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Phylogeny and Biogeography of the Genus *Ainsliaea* (Asteraceae) in the Sino-Japanese Region based on Nuclear rDNA and Plastid DNA Sequence Data

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- *Background and Aims* The flora of the Sino-Japanese plant region of eastern Asia is distinctively rich compared with other floristic regions in the world. However, knowledge of its floristic evolution is fairly limited. The genus *Ainsliaea* is endemic to and distributed throughout the Sino-Japanese region. Its interspecific phylogenetic relationships have not been resolved. The aim is to provide insight into floristic evolution in eastern Asia on the basis of a molecular phylogenetic analysis of *Ainsliaea* species.
- *Methods* Cladistic analyses of the sequences of two nuclear (ITS, ETS) and one plastid (*ndhF*) regions were carried out individually and using the combined data from the three markers.
- *Key Results* Phylogenetic analyses of three DNA regions confirmed that *Ainsliaea* is composed of three major clades that correspond to species distributions. Evolution of the three lineages was estimated to have occurred around 1·1 MYA during the early Pleistocene.
- *Conclusions* The results suggest that *Ainsliaea* species evolved allopatrically and that the descendants were isolated in the eastern (between SE China and Japan, through Taiwan and the Ryukyu Islands) and western (Yunnan Province and its surrounding areas, including the Himalayas, the temperate region of Southeast Asia, and Sichuan Province) sides of the Sino-Japanese region. The results suggest that two distinct lineages of *Ainsliaea* have independently evolved in environmentally heterogeneous regions within the Sino-Japanese region. These regions have maintained rich and original floras due to their diverse climates and topographies.

Key words: *Ainsliaea*, ETS, ITS, *ndhF*, phylogeography, rheophytes, Sino-Japanese region.

INTRODUCTION

The genus *Ainsliaea* DC. (Asteraceae) comprises about 40 species of perennial herbs widely distributed in the Sino-Japanese region (Mabberley, 1998). *Ainsliaea* belongs to tribe Mutisieae. The most closely related genera are *Pertya* Sch. Bip. and *Myripnois* Bunge, based on a molecular phylogeny of Mutisieae using the plastid *ndhF* gene (Kim *et al.*, 2002). *Ainsliaea* can be distinguished from other related genera by the pappus and flower heads; the pappus has plumose bristles and mostly three (one or five in some species) florets per flower head, whereas related genera have a scabrous pappus and one to more than ten floret(s) per head (Kitamura, 1981). The infrageneric classification of the genus recognizes three sections (Beauverd, 1909; Tseng, 1996) based on leaf arrangements on the stem and stem branching patterns: section *Ainsliaea* (*Scaposae sensu* Beauverd) has radical leaves and clustered scapes; section *Aggregatae* Beauverd has cauline leaves; section *Frondosae* Beauverd has branching aerial stems. *Aggregatae* and *Ainsliaea* do not have branching stems. However, this taxonomic subdivision has been controversial (e.g. Koyama, 1995; Peng *et al.*, 1998), partly due to the uniform reproductive characteristics and geographically continuous distribution of the genus. Most *Ainsliaea* species grow in the forest understorey or in rocky areas with humid and wet conditions, but some are confined to river- and streambeds where they experience strong water flow during floods.

The Sino-Japanese region covers over 5000 km in an east–west direction. The flora is rich in endemics (Takhtajan, 1969, 1986; Good, 1964; Wu and Wu, 1996; Qian, 2002). The floristic richness of the Sino-Japanese region is greater than that of the North American floristic region, even though these two regions have similar climates and floristic components (Qian and Ricklefs, 1999, 2000; Qian, 2002, 2004). Wu and Wu (1996) proposed the Sino-Japanese region as a floristic kingdom and recognized two environmentally heterogeneous subkingdoms: the Sino-Himalayan Forest subkingdom and the Sino-Japanese Forest subkingdom. South-west (SW) China is representative of the Sino-Himalayan subkingdom. Almost half of the total plant species in China are distributed in SW China with more than one-third of its species being endemic to this region (Wu, 1980; Wu and Wu, 1996). The floral richness of this region is attributed to its diverse climate and topography, and vegetation varies

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from tropical to boreal forest (Axelrod *et al.*, 1998; Qian, 2004; Xiang *et al.*, 2004). The characteristics of this region were created by the uplifting of the Himalayas and the Tibetan Plateau in the late Palaeocene (about 55 MYA) to the early Pleistocene (Committee of Chinese Academy of Sciences for Physical Geography of China, 1984). Moreover, the development of the Himalayas brought a large amount of rainfall to the region by forming a monsoon system in East Asia (Sun and Wang, 2005). Such a diverse geology and climate has significantly affected the evolutionary history of plants.

The eastern edge of the Sino-Japanese region, ranging from south-east (SE) China to mainland Japan and the Korean Peninsula through Taiwan and the Ryukyu Islands, is included in the Sino-Japanese Forest subkingdom. This region is also characterized by a remarkably rich flora and high rainfall due to the south-eastern monsoon. It encompasses >120 continental islands (Takhtajan, 1969, 1986). The biota of these islands experienced dramatic distribution changes attributed to topographical changes during the Quaternary climatic oscillations. A land-bridge once existed between SE China and mainland Japan through the Ryukyu Islands and Taiwan during glacial periods, but was fragmented during inter- and post-glacial periods (Kizaki and Oshiro, 1977, 1980; Ujiie, 1990; Kimura, 1996, 2000). The *Ainsliaea* species in this region may have evolved allopatrically due to the topogeographical events of the Quaternary.

Ainsliaea is endemic to this floristic region and is mainly distributed in the corridor-shaped area from the Himalayas to Japan and the Korean Peninsula, through SW to SE China, Taiwan and the Ryukyu Islands (Fig. 1). Quaternary climatic oscillations led to repeated migration, adaptation and extinction of plant and animal species; the present genetic structure of populations, species and communities was mainly formed during the Pleistocene (Bennett, 1997; Hewitt, 2000). Previous molecular phylogenetic studies have revealed evolutionary patterns sculpted by the Quaternary climatic oscillations (e.g. Xiang *et al.*, 2000, 2004; Wen, 2001; Xiang and Soltis, 2001). However, most of the studies addressed eastern Asian–eastern North American biogeography, and few phylogeographic studies have embraced the whole Sino-Japanese region. A recent phylogenetic study on varieties of *Spiraea japonica*, from SW and SE China to mainland Japan, found an allopatric evolutionary history in eastern and western areas of the Sino-Japanese region (Zhang *et al.*, 2006). However, this study analysed only 14 samples of the species across the wide distribution range and did not cover the entire Sino-Japanese region. Because *Ainsliaea* is widely distributed in the Sino-Japanese region, migration and evolution of *Ainsliaea* species may have occurred separately in several parts of this lengthy corridor. Reconstruction of *Ainsliaea* phylogenetic relationships and estimation of divergence times between outgroups and the major clades of the genus would provide a better insight into the floristic evolution in the Sino-Japanese region during the Quaternary.

One of the remarkable aspects of *Ainsliaea* is the occurrence of ‘rheophyte’ species (van Steenis, 1981, 1987). Rheophytes are adapted to river-bank environments in which plants are periodically covered with floodwater after heavy rains and they generally have narrow leaves. In the region from SE China to the Ryukyu Islands, at least four species (*A. walkeri* and *A. trinervis* in SE China, *A. oblonga* and *A. linearis* in the Ryukyu Islands) are adapted to this type of environment and exhibit this distinctive morphology. Because of the unusual ecology of these species, they are restricted to river banks. These rheophyte species are distributed in places that are characterized by high annual rainfall, e.g. *A. linearis* on Yakushima Island of the Ryukyus, where annual rainfall ranges from 3000 to 7000 mm year^{-1} (Takahara and Matsumoto, 2002). Plant genera that include several rheophyte species rarely occur in temperate regions (van Steenis, 1981, 1987). The occurrence of four rheophyte species of *Ainsliaea* is quite unusual. The unique suite of morphological features characteristic of rheophytes may be an adaptation to the rich environment and high annual rainfall in the region from SE China to the Ryukyu Islands. A molecular phylogenetic analysis of these species would contribute to our understanding of the evolution of rheophytes, i.e. whether they arose convergently after allopatric diversification to adapt to their rheophitic environments or were derived from a common ancestral rheophytic species. For example, two rheophytes, *A. walkeri* from SE China and *A. linearis* from Yakushima Island, were estimated to be the same or closely related species based on their similar morphology (Kitamura, 1981); however, their phylogenetic relationships and evolutionary history are still uncertain. Phylogenetic analysis of *Ainsliaea* would clarify the taxonomic situation and would provide evolutionary insights.

The aim of the present research was to resolve interspecific phylogenetic relationships and to estimate the divergence time of *Ainsliaea* by analysing three DNA sequence datasets. The nuclear ribosomal internal transcribed spacer (ITS) and nuclear ribosomal external transcribed spacer (ETS) regions have been shown to be useful sources of information for resolving the interspecific phylogenies in Asteraceae (Baldwin, 1992, 1993; Kim and Jansen, 1994; Sang *et al.*, 1994, 1995; Clevinger and Panero, 2000; Linder *et al.*, 2000), and the plastid *ndhF* region has been used previously to reconstruct the phylogeny of Asteraceae (Kim and Jansen, 1995; Kim *et al.*, 2002, 2005).

MATERIALS AND METHODS

Taxonomic sampling

Twenty-eight species and six varieties of *Ainsliaea*, four species of *Pertya* and *Diaspananthus uniflorus* (all Asteraceae) were sampled. The broadly distributed species and their subspecies or varieties (*A. henryi*, *A. macroclinidioides* and *A. macroclinidioides* var. *okinawensis*) were sampled from several points to examine their monophyly. *Diaspananthus*, a monotypic genus endemic to Japan, is almost identical to *Ainsliaea* except for floral characteristics (*D. uniflorus* has only one floret per flower head); however, a previous phylogenetic analysis

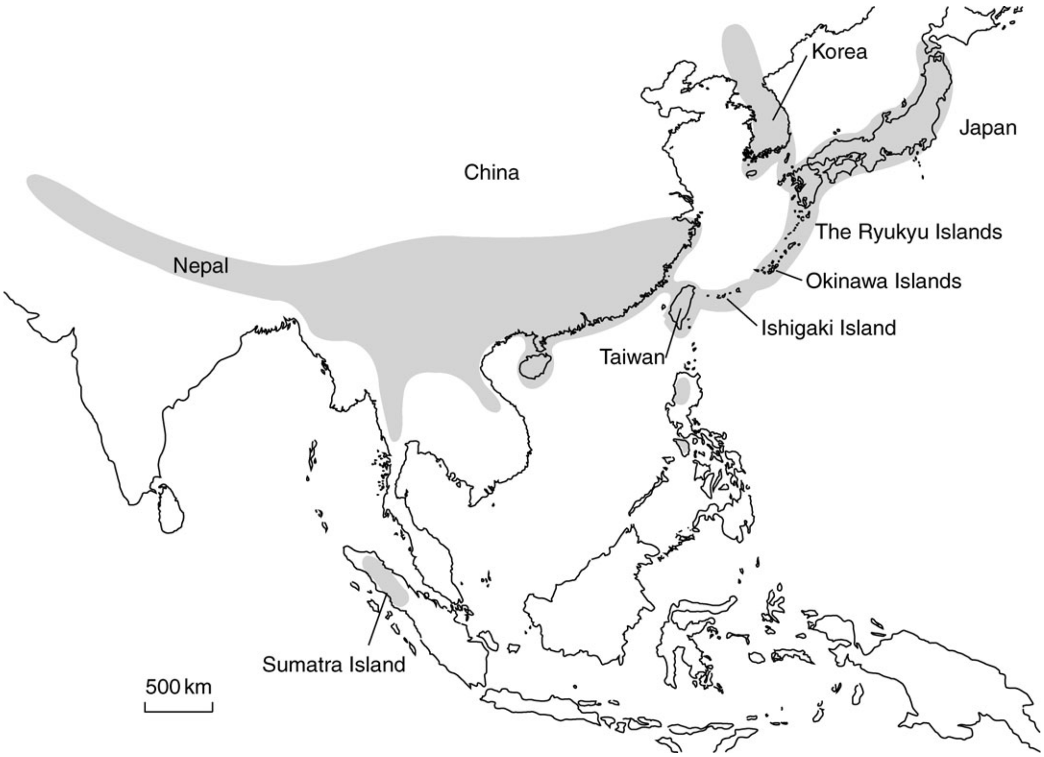

FIG. 1. Distribution of the genus *Ainsliaea*. Only *A. latifolia* and its close relatives are found in the temperate area of south-east Asia. Four taxa (*A cordifolia, A. acerifolia* var. *acerifolia, A. acerifolia* var. *subapoda* and *A. apiculata*) are shared between mainland Japan, northern China and the Korean Peninsula.

of plastid DNA sequences for Mutisieae (Kim *et al.*, 2002) did not include this genus. *Pertya* and *Diaspananthus* were used as outgroups in this study. Samples of *Ainsliaea* and putative outgroups were collected from their natural habitats, cultivated stocks at botanical gardens and from herbarium specimens. The taxa, GenBank accession numbers and voucher information are listed in Table 1.

DNA extraction, amplification and sequencing

Silica gel-dried or fresh leaf material was frozen in liquid nitrogen and ground to a powder. After the polysaccharides were removed from this powder with Hepes buffer (pH 8·0; Setoguchi and Ohba, 1995), total DNA was extracted using the 2× CTAB method (Doyle and Doyle, 1987). The extracted DNA was dissolved in 100 μL of TE buffer and used for polymerase chain reaction (PCR). The primers used to amplify ITS1 and ITS2, the 3′ end of ETS and the 3′ end of the plastid *ndhF* gene are listed in Table 2, with primer sequences and their original references. PCR was conducted in a total reaction volume of 25 μL containing 18·5 μL of autoclaved ion-exchanged water, 0·2 mM dNTP mixture, 2·0 mM 10× Ex*Taq* Buffer (Takara Ex*Taq*), 0·625 U Takara Ex*Taq* (Takara Bio, Ohtsu, Japan), 0·2 μM of each primer, and 1·25 μL of DNA. PCR was performed in 35 cycles with the following conditions for each region: ITS1 and ITS2 (1 min at 94 °C, 1 min at 48 °C, 1 min at 72 °C), ETS and *ndhF* (1 min at 94 °C, 1 min at 56 °C, 1 min at 72 °C).

The PCR products were sequenced in both directions using the standard methods of the BigDye™ Deoxy Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) using the same primers as above on an ABI 3100 Genetic Analyser (Applied Biosystems).

Sequence alignment and phylogenetic analysis

ITS sequences were determined for 37 taxa, ETS sequences for 36 taxa and *ndhF* sequences for 33 taxa. All sequences have been deposited in a DNA database (DDBJ/EMBL/GenBank; accession numbers AB288427–AB288547). Sequence data were edited and assembled using AutoAssembler™ (Applied Biosystems). Parsimony analyses were performed for four data sets: ITS alone, ETS alone, *ndhF* alone and a combined dataset of all three DNA regions (ITS + ETS + *ndhF*). Phylogenetic analyses were conducted using PAUP* 4.0b10 (Swofford, 2002). Parsimony analyses were performed with heuristic searches of 1000 replicates of random stepwise addition using tree bisection and reconnection (TBR) branch swapping. The gaps caused by mononucleotide repeat units were removed from consideration in the phylogenetic analysis, because homology can be highly uncertain for these repeated nucleotides (Kelchner, 2000). All characters and character state transformations were equally weighted. The consistency index (CI) excluding uninformative characters and retention index (RI) (Kluge and Farris, 1969,

TABLE 1. *Materials used in the present study*

Taxa	Distributions	GenBank accession no.			Voucher information
		ITS	ETS	*ndhF*	
Ainsliaea acerifolia Sch. Bip.					
var. *acerifolia* Sch. Bip.	Mainland Japan, Korean Peninsula	AB288427	AB288469	AB288510	A. Fujisaki 93–353, KYO
var. *subapoda* Nakai	Mainland Japan, Korean Peninsula	AB288428	AB288470	AB288511	H. Setoguchi JP2448, KYO
A. apiculata Sch.Bip	Yakushima Island, Minaland Japan, Korean Peninsula	AB288429	AB288471	AB288512	H. Setoguchi JP2438, KYO
var. *acerifolia* Masam.	Yakushima Island	AB288430	AB288478	AB288513	H. Setoguchi JP2434, KYO
A. aptera DC.	The Himalayas to Tibet	AB288431	AB288472		H. Tabata 20758, KYO
A. apteroides (C. C. Chang) Y. C. Tseng	SW China	AB288432	AB288473	AB288514	H. Chuang 0731465, KYO
A. cordifolia Franch. et Savat.	Mainland Japan, Korean Peninsula	AB288433	AB288474	AB288515	H. Setoguchi JP2446, KYO
A. dissecta Franch. et Savat.	Mainland Japan	AB288434	AB288475	AB288516	T. Kobayashi 32365, KYO
A. elegans Hemsl.					
var. *strigosa* Mattf.	SW China	AB288435	AB288476	AB288517	H. Chuang 0731544, KYO
A. foliosa Hand.-Mazz.	SW China	AB288437	AB288479		H. Chuang 3589, KYO
A. fragrans Champ. ex Benth	SE China, Taiwan	AB288438	AB288480	AB288519	Y. Mitsui C06801, KYO
var. *integrifolia* (Maxim.) Kitam.	Mainland Japan	AB288439	AB288481	AB288520	H. Setoguchi JP 2462, KYO
A. fulvipes Jeffrey et W. W. Sm.	SW China	AB288440	AB288482	AB288521	H. Chuang 1514, KYO
A. glabra Hemsl.	SW China	AB288441	AB288483	AB288522	B. L. Chen 0731626, KYO
A. gongshanensis H.Chung	SW China	AB288442	AB288484	AB288523	H. Chuang 0731637, KYO
A. gracilis Franch.	SE China	AB288443	AB288485	AB288524	Y. Mitsui C06850, KYO
A. grossedentata Franch.	SE China	AB288444	AB288486	AB288525	H. Chuang 16883, KYO
A. henryi Diels	Himalayas to Taiwan (Yunnan Prov.)	AB288445	AB288487	AB288526	Y. Fei 0731686, KYO
A. henryi Diels	Himalayas to Taiwan (Yunnan Prov.)			AB288530	S. Chen, H. Setoguchi 2004062
A. henryi Diels	Himalayas to Taiwan (Sumatra Island)	AB288449	AB288491	AB288529	H. Nagamasu 3705, KYO
A. henryi Diels	Himalayas to Taiwan (Taiwan)	AB288450	AB288492	AB288531	H. Setoguchi 04T-M2310, KYO
A. heterantha Hand.-Mazz.	SW China, Tibet	AB288446	AB288488	AB288527	S. Chen, H. Setoguchi 2004006, KUN, KYO
A. lancifolia Franch.	SW China	AB288447	AB288489		W. H. Wang 8078, KYO
A. latifolia (D. Don) Sch. Bip.	The Himalayas to Taiwan (Yunnan Prov.)	AB288448	AB288490	AB288528	H. Setoguchi C200605, KYO
A. lijiangensis H. Chung	SW China	AB288451	AB288494	AB288532	S. Chen, H. Setoguchi 2004005, KUN, KYO
A. linearis Makino	Yakushima Island	AB288436	AB288477	AB288518	H. Setoguchi JP2436, KYO
A. macrocephala (Mattf.)Y. C. Tseng	SW China	AB288452	AB288493	AB288533	S. Chen, H. Setoguchi 2004241, KUN, KYO
A. macroclinidioides Hayata	SE China, Taiwan (Guangdong Prov.)	AB288453			H. Migo no number, KYO
A. macroclinidioides Hayata	SE China, Taiwan (Taiwan)	AB288454	AB288495	AB288534	H. Setoguchi 04T-M2311, KYO
var. *okinawaensis* (Hayata) Kitam.	The Ryukyu Islands (Ishigaki Island)	AB288455	AB288496	AB288535	H. Setoguchi JP2605, KYO
var. *okinawaensis* (Hayata) Kitam.	The Ryukyu Islands (Okinawa Island)	AB288456	AB288497	AB288536	H. Setoguchi JP2448, KYO
A. oblonga Koidz	Okinawa Island	AB288457	AB288498	AB288537	H. Setoguchi JP2449, KYO
A. pertyoides Franch.	SW China	AB288458	AB288499	AB288538	H. Chuang 0731991, KYO
A. spicata Vaniot	SW China	AB288459	AB288500	AB288539	S. Chen, H. Setoguchi 2004092, KUN, KYO
A. sutchuenensis Franch.	SW China	AB288460	AB288501	AB288540	H. Chuang 0732127, KYO
A. trinervis Y. C. Tseng	SE China	AB288461	AB288502	AB288541	H. Chuang 0732148, KYO
A. walkeri Hook. f.	SE China	AB288464	AB288503		Z. Huang 604507, IBSC
A. yunnanensis Franch.	SW China	AB288462	AB288504	AB288542	S. Chen, H. Setoguchi 2004137, KUN, KYO
Diaspananthus uniflorus (Sch. Bip.) Kitam.	Mainland Japan	AB288468	AB288509	AB288547	H. Setoguchi JP2444, KYO
Pertya glabrescens Sch. Bip.	SE China, Mainland Japan	AB288463	AB288505	AB288543	T. Shimizu 11, KYO
P. rigidula (Miq.) Makino	Mainland Japan	AB288465	AB288506	AB288544	S. Yasuda 527, KYO
P. robusta (Maxim.) Beauv.	Mainland Japan	AB288466	AB288507	AB288545	S. Tsugaru, T. Takahashi 72310, KYO
P. scandens (Thunb. ex Murray) Sch. Bip.	Mainland Japan	AB288467	AB288508	AB288546	H. Setoguchi JP2464, KYO

Localities of widely distributed species are shown in parentheses after each distribution.

Voucher specimens are deposited in the following herbaria: KYO, Kyoto University; KUN, Kunming Institute of Botany, Chinese Academy of Science; IBSC, South China Botanical Garden.

Farris, 1989) were calculated. Statistical support was evaluated through nonparametric bootstrap analysis (Felsenstein, 1985) with 1000 replicates using NNI branch swapping for each data set and decay indices (DI; Bremer, 1988, 1994) with the Parsimony Ratchet Analyses using PAUP (PRAP; Muller, 2004). An incongruence length difference (ILD) test (Farris *et al.*, 1995) was conducted to investigate character congruence between taxonomically equivalent ITS, ETS and *ndhF* partitions.

Relative rate tests (Sarich and Wilson, 1973; Wu and Li, 1985) were used to examine the heterogeneity of the ITS and ETS sequence divergence rates between major clades. The divergence rate of *ndhF* was not calculated due to the small number of substitutions. *Pertya robusta* was used as the reference outgroup taxon in substitution rate comparisons. Sequence divergences for the tests were calculated only from substitutions based on the method of Jukes and Cantor (1969), implemented in RRTree (Relative-Rate Tests between groups of sequences on a phylogenetic tree; Robinson-Rechavi and Huchon, 2000).

The estimation of divergence time for selected lineages was calculated as $T = D_A/2\lambda$ (Nei, 1987). For ITS, the rate of nucleotide substitutions per site per year (λ) of *Eupatorium* was used, which has been calculated as $1{\cdot}95 \times 10^{-8}$ substitutions site^{-1} year^{-1} (Schmidt and Schilling, 2000) as an example of perennial herbs in Asteraceae in temperate regions.

TABLE 2. *Primer sequences used for PCR and cycle sequencing*

Name	Sequence (5′–3′)	Source
ITS5	GAAAGTAAAAGTCGTAACAAGG	White *et al.*, 1990
ITS2	GCTGCGTTCTTCATCGATGC	White *et al.*, 1990
ITS3	GCATCGATGAAGAACGCAGC	White *et al.*, 1990
ITS4	TCCTCCGCTTATTGATATGC	White *et al.*, 1990
ETS1f	CTTTTTGTGCATAATGTATATATAGGGGG	Linder *et al.*, 2000
18S-2L	TGACTACTGGCAGGATCAACCAG	Linder *et al.*, 2000
2L-2	CGYATCGTTCGGTRCATTCTGG	Designed for this study
ndhF-F	CTTAATGATAGTTGGTTGTATTCACC	Designed for this study
P14	ACCAAGTTCAATGTTAGCGAGATTAGTC	Jansen, 1992

RESULTS

Sequencing comparison

DNA site variation for each of the three regions and the combined data (ETS + ITS + *ndhF*), as well as tree statistics, are shown in Table 3. Within *Ainsliaea*, the nucleotide sequence length of the three regions was 646–650 bp for the ITS region, 475–481 for the ETS region and 947 for the *ndhF* region. The percentage of potentially parsimony-informative sites in the ITS region was 17·15 % from 649 characters. The ETS data matrix contained proportionally more informative sites (19·50 %) from 481 characters than the other data sets. The *ndhF* sequences included only 36 potentially parsimony-informative sites (3·91 %) from 947 characters. The number of indels was small. These indels were excluded from the analyses because they did not affect any results of the phylogenetic analyses. There were two indels which distinguish *Ainsliaea* and *Diaspananthus* from *Pertya* in the ITS and ETS. Within *Ainsliaea* species, two and one indels were found in ITS and ETS, respectively. In *ndhF*, there was one indel which distinguishes *Diaspananthus* from the others and no indels were found within *Ainsliaea*.

Phylogenetic analysis

Of the most parsimonious ITS trees 3323 were recovered; the strict consensus is shown in Fig. 2 with bootstrap support and DI. The most parsimonious trees had a length of 316 steps (CI 0·56, RI 0·83; Table 3).

Ainsliaea was paraphyletic, with *Diaspananthus uniflorus* falling in the ingroup, whereas the four species of *Pertya* were strongly supported as a monophyletic group (bootstrap value of 100 %, DI of 14). The infrageneric taxonomy recognizing three sections (*Ainsliaea*, *Aggregatae* and *Frondosae*) was inconsistent with the molecular phylogenetic trees. The ITS data supported three major clades of *Ainsliaea*, which were correlated with regional distributions.

Clade A was strongly supported (bootstrap 95 %, DI 2). All of the species in this clade are distributed from SE China to Japan through Taiwan and the Ryukyu Islands. Within this clade, *A. macroclinidioides* (distributed in SE China and Taiwan) did not form a monophyletic group and the Taiwanese species was placed within a polytomy of Japanese–Taiwanese species along with *A. macroclinidioides* var. *okinawensis*. Monophyly was

TABLE 3. *DNA site variation and tree statistics of* Ainsliaea *species for the three data sets used in the cladistic analyses presented in this study*

DNA region	No. taxa	No. characters (mean)	No. variable sites	No. informative sites	Informative sites (%)	No. trees	Tree length	CI	RI	RC
ITS	37	649	169	112	17·15	3323	316	0·5630	0·8348	0·5416
ETS	36	480	147	94	19·50	88	258	0·6332	0·8385	0·6012
ndhF	33	947	57	37	3·91	6876	76	0·7818	0·8776	0·7390
ITS + ETS + *ndhF*	32	2076	368	239	11·51	367	668	0·5758	0·7947	0·5282

CI = consistency index; RI = retention index; RC = rescaled consistency index (excluding uninformative characters).

also not supported for *A. macroclinidioides* var. *okinawensis* from Okinawa and Ishigaki Island of the Ryukyu Islands. The four rheophytic species, *A. linearis*, *A. oblonga*, *A. trinervis* and *A. walkeri* (indicated by * in Fig. 2) were included in a monophyletic group with ten OTUs (operational taxonomic units) of inland taxa (bootstrap 100 %, DI 9). The Chinese and Japanese rheophytes were divided into two clades, and the two Chinese rheophytes did not form a clade.

Clade B was weakly supported (bootstrap 66 %, DI 2). The species in this clade also showed a regional pattern, ranging between SW China and the Himalayas, except the outgroup *Diaspananthus uniflorus*, which is endemic to Japan. Within this clade, *A. henryi* from China and Sumatra was monophyletic, but the sample from Taiwan did not fall with the other samples, making *A. henryi* paraphyletic, albeit with low bootstrap support (<50 %).

Clade C was strongly supported (bootstrap 100 %, DI 6) and comprised three species (*A. glabra*, *A. lancifolia* and *A. sutchuenensis*) mainly distributed in Sichuan Province of South China. *Ainsliaea sutchuenensis* and *A. lancifolia* were strongly supported as sister species.

Parsimony analysis based on ETS sequence data resulted in 88 equally parsimonious trees of 258 steps, CI 0·63 and RI 0·84. The consensus of the 88 most parsimonious trees is shown in Fig. 3. The ETS tree had some incongruence with the ITS tree. Four species of *Pertya* were strongly supported as outgroups (bootstrap 100 %, DI 17). In the ETS tree, *Diaspananthus* was weakly supported as sister to *Ainsliaea* (bootstrap 54 %, DI 1). The three traditional

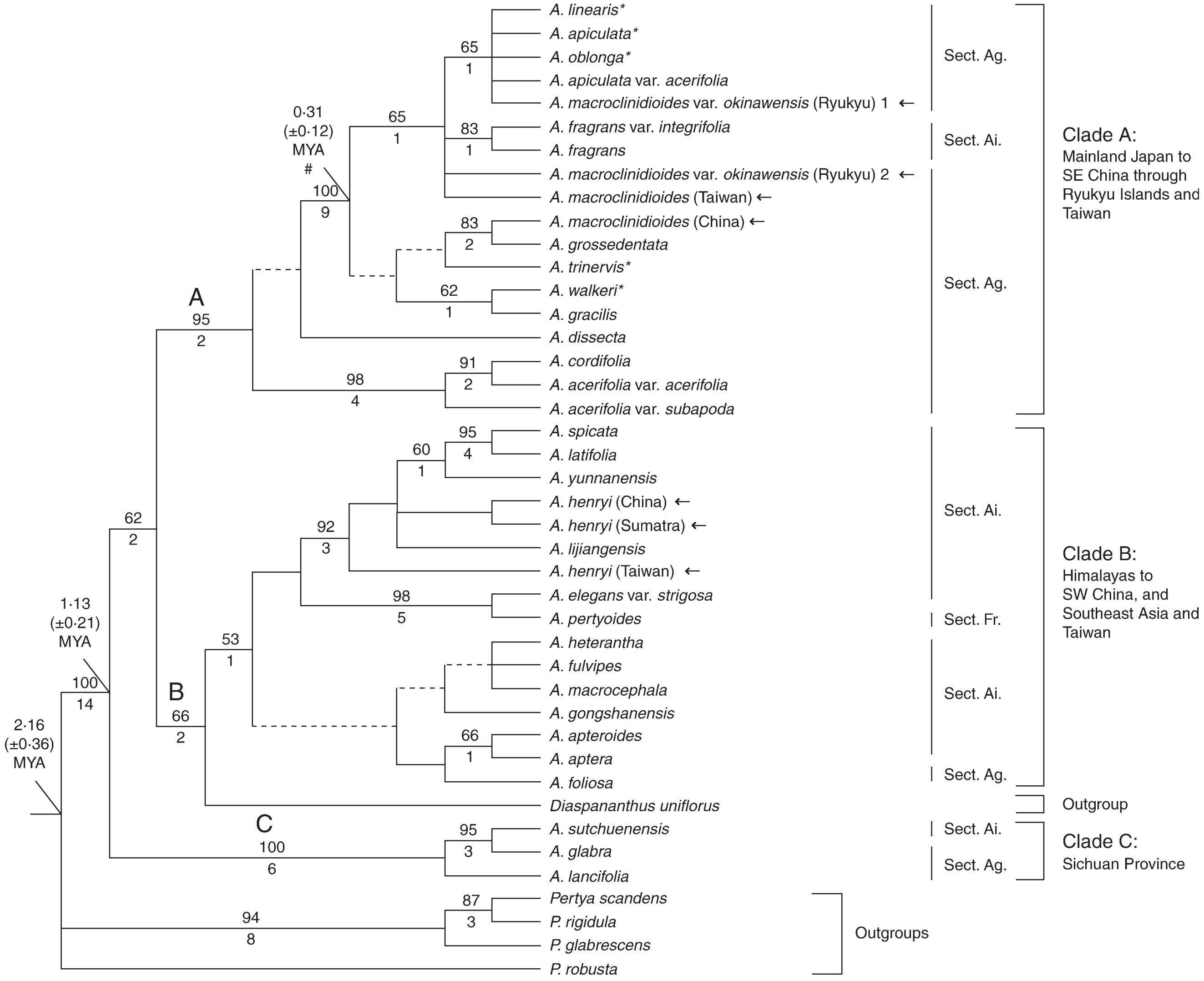

FIG. 2. Strict consensus of the 3323 most parsimonious trees from ITS sequences for 37 taxa and five outgroups. Tree length = 316. Numbers above and below branches indicate bootstrap support and decay index, respectively. Branches with broken lines are only in the 50 % majority rule consensus. Estimated divergence times among the major clades are indicated above branches with standard deviations (shown in parentheses). The four rheophyte species are indicated by*. The broadly distributed taxa *Ainsliaea macroclinidioides* var. *okinawensis*, *A. macroclinidioides* and *A. henryi* are represented by arrows. The monophyletic group of taxa from SE China, Taiwan and the Ryukyu Islands, including the four rheophytes in clade A, is indicated by #. Infrageneric classification by Beauverd (1909) is provided alongside each taxon. Abbreviations: Ai, section *Ainsliaea*; Ag, section *Aggregatae*; Fr, section *Frondosae*.

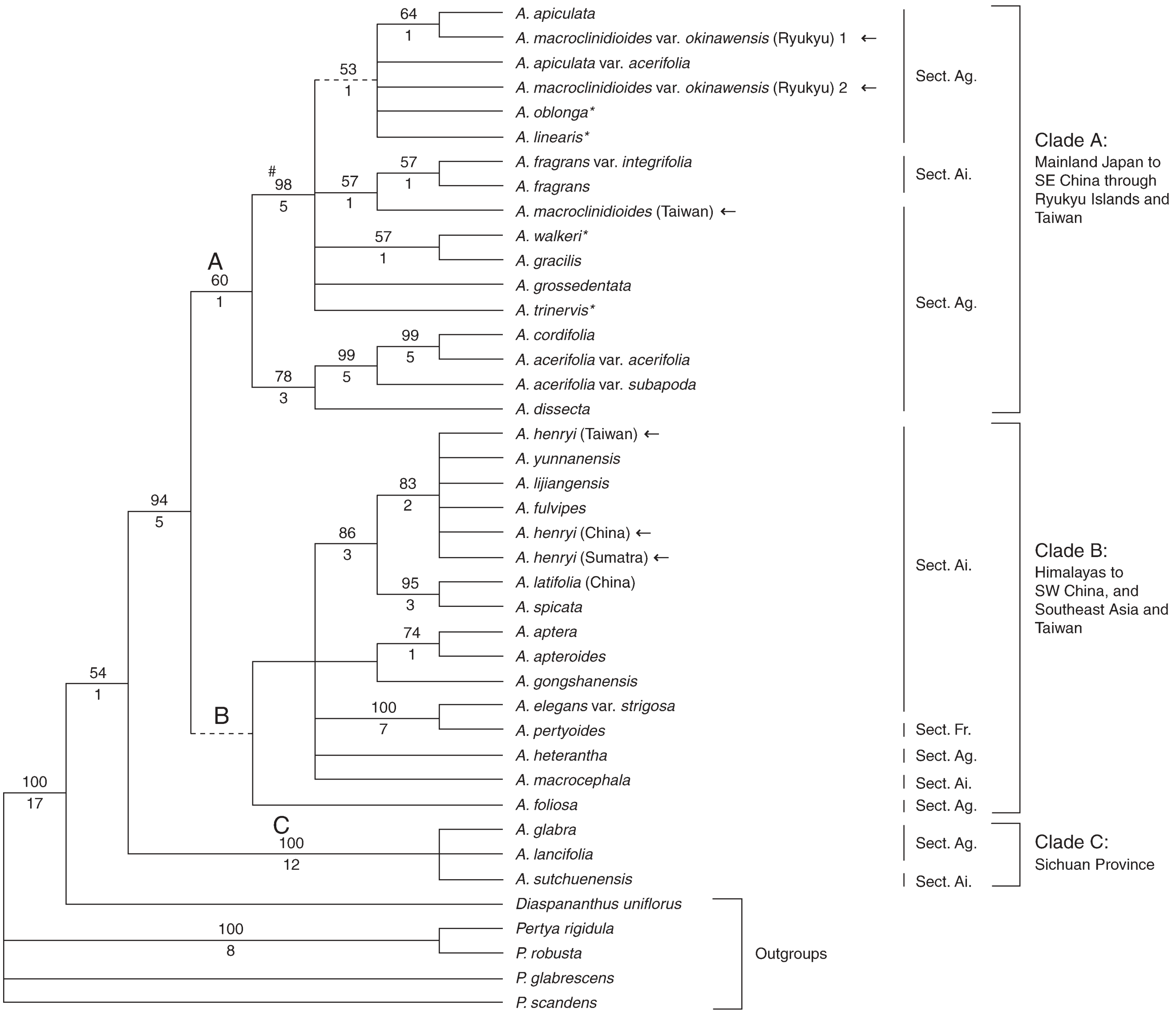

FIG. 3. Strict consensus of the 88 most parsimonious trees from the ETS sequences for 36 taxa and five outgroups. Tree length = 258. The four rheophyte species are indicated by *. The broadly distributed taxa *A. macroclinidioides* var. *okinawensis*, *A. macroclinidioides*, *A. henryi* are represented by arrows. The monophyletic group of taxa from SE China, Taiwan and the Ryukyu Islands, including the four rheophytes in clade A, is indicated by #. Infrageneric classification by Beauverd (1909) is provided alongside each taxon. Abbreviations: Ai, section *Ainsliaea*; Ag, section *Aggregatae*; Fr, section *Frondosae*.

sections of *Ainsliaea* were again inconsistent with the ETS phylogeny. Three species from Sichuan (clade C) formed a strongly supported clade (bootstrap 100 %, DI 12), as in the ITS trees. However, the other two major regional clades, A and B, were only weakly supported. Clade B, which included species distributed from SW China to the Himalayas, was poorly supported (bootstrap <50 %). Samples of the widely distributed *A. henryi* from China, Sumatra and Taiwan were included in a polytomy with *A. yunnanensis*, *A. lijiangensis* and *A. fulvipes*. Clade A, comprising species ranging from SE China to mainland Japan through Taiwan and the Ryukyu Islands, was weakly supported (bootstrap 60 %, DI 1). Within this clade, the Japanese and Korean endemic taxa *A. cordifolia, A. acerifolia* var. *acerifolia, A. acerifolia* var. *subapoda* and *A. dissecta* comprised a monophyletic group (bootstrap 78 %, DI 3). The four species of rheophytes fell in a monophyletic group with inland taxa. As in the ITS tree, they were polyphyletic in the ETS tree.

The most parsimonious trees based on *ndhF* did not resolve the relationships among *Ainsliaea* species well because the number of potentially parsimony-informative characters was only 3·9 %, the lowest among the three DNA regions. The phylogenetic analysis resulted in 6876 most parsimonious trees of 76 steps, CI 0·78 and RI 0·88. The consensus tree is shown in Fig. 4. In this case, monophyly of the genus *Ainsliaea* was weakly supported (bootstrap 69 %, DI 1), whereas the three clades (A, B and C) found in the ITS and ETS phylogenies formed a polytomy.

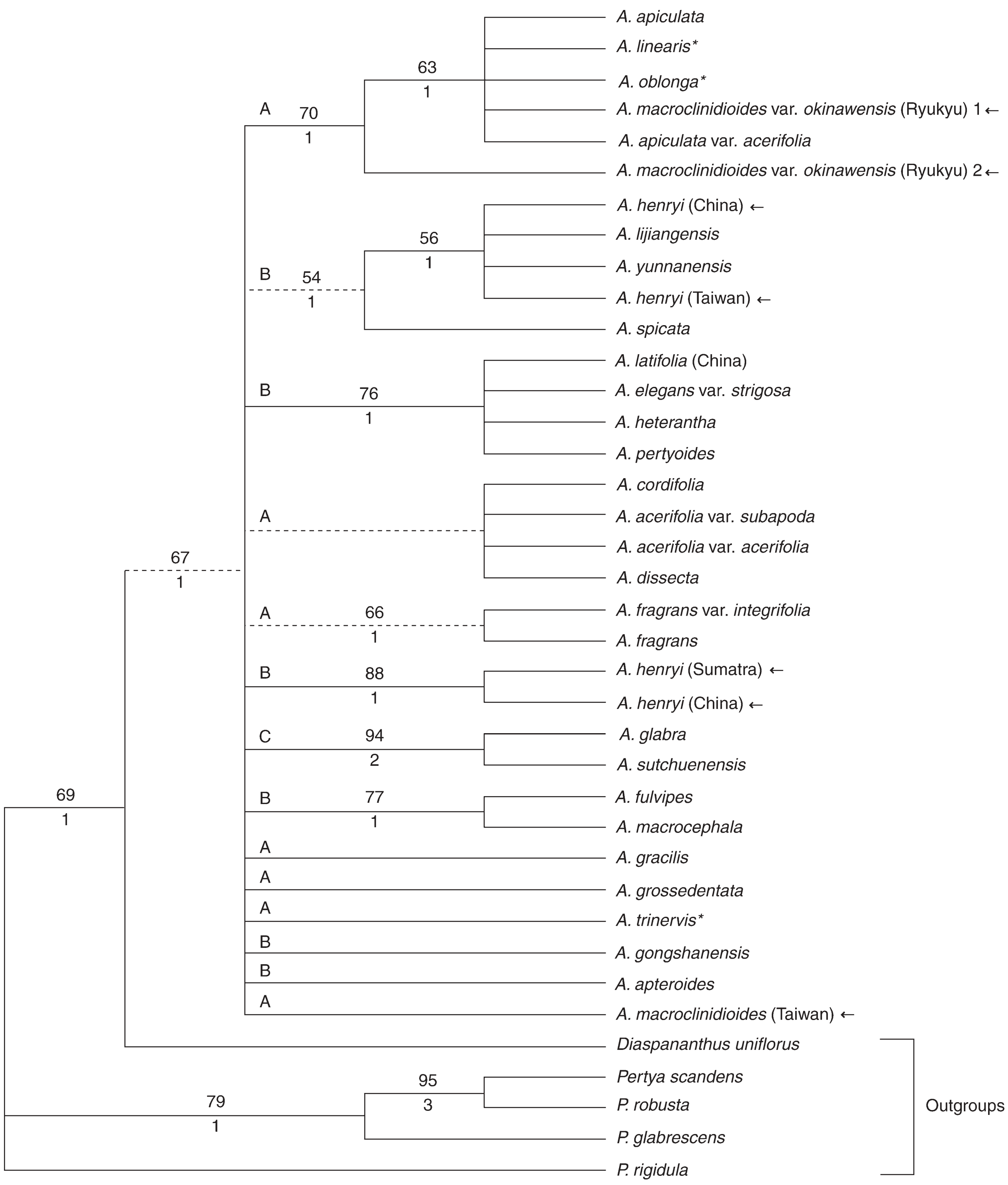

FIG. 4. Strict consensus of the 6876 most parsimonious trees from *ndhF* sequences for 33 taxa and five outgroups. Tree length = 76. Upper-case letters A, B and C represent the three major clades shown in the ITS and ETS trees. The three rheophyte species are indicated by *. The broadly distributed taxa *A. macroclinidioides* var. *okinawensis*, *A. macroclinidioides*, *A. henryi* are represented by arrows.

Although the ILD test identified incongruence between the ITS and ETS ($P < 0{\cdot}001$), between the three regions ($P < 0{\cdot}001$), the combined tree from the three data sets is shown because the topologies of the ITS, ETS and *ndhF* trees did not conflict with respect to the major clades, and the combined data resolved the relationships among the major lineages of *Ainsliaea* and the outgroups (Fig. 5). The combined data set of 2076 bp generated 367 most parsimonious trees of 668 steps, CI 0·58 and RI 0·79. In the strict consensus tree, *Ainsliaea* taxa formed a monophyletic

FIG. 5. One of the 367 most parsimonious trees from the combined ITS, ETS and *ndhF* sequence data for 32 taxa and five outgroups. Tree length = 668. The branch length represents the number of the base substitutions. The numbers above branches indicate the bootstrap support and decay index. The three rheophyte species are indicated by *. The broadly distributed taxa *A. macroclinidioides* var. *okinawensis*, *A. macroclinidioides*, *A. henryi* are represented by arrows. The monophyletic group of taxa from SE China, Taiwan and the Ryukyu Islands, including the three rheophytes in clade A, is indicated by #. Infrageneric classification by Beauverd (1909) is provided alongside each taxon. Abbreviations: Ai, section *Ainsliaea*; Ag, section *Aggregatae*; Fr, section *Frondosae*.

group with moderate support (bootstrap 74 %, DI 4), and *Diaspananthus* was strongly supported as sister (bootstrap 100 %, DI 38). The three major clades of *Ainsliaea* were supported, with bootstrap support and DI, respectively, of 94 % and 5 for clade A, 86 % and 3 for clade B, and 100 % and 28 for clade C.

Relative rate test

There was no significant difference in substitution rates between clades A and B + C for the alignments of 649 ITS or 480 ETS bp. The mean substitution rates between the two lineages were similar (ITS: $K_A = 0{\cdot}0575319$, $K_{B+C} = 0{\cdot}058341$, $\Delta K = 0{\cdot}000814284 \pm 0{\cdot}00673774$, ETS: $K_A = 0{\cdot}115124$, $K_{B+C} = 0{\cdot}107004$, $\Delta K = 0{\cdot}00812007 \pm 0{\cdot}00672482$). Therefore, clade A (SE China to mainland Japan) and clade B + C (SW China and the surrounding areas) do not significantly differ in their mutation rates, and a molecular clock was not rejected.

Estimation of divergence time

The estimated divergence times among the major clades and outgroups were calculated based on the ITS sequence data. The estimated divergence time between *Pertya* and the other clades was 2·16 ± 0·36 MYA, i.e. from the end of Tertiary to the early Pleistocene. As for the three major clades of *Ainsliaea*, because the mean number of base substitutions between them was almost the same (29–34), their molecular divergence time converged at around 1·1 MYA in the early Pleistocene. The monophyletic group ranging from SE China to mainland Japan through the Ryukyu Islands and Taiwan (clade A), which encompassed the four rheophytes (the clade indicated by # in Fig. 2), was estimated to have diverged at 0·31 ± 0·12 MYA in the middle Pleistocene.

DISCUSSION

Utility of different DNA regions for resolving interspecific phylogeny

Some topological conflicts were found between the ITS and ETS trees, whereas *ndhF* provided less information for phylogenetic relationships in *Ainsliaea*. The ITS trees indicated that the supposed outgroup *Diaspananthus uniflorus* was placed within the ingroup; however, *D. uniflorus* was placed as sister to *Ainsliaea* in the ETS and combined phylogenetic trees. Moreover, there were some inconsistencies in interspecific relationships within clades A and B. This incongruence between the ITS and ETS trees indicates that there may have been an uneven rate of concerted evolution (gene conversion) within the *Ainsliaea* lineage, even though these two regions are adjacent in the nrDNA and are usually treated as parts of a single molecule with the same evolutionary history (Baldwin and Markos, 1998; Alvarez and Wendel, 2003). The results of the ILD test also indicated incongruence.

The topological conflict between the ITS and ETS trees may have been caused by introgression, which could be attributed to frequent hybridization among closely related species within the lineage (Okuyama *et al.*, 2005). Watanabe *et al.* (1992) reported hybridization between *Ainsliaea* species in Japan and suggested that genetic assimilation was occurring. The internal structure of clade B was weak and the bootstrap value for each branch was low. Considering the number of species in a fairly limited range in Yunnan Province and the surrounding areas, it is likely that recombination and introgression have occurred in some species during their evolution. Therefore, the effect of introgression among species could lead to the phylogenetic inconsistencies seen here.

Although there were some inconsistencies between the two regions, the ITS and ETS trees are consistent in having three major clades (A, B and C, in Figs 2 and 3). The bootstrap support for these major clades was higher in the ITS tree than in the ETS tree, although the ETS tree had a higher percentage of potentially parsimony-informative sites than the ITS tree. The present results differ from previous studies in which ETS was the more informative region for resolving interspecific phylogenetic relationships (Baldwin and Markos, 1998; Acevedo-Rosas *et al.*, 2004).

In contrast, analysis of *ndhF* resulted in uninformative trees and the three major clades seen in the ITS and ETS trees formed a polytomy. This may have been the result of fewer informative site changes, ascribed to a slow substitution rate in *ndhF*. However, each clade in the *ndhF* tree could be included in one of the major clades (A, B or C) of the ITS and ETS trees, and the major branches of the three data sets were not in conflict with each other. Therefore, the phylogeographical analysis in this study depended on the combined phylogenetic analyses of the three data sets.

Phylogeographical analysis of the Sino-Japanese region

The genus *Ainsliaea* is composed of three major clades, which correspond to species distributions within the Sino-Japanese region; (A) the eastern region from SE China to Japan, through Taiwan and the Ryukyu Islands, (B) the western region covering Yunnan and its surrounding areas, including the Himalayas; and (C) Sichuan (Fig. 1). Although the origin and past distributions of the three groups remain uncertain, it is plausible that *Ainsliaea* taxa evolved allopatrically and that their descendants have been isolated on the eastern (clade A) and western (clades B and C) sides of the Sino-Japanese corridor. The east and west regions are generally thought to possess rich floras with high endemism; SW China (including Sichuan) possesses especially high diversity (Axelrod *et al.*, 1998; Qian, 2004; Xiang *et al.*, 2004), and the region from SE China to Japan through Taiwan and the Ryukyu Islands also encompasses a rich flora (Takhtajan, 1986; Wu and Wu, 1996). Floristic studies (Wu, 1979; Wu and Wu, 1996) suggest that the Sino-Japanese plant region should be divided into two subkingdoms: the Sino-Himalayan Forest subkingdom and the Sino-Japanese Forest subkingdom. The present phylogenetic findings are highly geographically structured and recognize each area as an independent evolutionary unit in the Sino-Japanese region that is consistent with a floristic unit, the eastern and western divisions. Moreover, these phylogeographical units agree with the infraspecific relationships in *Spiraea japonica*, with two clades corresponding to the eastern and western divisions (Zhang *et al.*, 2006).

Origin of the three lineages of *Ainsliaea* was estimated at around 1·1 MYA, or the early Pleistocene. In this era, the uplift of the Himalayas occurred (Committee of Chinese Academy of Sciences for Physical Geography of China, 1984). The present moist monsoon climate of the Sino-Japanese region was formed by uplift of the Himalayan Range as a result of the collision of Eurasia, the Indian subcontinent and the Burma–Malaya Geoblock (Zhang *et al.*, 1984; Sengor and Natal'in, 1996), estimated to have occurred during the late Pliocene (Fort, 1996) or the late Pliocene and Pleistocene (Hsü, 1978). Therefore, the present findings suggest that topogeographical and/or palaeoclimatic barriers sculpted the phylogeographical lineages in the early Pleistocene. The climatic and topographical changes may have led to allopatric speciation in *Ainsliaea* in three geographic areas. Previous studies indicated the presence of varied vegetation, from tropical to frigid forests, and these rich environments would have supplied refugia during Quaternary climatic oscillations, harbouring species diversity and acting as important centres for survival, speciation and evolution (Axelrod *et al.*, 1998; Qian, 2004; Xiang *et al.*, 2004). The three regions occupied by clades A, B and C might represent refugia, and the plants occurring there could have been sources for later speciation and evolution in each area.

On the western side of the Sino-Japanese region, *Ainsliaea* species have diverged in two regions in SW China, i.e. Sichuan (clade C) and the adjacent area represented by Yunnan (clade B). Most of the species included in these two clades are restricted to a fairly narrow area of Sichuan and Yunnan. Several factors could have contributed to allopatric speciation: the presence of effective topographic barriers (e.g. splitting the ranges of ancestral species, preventing gene flow or enhancing the founder effect), strong sexual selection (e.g. leading to divergence in mate recognition systems), ecological specialization (e.g. limiting population distribution and prompting divergence), low dispersal rates (e.g. reducing gene flow among populations) and bottlenecks in population size facilitating genetic peak shifts (Barraclough *et al.*, 1998; Schluter, 2001; Turelli *et al.*, 2001). In SW China, topographic complexity can be ascribed to the uplift of the Himalaya-Tibetan massif from the late Paleocene to the early Pleistocene. The Himalayan movements during the early Pleistocene created a mosaic of plateaux, mountains, basins and gorges in this small area of Yunnan and its surrounding areas (Committee of Chinese Academy of Sciences for Physical Geography of China, 1984). We propose this environmental complexity restricted the movement of *Ainsliaea* species due to the geographic barriers, causing limited gene flow among populations. The fractured populations with restricted gene flow and higher selection pressure led to species differentiation by adaptation to local environments. In fact, species in clade C are from Emei Mountain, which is part of a large mountain chain and is extraordinarily rich in endemics. Considering that the three major clades diverged at around 1·1 MYA and speciated through the Pleistocene, the present results support the idea that complex topography and varied environments, linked with the continual movements of the Himalayas, functioned as major factors leading to species differentiation in this area.

The present results indicate that clade A is geographically restricted and suggest that this area would have been a centre of diversification for the genus *Ainsliaea*. The estimated divergence time between the species of SE China and the Ryukyu Islands (Fig. 5, represented by #) was about 0·31 MYA, i.e. the middle Pleistocene. A similar divergence time, approx. 0·25 MYA, was also estimated for *Cardiandra* (Hydrangeaceae) species with similar geographic distribution (Setoguchi *et al.*, 2006). During this period, the Ryukyu Islands and Taiwan repeatedly formed a land-bridge from SE China to mainland Japan (Kizaki and Oshiro, 1977, 1980; Ujiie, 1990; Kimura, 1996, 2000). The phytogeographical structure of some plant taxa and traces of introgressive hybridization between allopatric species in the Ryukyu Islands have also been reported (e.g. Setoguchi and Watanabe, 2000; Chiang *et al.*, 2001; Hiramatsu *et al.*, 2001). The present distribution of *Ainsliaea* species is likely to have been sculpted by these historical geographic events, and allopatric speciation due to insular isolation might have been promoted in this region.

Among the species on the Ryukyu Islands, including the rheophytes *A. linearis* and *A. oblonga*, little differentiation in the DNA sequences was found (0–2 bp out of 649 bp of ITS), whereas they are morphologically very different (e.g. Watanabe *et al.*, 1992), possibly indicating rapid diversification. Genetic polymorphism was also found within *A. macroclinidioides* var. *okinawensis* located on Okinawa and Ishigaki Islands. The genetic distances among *A. macroclinidioides* var. *okinawensis* populations were larger than those among other species from the Ryukyu Islands. This might be attributable to isolation on islands and subsequent adaptive radiation to their environment following migration over land-bridges in the Quaternary glacial periods.

Zhang *et al.* (2006) pointed out several distinct geographic and population characteristics and a slower divergence rate in SE China (as comparedwith SW China): fewer geographical barriers, weaker divergent selection pressure, higher gene flow and fewer bottleneck events. However, the relative rate tests suggest almost the same divergence rates in the eastern (SE China to Japan) and western (SW China and its surrounding areas) sides of the Sino-Japanese region for *Ainsliaea*. Moreover, it was found that topographical events including repeated transgression and regression significantly accelerated species differentiation through immigration and isolation in this region of numerous continental islands. Therefore, it is conceivable that the region from SE China to mainland Japan through Taiwan and the Ryukyu Islands had functioned as a floristic unit maintaining original lineages during the topographical and climatic changes throughout the Quaternary.

Circumscription and taxonomy of Ainsliaea

The monophyly of the genus *Ainsliaea* was supported based on analyses of ETS, *ndhF* and the combined data (Figs 3, 4 and 5, respectively), whereas *Diaspananthus uniflorus* was embedded in *Ainsliae*a in the ITS analysis (Fig. 2). The classification of the two genera is solely based on floral characters: the genus *Ainsliaea* has three florets (occasionally one or five), whereas *Diaspananthus* has one floret and *Diaspananthus* is sometimes included in *Ainsliaea* (Mabberley, 1998). The phylogenetic relationships between *Ainsliaea* and *Diaspananthus* should be further examined using larger DNA sequence data sets.

The three sections proposed by Beauverd (1909) were not supported by the present molecular analyses. The present results indicate that the genus consists of three monophyletic groups, corresponding to geographical distributions of species, not vegetative morphological characteristics. These three groups should be recognized as infrageneric taxonomic units, but no particular morphological characteristics have been identified for each clade. The chromosome number is still uncertain in many species, and microstructural characters such as pollen features have not been observed. Based on the three lineages found in this study, further analyses of comparative morphology are needed to suggest an infrageneric system for the genus.

The present study also suggests species boundaries with wide distributions. *Ainsliaea henryi* (samples from China, Sumatra and Taiwan) was not monophyletic in clade B. For *A. macroclinidioides* and its relatives in clade A, disagreement over the traditional classification and genetic structure was again observed and further analysis is necessary to reconsider their classification. In contrast, *A. fragrans* and *A. fragrans* var. *integrifolia*, disjuncts in SE China and mainland Japan, were monophyletic with little sequence differentiation. These two taxa are distinguished by differences in leaf pubescence, but this characteristic also varies among habitats even within the same population (personal observations in China and Japan). Therefore, the present molecular phylogenetic analysis supports the monophyly of *A. fragrans* among allopatric localities in eastern Asia, whereas *A. henryi* and *A. macroclinioides* are not monophyletic, and further taxonomic studies are needed to clarify their circumscription.

Diversification of rheophyte species

The phylogenetic relationships of the four rheophyte species were inferred. These grow along river banks that are occasionally flooded by violent river flow. *Ainsliaea linearis* and *A. oblonga* are distributed on Yakushima and Okinawa Islands of Japan, respectively, and *A. trinervis* and *A. walkeri* are found in SE China. These four species are included in a clade with ten OTUs of inland taxa. The Japanese *A. linearis* and *A. oblonga* are monophyletic, whereas the Chinese *A. trinervis* and *A. walkeri* are polyphyletic. Almost no base substitutions were seen between *A. linearis* and *A. oblonga*, whereas there were ten substitutions in ITS and ETS between Japanese and Chinese species, and five substitutions were found between the two Chinese species. Kitamura (1981) speculated that the Japanese *A. linearis* and the Chinese *A. walkeri*, which are morphologically similar, might be the same species. We agree with the existing classification recognizing *A. walkeri* and *A. linearis* as independent species, because the present analysis suggests that there is no correlation between morphological similarity and genetic relatedness. Differentiation of ITS sequences, with 15 substitutions, indicates that they have experienced different evolutionary processes. The morphological similarity was not caused by genealogy but represents convergent evolution for the peculiar rheophytic environment.

The two rheophytes in SE China may have evolved independently, whereas it appears that the Japanese *A. linearis* and *A. oblonga* are derived from a single ancestor. These findings suggest that allopatric evolution of these rheophyte species, both in mainland China and the Ryukyu Islands, was accomplished by adapting to the rheophytic environment (river banks subject to periodic flooding). The estimated divergence time of approx. 0·31 MYA in the mid-Pleistocene suggests that river-bank adaptations may have occurred during the drastic climatic oscillations and land-bridge formation. van Steenis (1981, 1987) suggested that most rheophytic plants are endemics confined to particular areas. Moreover, migration (gene flow) between river systems is thought to be fairly limited (Liao and Hsiao, 1998). Therefore, the independent evolution of rheophytic *Ainsliaea* species is due to adaptation to the environment on the eastern edge of the Sino-Japanese region, where annual rainfall is high (e.g. 3000–7000 mm on Yakushima Island; Takahara and Matsumoto, 2002).

Conservation aspects

SE China and the Ryukyu Islands are characterized by high annual rainfall and a large number of mountain streams. Rheophytic *Ainsliaea* species have adapted to such mountain stream environments over a relatively short time period. However, these environments have rapidly declined due to the construction of dams and electricity-generating stations in these areas. Rheophytic plants cannot breed away from their riparian (inland) habitats, because heavy water flow is necessary for excluding competitors. Once a dam is constructed and river flow is controlled, seasonal flooding does not occur, leading to a loss of natural populations and genetic diversity. The region from SE China to the Ryukyu Islands is a centre of species differentiation in the Sino-Japanese region. The mountain stream environment contributes to the biodiversity of this region, and conservation of the mountain stream environment is needed to sustain the unique plants of this region.

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