Biochemical Systematics and Ecology 38 (2010) 911–916

Contents lists available at ScienceDirect

Biochemical Systematics and Ecology

journal homepage: www.elsevier.com/locate/biochemsyseco

Genetic diversity in endangered *Notopterygium forbesii* Boissieu based on intraspecies sequence variation of chloroplast DNA and implications for conservation

Guoying Zhou [a,b,*,1], Lucun Yang [a,b,1], Chunli Li [a,b], Wenhua Xu [a], Guichen Chen [a]

[a] Northwest Institute of Plateau Biology, the Chinese Academy of Sciences, Xining 810008, China
[b] Graduate School of the Chinese Academy of Sciences, Beijing 100039, China

ARTICLE INFO

Article history:
Received 1 March 2010
Accepted 24 September 2010
Available online 3 November 2010

Keywords:
Genetic diversity
cpDNA
*trn*H-*psb*A
Notopterygium forbesii

ABSTRACT

A thorough understanding of the levels and partitioning of genetic variation across populations and geographical regions of endangered species is a prerequisite to ensure effective conservation and/or restoration activities. Here, we examined chloroplast DNA (cpDNA) *trn*H-*psb*A intergenic spacer sequences variation within *Notopterygium forbesii*, an endangered and endemic perennial herb in China. Sequence data obtained from 141 individuals in 14 populations revealed twenty-two haplotypes. A high level of haplotype diversity ($Hd = 0.81$) and low level of nucleotide diversity ($Pi = 0.0047$) were detected. Low genetic differentiation among populations and also among regions was consistently indicated by both hierarchical analyses of molecular variance (AMOVA) and the structure of a neighbor-joining tree. Low level of population differentiation between populations or between regions in cpDNA sequences may be due to effects of the abundance of ancestral haplotype sharing and the high number of private haplotypes fixed for each population. Based on our results, we proposed some conservation strategies.

1. Introduction

Notopterygium forbesii Boissieu, belonging to the *Notopterygium*, Umbelliferae, is an endangered perennial herb endemic to China. According to the historical record, it had a relatively wide distribution throughout alpine forest and sub-alpine shrubs between 1700 m and 4500 m in China (Zhou et al., 2003). As a traditional Tibetian medicine, “Zhuma” was used by local people in small quantities. However, in recent decades, wild populations of *N. forbesii* in China have rapidly decreased due to habit fragmentation, excessive exploitation, and a low rate of natural regeneration. At present, wild populations of *N. forbesii* can only be found above 4000 m. Consequently, the species is regarded as endangered in the Chinese Plant Red Book (Wang and Xie, 2004).

The long-term survival and evolution of species depends on the maintenance of sufficient genetic variability within and among populations to accommodate new selection pressures brought about by environmental changes (Barrett and Kohn, 1991). Therefore, knowledge about the genetic diversity and variation within and between populations in rare and endangered plants not only enhances our understanding of population dynamics, adaptation and evolution but also provides information useful for developing conservation strategies (Schaal et al., 1991).

* Corresponding author. Northwest Institute of Plateau Biology, the Chinese Academy of Sciences, Xining 810008, China. Tel.: +86 971 615 9630; fax: +86 971 614 3282.
E-mail address: zhougy@nwipb.cas.cn (G. Zhou).
[1] These authors contributed equally to this work.

0305-1978/$ – see front matter
doi:10.1016/j.bse.2010.09.012

To date, previous studies of *N. forbesii* have mainly focused on its morphology, anatomy (Wang et al., 1996; She and Pu, 1997), systematics (Pu et al., 2000), ecology (Jiang et al., 2005) and pharmacognosy (Yang et al., 2006). The genetic diversity of different populations of *N. forbesii* have not yet been reported. In the present study, we used chloroplast DNA (cpDNA) trnH-psbA to examine the genetic diversity of *N. forbesii*. Our aims were to reveal the genetic diversity and the partitioning of genetic diversity within and among populations and to provide baseline genetic information pertinent to the conservation and restoration of this endangered species.

2. Material and methods

2.1. Plant materials

A total of 140 individuals, which corresponded to 14 populations of *N. forbesii*, were sampled across 3 provinces in China; Qinghai, Gansu, and Sichuan Provinces (Table 1; Fig. 1). Due to the limited availability of individuals in wild populations, samples sizes of populations in this study were relatively small, from 8–12 individuals. Neighboring sampled individuals were at least 10 m apart to avoid resampling from the same individual. Leaf material was dried in silica gel and stored at room temperature. Parameters such as longitude, latitude and altitude were recorded for each population (Table 1).

2.2. DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted with the CTAB method (Doyle, 199l). DNA quality was checked on a 1% agarose gel. The intergenic spacer between *trn*H and *psb*A genes of the cpDNA was amplified with a pair of universal primers (5′-ACTGCCTTGATCCACTTGGC-3′; 5′-CGAAGCTCCATCTACAAATGG-3′) (Hamilton, 1999). Polymerase chain reaction (PCR) was performed in a 25 μL volume, which contained 1 μL (10–40 ng) plant DNA, 2.5 μL 10×reaction buffer, 1.5 μL $MgCl_2$ (25 mM), 0.5 μL dNTP mix (10 mM), 1 μL of each primer (10 pmol), and 1 U of *Taq* polymerase (Promega, MBI, USA). All reactions were performed with the following program: 5 min at 95 °C, 35 cycles of 1 min at 94 °C, annealing for 1 min at 52 °C, 1.5 min at 72 °C, a final extension for 7 min at 72 °C, and then the reactions were held at 4 °C until further steps. PCR products were resolved electrophoretically on 1.5% agarose gels run at 200 V in 1×TAE, visualized by staining with ethidium bromide, and photographed under ultraviolet light.

All successfully amplified DNA fragments were purified using a TIANquick Midi Purification Kit according to the recommended protocol (TIANGEN), prior to sequencing. Then, PCR products were sequenced directly using an ABI3730XL automated DNA sequencer, applying the PCR-primers as sequencing primers. Sequence electropherograms were edited using Chromas version 2.33 (http://www.technelysium.com.au/chromas.html).

2.3. Data analysis

DNA sequences were aligned with the CLUSTAL X program (Thompson et al., 1997) and then manually refined. Arlequin, version 3.01 (Excoffier et al., 2006), DnaSP, version 4.0 (Rozas et al., 2003), and MEGA, version 4.0 (Tamura et al., 2007), were used to calculate statistical values, such as the nucleotide composition, number of polymorphic sites (S), haplotype diversity (*Hd*), nucleotide diversity (*Pi*), and average number of pairwise nucleotide differences (*k*). These values were calculated for each geographic population and geographic region. The phylogenetic trees were constructed by the neighbor-joining (NJ)

Table 1
Location data, number of individuals analyzed for *trn*H-*psb*A (n) and haplotype diversity (*Hd*), nucleotide diversity (*Pi*) of the investigated populations of *Notopterygium forbesii*.

Code	Population	Voucher	Location	Altitudem (m)	Sample number	Hd	Pi
HY	Huangyuan, QH[a]	G.Y. Zhou001	101.36°E36.73°N	3032	9	0.69 ± 0.147	0.0070 ± 0.00243
HZH	Huangzhong, QH[a]	G.Y. Zhou002	101.69°E36.30°N	2912	11	0.60 ± 0.154	0.0023 ± 0.00020
PA	Pingan, QH[a]	G.Y. Zhou003	101.90°E36.31°N	2945	12	0.53 ± 0.136	0.0036 ± 0.00274
LD	Ledu, QH[a]	G.Y. Zhou004	102.40°E36.69°N	2704	9	0.75 ± 0.079	0.0029 ± 0.00410
MH	Minhe, QH[a]	G.Y. Zhou005	102.64°E36.17°N	2405	8	0.46 ± 0.200	0.0020 ± 0.00189
HZ	Huzhu, QH[a]	G.Y. Zhou006	102.43°E36.87°N	2800	8	0.46 ± 0.200	0.0014 ± 0.00154
MY1	Mengyuan1, QH[a]	G.Y. Zhou009	101.40°E37.45°N	2945	12	0.86 ± 0.079	0.0064 ± 0.00429
MY2	Mengyuan2, QH[a]	G.Y. Zhou010	102.00°E37.28°N	2770	10	0.53 ± 0.180	0.0022 ± 0.00196
ZK	Zeku, QH[a]	G.Y. Zhou014	101.94°E35.31°N	2907	11	0.69 ± 0.086	0.0024 ± 0.00208
TZ1	Tianzhu1, GS[b]	G.Y. Zhou007	102.66°E36.97°N	2923	10	0.51 ± 0.164	0.0031 ± 0.00249
TZ2	Tianzhu2, GS[b]	G.Y. Zhou008	102.61°E36.93°N	2693	9	0.83 ± 0.098	0.0050 ± 0.00360
XH	Xiahe, GS[b]	G.Y. Zhou011	102.90°E35.37°N	2234	10	0.36 ± 0.159	0.0010 ± 0.00122
HZU	Hezuo, GS[b]	G.Y. Zhou012	102.91°E35.06°N	2919	10	0.00 ± 0.000	0.0000 ± 0.00000
REG	Reergai, SC[c]	G.Y. Zhou013	103.18°E33.60°N	3254	11	0.60 ± 0.154	0.0030 ± 0.00244
Total					140	0.81 ± 0.021	0.0047 ± 0.00308

[a] QH: Qinghai Province China.
[b] GS: Gansu Province, China.
[c] SC: Sichuan Province, China.

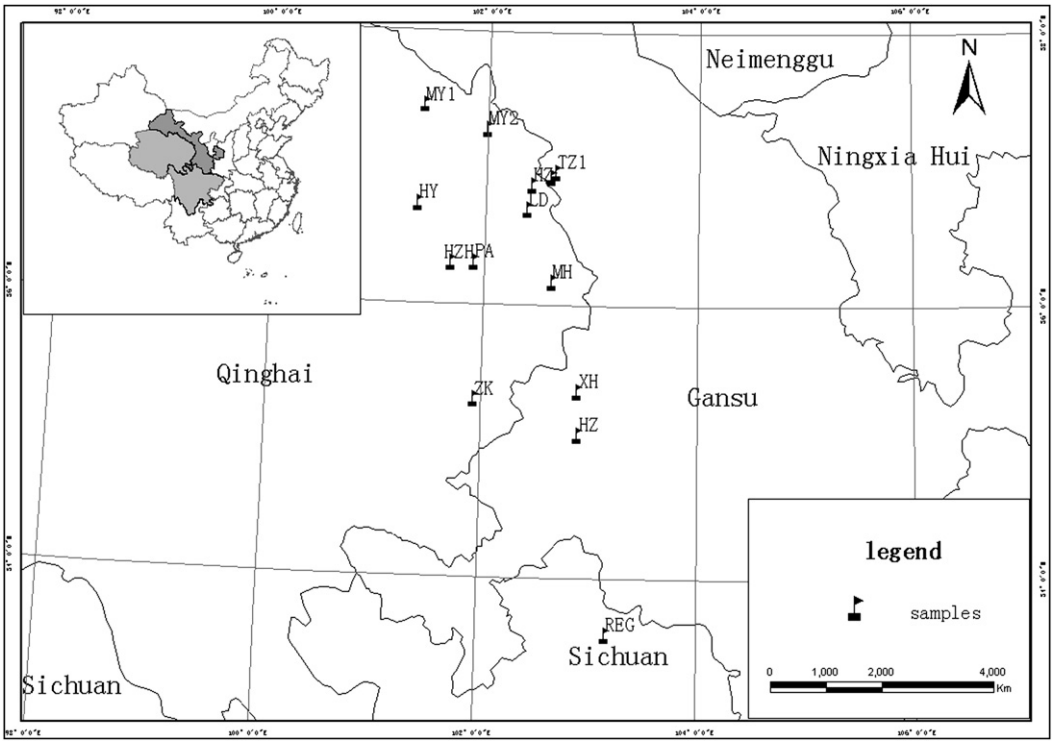

Fig. 1. Geographic locations of sampling sites for plants in the present study.

method using the Kimura-two-parameter (K2P) model in MEGA, version 4 (Tamura et al., 2007). The statistical robustness in the nodes of the resulting tree was determined by 1000 bootstrap replicates.

A hierarchical analysis of molecular variance (AMOVA) was performed with ARLEQUIN (version 3.01, Excoffier et al., 2006), with significance estimated based on 1000 permutations. Variance was apportioned to the following components: among individuals within population, among populations within region, and among regions. Patterns of geographical subdivision and gene flow (*Nm*) were also estimated hierarchically with the DnaSP 4.0 program. Gene flow (*Nm*) within and among populations was estimated using the expression $F_{ST} = 1/(1 + 2Nm)$ where N is the female effective population size and m is the female migration rate.

3. Results

3.1. Haplotype distribution and genetic diversity

Sequences of the cpDNA *trn*H-*psb*A intergenic spacer varied from 346 bp to 348 bp in length. The aligned sequences were 351 bp long, with 16 nucleotide substitutions and five indels. In total, 22 haplotypes were identified among the 140 individuals (accession numbers HQ269393–HQ269414), with a haplotype diversity (*Hd* ± SD) of 0.81 ± 0.021. Haplotype H6 was the most abundant haplotype, occurring in 47 samples, followed by haplotypes H11 and H2, which occurred in 29 and 26 samples, respectively. H1, H10 and H4 were found in eight, eight and three samples, respectively. Three haplotypes occurred in each of two samples, and the remaining haplotypes were found in only a single sample (Table 2).

Overall haplotype diversity (*Hd*) and nucleotide sequence (*Pi*) diversity for *N. forbesii* were 0.81 ± 0.021 and 0.0047 ± 0.00308 respectively. At the region level, haplotype diversity and nucleotide diversity among three regions varied between 0.47 (Gansu) and 0.80 (Qinghai), and between 0.0024 (Gansu) and 0.0046 (Qinghai), respectively (Table 3). Qinghai had the highest haplotype diversity and nucleotide diversity among the three regions. At the population level, the highest level of haplotype diversity occurred in population MY1, while the highest level of nucleotide diversity appeared in population HY.

3.2. Phylogeny and patterns of population structure

The phylogenetic analyses conducted using the NJ method revealed two strongly supported groups (a and b) (Fig. 2). However, it revealed that two well-defined clusters were not consistent with the two main geographical regions (Qinghai and

Table 2
Geographical distribution of haplotypes in populations of *N. forbesii*.

Haplotype	Population														Total number of individuals
	HY	HZH	PA	LD	MH	HZ	MY1	MY2	ZK	TZ1	TZ2	XH	HZU	REG	
H1						1				2	1	2		2	8
H2	1	2	8	3	6			1	5						26
H3														1	1
H4				3											3
H5			1												1
H6		1				6	3		2	7	3	8	10	7	47
H7							1							1	2
H8					1										1
H9					1										1
H10						1	4				3				8
H11	5	7	3	3				7	4						29
H12								1							1
H13											1				1
H14		1													1
H15							1	1							2
H16							1								1
H17	2														2
H18										1					1
H19	1														1
H20							1								1
H21											1				1
H22							1								1
Total number of individuals	9	11	12	9	8	8	12	10	11	10	9	10	10	11	140

Gansu). Moreover, haplotypes from different geographic regions (Qinghai, Gansu and Sichuan) were clustered into the same clade, whereas haplotypes from the same population are not grouped within the same clade (e.g., H10, H15, H16, H20, H22). That is, haplotypes lack a distinct geographic distribution structure and disperse into different clades.

The analysis of molecular variance (AMOVA) showed that the proportion of genetic variation attributable to differences among populations was low but significant (36.8%), with most of the total genetic variation residing within populations (Table 4). When the populations were divided into three groups (Qinghai, Gansu and Sichuan), the hierarchical AMOVA showed that genetic variation among groups and among populations within regions are small (25.2% and 18.9% of the total, respectively), a larger significant amount (55.9% of the total) is due to differences within populations. The overall level of inferred gene flow (*Nm*) was estimated as 0.42 individuals per generation among populations, indicating limited gene flow between the populations of this species.

4. Discussion

Many studies have demonstrated that endangered and endemic species tend to possess low levels of genetic diversity (e.g., Xiao et al., 2004), whereas some others have shown opposite findings (e.g., Luan et al., 2006). Compared to the values of haplotype diversity previously found in endangered plants (Su et al., 2004, 2005; Huang et al., 2001, 2005), the value of haplotype diversity in this study could be categorized as high. However, the level of nucleotide diversity within the whole species ($Pi = 0.0047$) of *N. forbesii* was lower than that of the endangered plants mentioned above. Such a high *Hd* value and low *Pi* value indicated that this species experienced a fast expansion from a small effective population size. In this case, most polymorphic sites could have emerged during a demographic expansion and haplotype polymorphism has been accumulated

Table 3
Descriptive statistics for the studied *N. forbesii* samples.

Sampling region	n[a]	H[b]	S[c]	*Hd*[d]	K[e]	*Pi*[f]
Qinghai	90	18	15	0.80 ± 0.027	1.61 ± 0.964	0.0046 ± 0.00305
Gansu	39	8	6	0.47 ± 0.092	0.83 ± 0.604	0.0024 ± 0.00193
Sichuan	11	4	3	0.60 ± 0.154	1.06 ± 0.756	0.0030 ± 0.00244

[a] n, sample size.
[b] H, number of haplotypes.
[c] S, number of polymorphic sites.
[d] *Hd*, haplotype diversity.
[e] k, mean pairwise nucleotide differences.
[f] *Pi*, nucleotide diversity.

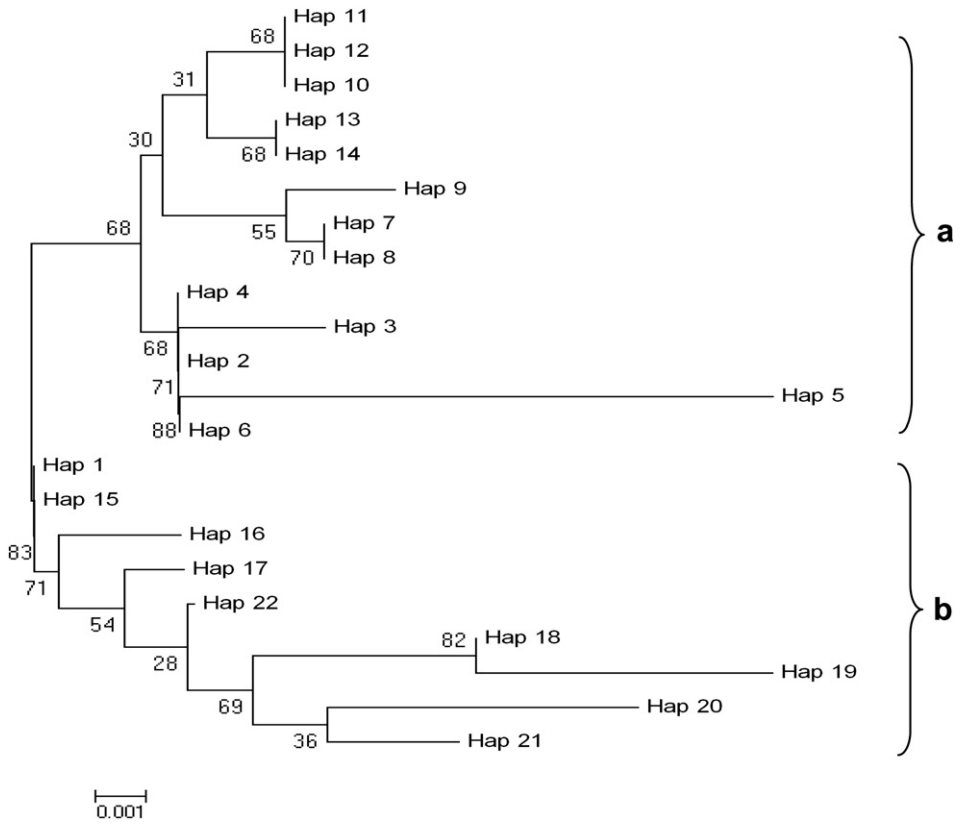

Fig. 2. Neighbor-joining tree of *Notopterygium forbesii* based on sequences of haplotypes of the trnH-psbA noncoding spacers of cpDNA. Numbers above branches indicate the bootstrap values of 1000 replicates.

by mutations, but it failed to accumulate nucleotide sequence diversity (Avise, 2000). In addition, the low nucleotide diversity of the species also may be due to habitat fragmentation.

Essentially low population differentiation was found by the AMOVA, which is concordant with the result of the NJ tree, in which the distribution of haplotypes mixed and did not cluster according to populations or geographic regions. The low level of population differentiation may have resulted from an abundance of ancestral haplotype sharing. Nine out of 14 populations of *N. forbesii* shared one haplotype, H6. According to coalescence theory (Posada and Crandall, 2001), the common haplotype most likely represents the oldest lineage. The basal position of H6 (in clade a) in the NJ tree (Fig. 2) supports this view. Thus, under conditions of co-ancestry due to recent common ancestry, the newly established populations would be less differentiated from the ancestral populations even though restricted gene flow ($Nm = 0.42$) existed after isolation (Huang et al., 2005). Meanwhile, due to insufficient evolutionary time for coalescence, the allele composition within the offspring populations would have a low probability of attaining homogeneity (Futuyma, 1998). During the lineage sorting stages, with ancestral polymorphisms maintained within populations, the cpDNA genealogy is less likely to reflect population structure and ongoing gene flow (Whitlock and MaCauley, 1999). Furthermore, the low level of genetic variation among populations may be caused by the high number of unique haplotypes fixed in each population (Table 2).

Information about the spatial organization of genetic variability is essential for the conservation of genetic resources (Bawa, 1993). Our study gives insight into genetic variation at the intra- and interpopulation levels of *N. forbesii*. Hamrick (1993) suggested that as >80.0% of the total genetic diversity resides within populations, five strategically placed

Table 4
Analysis of molecular variance (AMOVA) for populations of *N. forbesii* based on sequences of cpDNA trnH-psbA regions.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation (%)	F	*p* value
Among groups	2	21.730	0.240	25.2	$F_{SC} = 0.252$	<0.001
Among populations within groups	11	25.485	0.180	18.9	$F_{ST} = 0.441$	<0.001
Within populations	126	67.213	0.533	55.9	$F_{CT} = 0.252$	<0.01
Among populations	13	47.216	0.310	36.8		
Within populations	126	67.213	0.533	63.2	$F_{ST} = 0.368$	<0.01

d.f., degrees of freedom.

populations should maintain 99.0% of their total genetic diversity. However, in our study, genetic diversity of 63.2% was observed within populations of *N. forbesii*, which implies a need to conserve more populations to maintain genetic diversity within this species. Furthermore, habitat protection is recommended because its suitable habitats have been seriously destroyed and fragmented due to human overexploitation and denudation. At the same time, some policies must be promulgated to ban overexploitation and denudation. Additionally, as a traditional medicinal herb, *N. forbesii* has long been subject to excessive collection, which partly contributes to the declining of effective population sizes. To meet the commercial demand for this herb, cultivation facilities can be established as an alternative source of raw materials.

This information about genetic structure has important implications for creating conservation strategies for *N. forbesii*. However, sound and effective conservation and management strategies must be founded on well-rounded genetic information, so it is particularly important to understand the genetic structure of extant populations over the entire range of the species' distribution. Therefore, additional population samples of *N. forbesii* should be tested to clarify the genetic structure of *N. forbesii* from the entire range of the species' distribution in future work.

Acknowledgments

We thank Dr. Gonghua Lin for his help. This study was supported by the West Light Foundation of the Chinese Academy of Sciences (2007).

References

Avise, J.C., 2000. Phylogeography: The History and Formation of Species. Harvard University Press, Cambridge, MA.
Barrett, S.C.H., Kohn, J.R., 1991. Genetic and evolutionary consequences of small population size in plants: implications for conservation. In: Falk, D.A., Holsinger, K.E. (Eds.), Genetics and Conservation of Rare Plants. Oxford University Press, New York, pp. 3–30.
Bawa, K.S., 1993. Effects of deforestation and forest fragmentation on genetic diversity in tropical tree populations. In: Drysdale, R.M., John, S.E.T., Yapa, A.C. (Eds.), Genetic Conservation and Production of Tropical Forest Seed. ASEAN−Canada Forest Tree Seed Centre, Chiang Mai, Thailand, pp. 10–16.
Doyle, J., 1991. DNA protocols for plants–CTAB total DNA isolation. In: Hewitt, G.M., Johnston, A. (Eds.), Molecular Techniques in Taxonomy. Springer, Berlin, pp. 283–293.
Excoffier, L., Laval, G., Schneider, S., 2006. Arlequin 3.01: An Integrated Software Package for Population Genetics Data Analysis. Institute of Zoology, University of Berne, Berne.
Futuyma, D.J., 1998. Evolutionary Biology, third ed. Sinauer, Sunderland.
Hamilton, M.B., 1999. Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. Mol. Ecol. 8, 521–523.
Hamrick, J.L., 1993. Genetic diversity and conservation in tropical forest. In: Drysdale, R.M., John, S.E.T., Yapa, A.C., Yapa, A.C. (Eds.), Proceeding of the ASEAN–Canada Symposium on Genetic Conservation and Production of Tropical Tree Seed. ASEAN–Canada Forest Tree Center, Saraburi, Thailand, pp. 1–9.
Huang, S., Chiang, Y.C., Schaal, B.A., Chou, C.H., Chiang, T.Y., 2001. Organelle DNA phylogeography of *Cycas taitungensis*, a relict species in Taiwan. Mol. Ecol. 10, 2669–2681.
Huang, J.C., Wang, W.K., Peng, C.I., Chiang, T.Y., 2005. Phylogeography and conservation genetics of *Hygrophila pogonocalyx* (Acanthaceae) based on atpB–rbcL noncoding spacer cpDNA. J. Plant Res. 118, 1–11.
Jiang, S.Y., Sun, H., Huang, X.J., Zhou, Y., Ma, X.J., Yang, Z.R., 2005. Environmental pedology of Notopterygium incisum and N.forbesii. Chin. Tradit. Herb. Drugs 36, 918–921.
Luan, S., Chiang, T.Y., Gong, X., 2006. High genetic diversity vs. low genetic differentiation in *Nouelia insignis* (Asteraceae), a narrowly distributed and endemic species in China, revealed by ISSR fingerprinting. Ann. Bot(Lond) 98, 583–589.
Posada, D., Crandall, K.A., 2001. Intraspecific gene genealogies: trees grafting into networks. Trends Ecol. Evol. 16, 37–45.
Pu, F.T., Wang, P.L., Zheng, Z.H., Wang, Y.P., 2000. A reclassification of Notopterygium Boissieu (Umbelliferae). Acta. Phytotaxon. Sin 38, 430–436.
Rozas, J., Sanchez-DelBarrio, J.C., Messeguer, X., Rozas, R., 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19, 2496–2497.
Schaal, B.A., Leverich, W.J., Rogstad, S.H., 1991. A comparison of methods for assessing genetic variation in plant conservation biology. In: Falk, D.A., Holsinger, K.E. (Eds.), Genetics and Conservation of Rare Plants. Oxford University Press, New York, pp. 123–134.
She, M.L., Pu, F.D., 1997. A new species of Notopterygium de Boiss. from China. J. Plant.Resour.Environ 6, 41–42.
Su, Y.J., Wang, T., Zheng, B., Jiang, Y., Chen, G.P., Gu, H.Y., 2004. Population genetic structure and phylogeographical pattern of a relict tree fern, *Alsophila spinulosa* (Cyatheaceae), inferred from cpDNA atpB–rbcL intergenic spacers. Theor. Appl. Genet. 109, 1459–1467.
Su, Y.J., Wang, T., Zheng, B., Jiang, Y., Yang, P.Y., Chen, G.P., 2005. Genetic variation and phylogeographical patterns in *Alsophila podophylla* from Southern China based on cpDNA atpB–rbcL sequence data. Am. Fern J. 95, 68–79.
Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. Mega 4: molecular evolutionary genetics analysis (Mega) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.
Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acid. R. 25, 4876–4882.
Wang, Y.P., Pu, F.D., Wang, P.L., He, X.J., 1996. Studies on the systematics of the Chinese endemic genus Notopterygium. Acta Bot. Yunnan 18, 423–430.
Wang, S., Xie, Y., 2004. China Species Red List [M], vol. 1. Higher Education Press, Beijing.
Whitlock, M.C., MaCauley, D., 1999. Indirect measures of gene flow and migration, Fst $\neq$ 1/(4Nm + 1. Heredity 82, 117–125.
Xiao, L.Q., Ge, X.J., Gong, X., Hao, G., Zheng, S.X., 2004. ISSR variation in the endemic and endangered plant *Cycas guizhouensis* (Cycadaceae). Ann. Bot (Lond) 94, 133–138.
Yang, X.W., Zhang, P., Tao, H.Y., Jiang, S.Y., Zhou, Y., 2006. GC-MS analysis of essential Oil Constituents from Rhizome and Root of *Notopterygium forbesii*. J.Chin. Pharmaceu.Sci. 15, 200–205.
Zhou, Y., Jiang, S.Y., Ma, X.J., Sun, H., Pu, F.D., Wu, R., 2003. Resource crisis and protective measures on Rhizoma et Radix Notopterygii. Chin. Tradit. Herb. Drugs 34, 12–14.