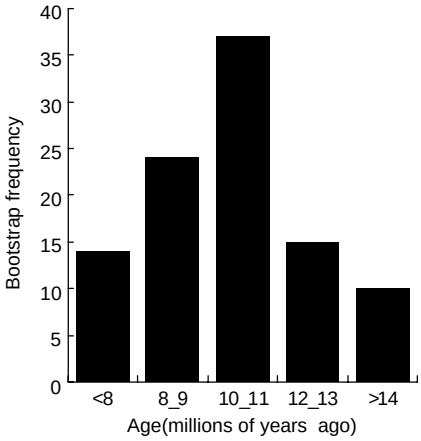

Fig. 5. The histogram of bootstrap results of dating the most recent common ancestor (MRCA) of the *Ligularia–Cremanthodium–Parasenecio* clade based on ML branching of ITS data when the assumed divergence time (4 million years) of two *Robinsonia* subgenera is used as the calibration point.

NPRS smoothing, and to 10.65 Ma using a similar MP tree. On average, *L–C–P*species have 41 substitutions from the MRCA node to the tip of each branches. We further used substitution rates of other taxa with similar habit or in the same sunflower family to estimate the onset of diversification. Table 2 is a summary of estimated time using these rates. The estimated times based on ITS substitution are between 10.41 and 27.17 Ma with a mean estimate of 19.51 Ma and most calibrations fall within 20 Ma (Table 2).

An overall divergence rate of approximately 0.05–0.07% per million year for *ndh*F was suggested by (Seelanen et al., 1997) and used by (Kim et al., 1998) to estimate divergence times in the Asteraceae. The *ndh*F branch distances from the MRCA node based on synonymous mutations were averaged as 0.00223 ± 0.0025. The diversification onset time was dated between 8.92 ± 1.00 and 6.37 ± 0.71 Ma when dividing the rates of 2.5×10^{-10} to 3.5×10^{-10} substitution per site per year, falling mostly within the ITS estimates (Table 2; Fig. 5).

3.6. Character-state mapping and biogeographic analysis

Neither capitulum nor chromosome characters examined exhibited unique synapomorphic character-state changes (Fig. 6). The chromosome numbers of the *L–C–P* complex varied from 2n = 48 to 60 (Liu, 2004). The chromosome number 2n = 60 was inferred to be ancestral and 2n = 58, 52, and 48 were independently derived from it (Fig. 6A). Eleven of the ingroup taxa (*L. shichuana*, *Cremanthodium discoideum*, *Ligularia cymbulifera*, *Parasenecio taliensis*, *P. cyclotus*, *P. hastiformis*, *P. deltophyllus*, *P. maowenensis*, *Syneilesis aconitifolia*, *M. makineana*, and *Dendrocacalia crepidifolius floridana*, *C. linifolia*, *C. gladiata*, and *C. paludosa*) have discoid capitula, and this is clearly a derived feature. Examination of the evolution of this feature results in one parsimonious reconstruction of nine steps (Fig. 6B). The discoid capitula have evolved independently from the radiate capitula with a reversal in *Sinacalia tangutica*. Optimizations of three areas of endemism onto ITS ML trees with standard Fitch parsimony analysis (Fig. 7) and DIVA analyses revealed similar results. Both infer that the Q-T region is ancestral and that central China and Japan are derived from it with 16 dispersals. DIVA analysis further revealed four vicariance events.

4. Discussion

A localized lack of phylogenetic signal and poorly resolved phylogenetic relationships has been interpreted as a signature of explosive speciation or rapid radiation in some floras (see Baldwin and Sanderson, 1998; Richardson et al., 2001a,b; Verboom et al., 2003). Our investigation into the evolution of a morphologically diverse group of species of *Ligularia*, *Cremanthodium*, *Parasenecio* and closely related taxa, most of which are endemic to the Q-T Plateau, revealed that a radiation occurred within this complex and that diversification began probably between 20 and 7 Ma, i.e., during periods of major uplift of the plateau since the early Miocene (Guo et al., 2002; Harrison et al., 1992; Li et al., 1995; Shi et al., 1998). This finding suggests, therefore, that the radiation within the component of the Q-T Plateau flora examined was most probably triggered by geophysical and climatic changes that played an important role in creating high species richness within the region.

Molecular calibration of branching time in phylogenetic trees is controversial and should be treated with caution (Sanderson, 1997), but when paleontological data are lacking, molecular estimates provide the only means of inferring the ages of lineage (Bromham and Penny, 2003; Li, 1997). Dating the MRCA of the *Ligularia–Cremanthodium–Parasenecio*

Table 2
Estimated timing of divergence for the onset of diversification of the *L–C–P* complex based on the average number of substitutions from the MRCA to each clade tip according to molecular clocks calibrated from a range of other taxa

Rate source	Habit	Genomic region	Calibrated rate (s/s/y)	Diversification onset time (Ma)
Aichryson, Crassulaceae	Annual or perennial herbs	ITS2	5.69×10^{-9}	14.33
Astragalus, Leguminosae	Annual or perennial herbs	ITS	3.5×10^{-9}	23.29
Dendroseris, Asteraceae	Woody perennials	ITS	$3.9–6.1 \times 10^{-9}$	13.36–20.9
Lupinus, Leguminosae	Annual or perennial herbs	ITS1/ITS2	$3.3–3.6 \times 10^{-9}$	22.64–24.0
Silverswords, Asteraceae	Woody perennials	ITS	3.0×10^{-9}	27.17
Robinsonia, Asteraceae	Woody pachycaul	ITS	7.9×10^{-9}	10.41
Gossypium, Malvaceae	Woody shrubs	*ndh*F	$2.5\text{-}3.5 \times 10^{-10}$	6.4–8.9

The ITS substitution rate sources and their related references were referred to Richardson et al. (2001a).

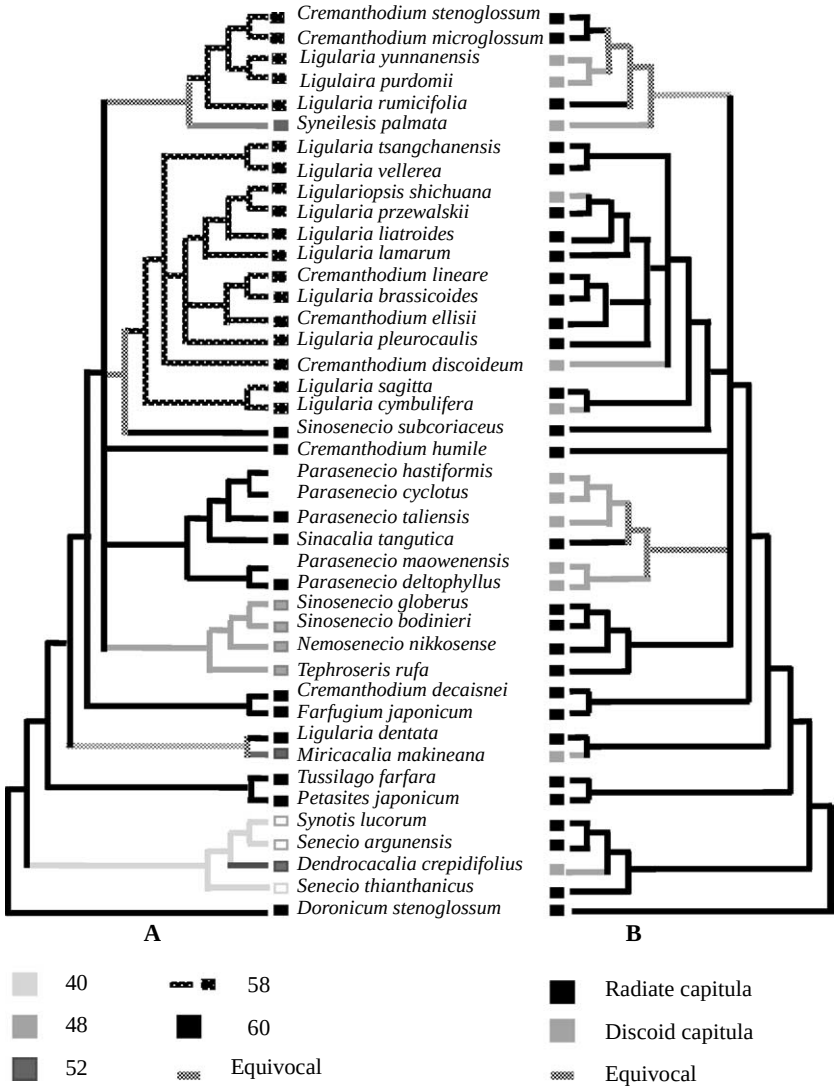

Fig. 6. Fitch parsimony optimization of capitulum and chromosome characters onto the consensus *L–C–P* ITS maximum likelihood topology. (A) Chromosome number. There is one optimal reconstruction of 8 steps (RI = 0.73; CI = 0.50; RC = 0.37). (B) Capitulum character. There is one optimal reconstruction of 9 steps (RI = 0.33; CI = 0.11; RC = 0.04).

radiation is crude for three reasons. The method used to calibrate the ITS phylogenetic tree according to the rate of Sang et al. (1995) is likely to have overestimated the date of the MRCA due to earlier appearance of islands than the occupation of plants. Second, the species examined represent only a sample of the total within the complex and it is feasible, therefore, that our estimates are not based on more divergent, ancient lineages. However, a pairwise distance analysis of 40 further species of *Ligularia* and *Cremanthodium* (unpublished data) indicate that nucleotide substitutions for these are within the range reported here for ITS, and addition of these species did not greatly change the estimated date of the MRCA presented here. Third, different ecological factors might influence substitution rates with increased aridity, for example, causing an acceleration (Arbogast et al., 2002; Bromham and Penny, 2003; Li, 1997). If this were the case in the Q-T Plateau, divergence times should be more recent than estimated here. Despite these concerns, we are reasonably confident that the radiation of the *L–C–P* clade occurred within the Miocene because: (i) several independent calibrations based on both nuclear and chloroplast DNA substitution provided a similar dating estimate, (ii) this estimated date corresponds well with other lines of evidence that the Q-T Plateau began several large-scale uplifts during the Miocene (An et al., 2001; Guo et al., 2002; Li et al., 1995; Shi et al., 1998), (iii) the dating of diversification of the Chinese sisorid catfish mainly occurring in the Qinghai-Tibetan Plateau also revealed a similar radiation time between the Oligocene and Miocene boundary (19–24 Ma) (Guo et al., 2005).

The bursts of speciation of the kind reported here within the *L–C–P* complex are most frequently recorded in island archipelago biomes, for example in *Arygyranthemum* (Asteraceae) in Macronesia (Francisco-Ortega et al., 1997) and in the lineage that gave rise to the Hawaiian silversword alliance (Baldwin and Sanderson, 1998). Rapid

Fig. 7. The inferred historical distribution of the *Ligularia–Cremanthodium–Parasenecio* clade using Macclade and DIVA. (A) Fitch parsimony optimization of distribution onto the consensus *L–C–P* ITS maximum likelihood topology with a construction of 16 steps (RI = 0.69; CI = 0.17; RC = 0.11). (B) The inferred historical distribution using DIVA. The optimal reconstruction required 16 dispersals. Equally optimal distributions are separated by slash.

colonization and recent speciation in these islands (Liem, 1990) is reflected in a low level of nucleotide distance between species. However, nucleotide differences in the ITS region between species pairs of the *L–C–P* clade investigated here ranged from 0.21 to 19.46% with a mean difference of 6.63%, which is far higher than corresponding figures for genera confined to islands (Panero et al., 1999). Nevertheless, the phylogenetic resolution of the *L–C–P* clade is far lower than that of archipelago genera (Baldwin and Sanderson, 1998; Francisco-Ortega et al., 1997; Panero et al., 1999). This is because many mutations in the clade (more than 54% in ITS, and more than 80% in plastid sequences) are autapomorphic and phylogenetically uninformative. This situation is similar to that found in the continental radiation of the neotropical legume genus, *Inga* (Richardson et al., 2001a). Among 609 ITS aligned sites examined in 32 species of *Inga*, only 104 (40%) of 260 variable sites were potentially informative; similarly of 1009

*trn*L-F aligned sites in *Inga*, only 16 (15.7%) of 102 variable sites were present in two or more taxa. We propose that deep valley formation and high mountain building, which has occurred in the Q-T Plateau region since the Miocene (Guo et al., 2002; Li et al., 1995), has most likely been a potent factor in causing a rapid burst of speciation that gave rise to the *L–C–P* complex. According to this hypothesis, the species so formed would have accumulated their autapomorphic mutations in isolation of each other following rapid formation of geographical barriers and fragmentation of habitat occupied by the ancestral lineage. Consequently, there is both a relatively low frequency of synapomorphic mutations and nested clades present in this complex relative to what might have been expected if speciation had occurred more gradually. Such a phenomenon may be a general rule in continental radiations.

This scenario of rapid allopatric speciation gains further support from the following. First, the restriction of most endemic species of the *L–C–P* to a single hill or valley in the Q-T Plateau clade (Chen, 1999; Liu, 1989; Liu et al., 1994, 2002b). Second, the absence of polyploids in more than 60 species of the complex, thus inferring that sympatric speciation through polyploidization has played a minor role in the diversification of the group (Liu, 2004). Third, while some traits appear to have been retained by species occupying very different habitats, the different states of other variable traits appear to have been fixed in different species irrespective of relationship or ecology. For example, long hairs at the plant base were recorded for two disjunct species, *L. vellera* and *L. rumicifolia* (Liu, 1989). The former species is distributed in the coniferous forests of northwest Yunnan and southwest Sichuan (the eastern plateau), while the latter occupies the alpine meadow in Tibet (the western plateau). In contrast, discoid capitula, which are used to discriminate several genera and some species of *Ligularia* and *Cremanthodium* (Chen, 1999; Liu, 1989; Liu et al., 1994, 2002b), appear in different taxa in the phylogenetic trees in a manner that indicates independent origins from radiate capitula among different lineages of the Senecioneae (Fig. 6A). Similarly the dysploid reduction of the chromosome basic number $x = 30$ to $x = 29$, 26, 24, and 20 has occurred in parallel several times in this tribe (Fig. 6B). Because of these and possible additional effects, e.g., convergent evolution due to rapid adaptation to similar ecological conditions, taxonomic delimitation above species level is difficult and the molecular phylogenies presented suggest that most genera of the Tussilagininae from eastern Asia seem to require generic re-circumscription. However, many other morphological characters, such as microfloral characters and pollen types, have not yet been scored in all taxa; moreover, the limited recorded types (Liu, 2001) do not show a clear correlation to the groups found from the molecular data. Therefore, it is difficult to find reliable characters that can be used for classification.

Geological evidence indicates that recent extensive uplifting of the Q-T Plateau occurred during at least four different periods since the early Miocene, i.e., 22, 15–13, 8–7, and 3.5–1.6 Ma (Harrison et al., 1992; Li et al., 1995; Shi et al., 1998; Spicer et al., 2003), and within each period new habitats may have been created while old ones became fragmented. It is feasible, therefore, that the more recent fragmentation of habitat intensified the isolation of species or populations produced by earlier alterations of habitat, thus fostering the accumulation of autapomorphic mutations. These repeated isolations produced high sequence divergence between some paired species, but provided no useful solution to differentiation of the internal clades (Fig. S3). In addition, new species may have originated through subdivision of the first wave of species produced. Consequently, a second radiation or more gradual allopatric speciation could have occurred, resulting in a discernible clustering of genetic mutations within phylogenetic trees. In the *L–C–P* complex, this type of gradual allopatric speciation might have happened in the *Sinosenecio* and *Parasenecio* groups.

It is also possible that as a consequence of migrations driven by climatic oscillation and the colonization of plants during relatively stable periods between uplifting, previously isolated species became sympatric in contact areas. If these species had experienced long isolation and accumulated sufficient mutations, they might have co-occurred without hybridizing. The current sympatric distribution of some species of the *L–C–P* clade supports this possibility, in that several species can be found growing together in contact zones, without evidence of intermediate individuals. On the other hand, if sympatric species were not fully reproductively isolated from each other, they might have undergone interspecific hybridization. Natural hybrids are commonly found in certain areas where the distributions of some *Ligularia* species currently overlap, e.g., *Ligularia dentata* × *veitchiana* and *Ligularia przewalskii* × *virgaurea* (unpublished results). It remains unknown whether such hybridization, if it occurred in the past, could have contributed to the radiation of the *L–C–P* complex. However, conflicts between ITS and chloroplast sequence phylogenetic trees (Figs. 2 and 3), and also the presence of ITS sequence additivity in some species, indicate that hybridization may have had some important consequences. In addition, this additivity found in more than one accession of each of these species suggests incomplete lineage sorting since a hybrid origin or the ongoing occurrence of introgression. Because all species are diploid, any hybrid species that may have evolved in the complex must be assumed to be homoploid. Homoploid hybrid speciation is rarely recorded in the angiosperms (Rieseberg, 1997), but is favored when a stabilized hybrid is able to occupy a different habitat from its parents (Abbott, 2003; Rieseberg, 1997). The complex topography and diverse habitats of the Q-T Plateau might have provided a suitable setting for homoploid hybrid speciation to have contributed to diversification within the *L–C–P* complex. Both ancient and recent introgression could cause replacements of cpDNA-types (Rieseberg and Carney, 1998), leading to identical or low divergence of *trn*L-F sequences. In addition, concerted

evolution after introgression and hybridization could cause homogenization of nuclear ITS sequences and therefore lead to low differentiation between some paired species with distinct morphology (Abbott, 2003), i.e., *Cremanthodium stenoglossum* vs. *C. microglossum*, which show a great difference in leaf morphology and belong to two different sections (Table 1).

The diversity anomaly (i.e., differences in species richness despite similar environmental condition) between eastern Asian and eastern North America has been repeatedly discussed in the literature (e.g., Qian and Ricklefs, 2000; Qian et al., 2005). Here, we propose that radiation and diversification within the *L–C–P* complex was triggered by uplift of the Q-T Plateau. Such effects may also have been involved in the production of high species diversity in adjacent areas, such as central China and Japan (Fig. 7), and may therefore be partly responsible for the Asian bias in plant richness. No such extensive geological changes have occurred in eastern North America during the same time period. A recent comparison of species diversity and molecular evolution between sister clades of disjunct genera from these two regions suggests greater net speciation in eastern Asia, which is accounted for by higher topographic heterogeneity and an accelerated rate of nucleotide substitution (Xiang et al., 2004). Our analyses indicate that species of the *L–C–P* complex occurring in central China and Japan might have originated from the Q-T Plateau through vicariance or dispersals (Fig. 7). This suggests that uplifts of the Q-T Plateau might have further contributed to the biodiversity in adjacent regions of the plateau in eastern Asia. It is feasible that a relatively high accumulation of nucleotide substitution in some species of the Asian groups might reflect genetic divergence in small isolated areas created by relatively early geological changes (e.g., the uplifts around 22 and 8 Ma, Guo et al., 2002; Harrison et al., 1992). Most of these substitutions might be autapomorphic mutations without phylogenetic significance but indicating continental radiation. Conversely, the morphological diversification and rapid radiation in some species or in other groups possibly triggered by the more recent uplift of the Q-T Plateau (i.e., 3.5–1.6 Ma) (Li et al., 1995; Shi et al., 1998) or by interspecific hybridisation, might not show a corresponding high rate of nucleotide substitution (e.g., *Senecio*, Wang et al., 2005a; Wang and Liu, 2004; *Rheum*, Wang et al., 2005b and *Rhododendron* subgen. *Hymenanthes*, Milne, 2004 and personal communication).

5. Conclusions

Several molecular phylogenetic studies conducted on species-rich plant groups that occur in biodiversity hotspots have now yielded similar findings with regard to species diversity being the product of recent bursts of speciation triggered most likely by geophysical and/or climatic changes within these regions since the middle Miocene (Richardson et al., 2001a). In the Neotropics, however, it has been argued that a mixture of both ancient and recent diversification should be involved to explain the extant high species richness of those speciose genera (Pennington et al., 2004). Whether the recent major uplift of the Q-T Plateau triggered bursts of speciation in temperate, species-rich genera distributed in this region other than those within the *L–C–P* complex remains to be demonstrated. It is possible, for example, that it played a role in the diversification of *Gentiana* sect. *Chondrophyllae*, although Yan and Kupfer (1997) have attributed high levels of diversity within this group to the biennial, herbaceous habit of species. In *Rhododendron*, more than 200 species are contained within subgenus. Hymenanthes, all of which occur in southeast Asia and many of which are native to the Q-T Plateau. Milne (2004; and personal communication) has recently shown that many members of subgenus *Hymenanthes* most likely originated during the rapid radiation of a clade sister to a southwest Eurasian species, *R. smirnovii*. The calibration of this clade based on fossils indicated that the radiation occurred approximately 4–6 Ma, i.e., shortly after diversification began in the *L–C–P* clade, based on the calibration presented here. In the case of *Rhododendron* subgen. *Hymenanthes*, the Q-T Plateau might have been one of several centers of diversification that existed in southeast Asia during the late Tertiary. Further studies of other species-rich plant groups are now required to establish if bursts of speciation triggered by the recent uplifts of the Q-T Plateau, possibly combined with hybridization because of secondary sympatry during relatively stable stages between different uplifts, are a common phenomenon and of major importance in generating the present-day high diversity of plants and other organisms within this region and adjacent areas.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2005.09.010.

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