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REGULAR PAPER

Population structure of *Nouelia insignis* (Asteraceae), an endangered species in southwestern China, based on chloroplast DNA sequences: recent demographic shrinking

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Abstract *Nouelia insignis*, an endangered species, is distributed in the Jinsha and Nanpan drainage areas in southwestern China. In this study, we examined the genetic diversity and population structure based on the sequences of the cpDNA *rp*L 16 intron. Low levels of genetic variation were detected within all populations of the endemic species. A gene genealogy of 11 haplotypes recovered two major lineages I and II, with haplotypes H1 and H6 nested as interior nodes, respectively. Haplotype H1 was widespread in all populations, while haplotype H6 was restricted to populations southern of the Jinsha River. Low levels of genetic differentiation were detected, as most F_{st} values between populations were zero. This result, however, contradicts previous studies based on allozymes and fingerprinting. Genetic analyses suggested that coancestry due to low evolutionary rates resulted in the lack of geographical subdivision. Molecular dating estimated that the two lineages split about 3.224 MYA (95% CI 1.070–6.089 MYA). Maintenance of ancestral polymorphisms was possibly attributable to a long-standing large effective population size until recently. Postglacial demographic expansion was supported by a unimodal mismatch distribution and star-like phylogenies.

Keywords *Nouelia insignis* · Endangered species · Ancestral polymorphisms · Effective population size · Demographic expansion · Star-like phylogenies

Introduction

One of the focuses of population genetics is the study of the level of genetic variation and spatial apportionment of the genetic polymorphisms within and among populations. A particular population genetic structure and the levels of genetic diversity within populations/species are usually results of various evolutionary forces acting in concert through time and space, and may therefore reflect effects of both historical and contemporary evolutionary events (Wu et al. 2003). To distinguish the effects of these events, an endeavor has been accomplished by the use of gene genealogies upon which geographical information is incorporated in the search of association between genetic variation and geographical distribution (Avise 1998).

Shaped by random genetic drift, a rare species tends to possess low levels of genetic variation, and display evident geographical structuring due to the stochastic loss of genetic polymorphisms from populations. Recovering a demographic history of a species experiencing bottlenecks has recently become academically interesting, especially

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for studies of speciation, domestication, and conservation (e.g., Zhu et al. 2007). The amount of neutral genetic variation in a species is largely shaped by two primary counteracting factors, i.e. random genetic drift and mutation, which decreases and increases variability, respectively. A species that experiences a dramatic reduction in size will lose genetic variation as a function of its population size, growth rate, and the duration of the population contraction.

Nouelia insignis Franch, a monotypic genus of the Mutisieae (Asteraceae), is characterized by an unusual, woody growth form. *N. insignis* grows in dry valleys at 1,000 to 2,800 m alt. in the Jinsha and Nanpan drainage areas in southwestern China (Wang 1989). The diploid species is a shrub with abundant branches and a height of 3–5 m (Peng et al. 2003). Morphological characteristics of numerous, solitary, terminal, and radiate capitula, and fertile florets with marginal uniseriate, bilabiate florets, and central tubular florets distinguish this genus from its sister Leucomeris that possesses capitula in a dense terminal cyme, and tubular florets (Kubitzki 2007). This species used to be common across the distributional range; only until the last half century, a period fewer than 10 generations, *N. insignis* has become endangered due to rapid habitat destruction and fragmentation as well as its usage as firewood (Luan et al. 2006). Our recent observations found that most of the extant populations consist of fewer than 80–100 individuals. *N. insignis* is primarily pollinated by a small bee-fly, which largely promotes outcrossing (Luan et al. 2006). In addition, calyxes of this species develop specialized pappi, a structure facilitating fruit dispersal via wind. Both mechanisms help to maintain genetic polymorphisms across populations, even in this rare species (Luan et al. 2006). Nevertheless, in addition to human disturbances, the species suffers from reproductive failure because of low seed productivity and seed germination rates due to serious drought, especially along the Jinsha River drainage (Peng et al. 2003). Therefore, very few seedlings could be located in the natural habitats.

In the field, all Nouelia populations are fragmented and patchy and distributed mostly at steep-slope habitats, with poorly developed soil, along the Jinsha and Nanpan drainage areas, where biodiversity hotspots for many endemic plants, nevertheless, have been identified (Luan et al. 2006). Jinsha River, the upper stream of the Yangtzejiang River, runs eastward; while Nanpan Rives runs southward (Fig. 1). The two rivers are separated by Mt. Wumeng of about 4,200 m in elevations, a natural barrier for both seed and pollen dispersal. A dramatic demographic decline, plus geographical isolation, may have played a predominant role in shaping the levels of genetic variation. Such historical events would leave imprints on contemporary genetic composition, likely with reduced genetic variation within populations and elevated genetic differentiation among populations (Schaal and Olsen 2000). Previous studies based on allozymes and ISSR fingerprinting, however, detected a pattern with excessive heterozygosity within species/populations of *N. insignis* (Peng et al. 2003), seemingly implying a very large population size before the recent demographic decline, which, in turn, may have maintained the genetic diversity within populations (Luan et al. 2006).

In this study, we examined the genetic diversity and spatial apportionment of cpDNA variation in the endangered species. At the intraspecific level, genetic structure may reflect the historical sequence of population fragmentation and expansion, as well as the pattern of gene exchange among existing populations. In the present study, we sequenced the *rp*L16 intron of cpDNA for examining the demographics and phylogeographical pattern of *N. insignis*. Several questions are addressed.

(1) As a rare species, should cpDNA display low levels of genetic diversity, as generally expected? Otherwise, as an element of the biodiversity hotspots and given a short history of disturbance, can the effects of random genetic drift that lead to loss of genetic diversity possibly be delayed, like in the nuclear genome?
(2) Given a natural barrier of Mt. Wumeng for genetic exchanges, are populations of Jinsha and Nanpan River drainages genetically differentiated?
(3) When did *N. insignis* diverge? Did the species experience demographic shrinking over the glacial maxima followed by postglacial expansion?

Materials and methods

Plant materials and DNA extraction

Plant materials of *N. insignis* from 14 populations, i.e. 11 from Jinsha drainage, and three from Nanpan drainage, were collected (Fig. 1; Table 1). About one-fifth of the individuals of each population were randomly sampled. Accordingly, for cpDNA sequencing, 8–22 individuals of each population were surveyed. In total 176 individuals were sampled for estimating the population structure of the rare species. Three individuals of *Leucomeris decora* Kurz., the sister genus of Nouelia, were chosen as outgroups (Kubitzki 2007). Healthy leaflets were dried with silica gel. Leaf tissue of the above materials was ground to powder in liquid nitrogen. Genomic DNA was extracted from the tissue powder following the CTAB protocol of Doyle (1991).

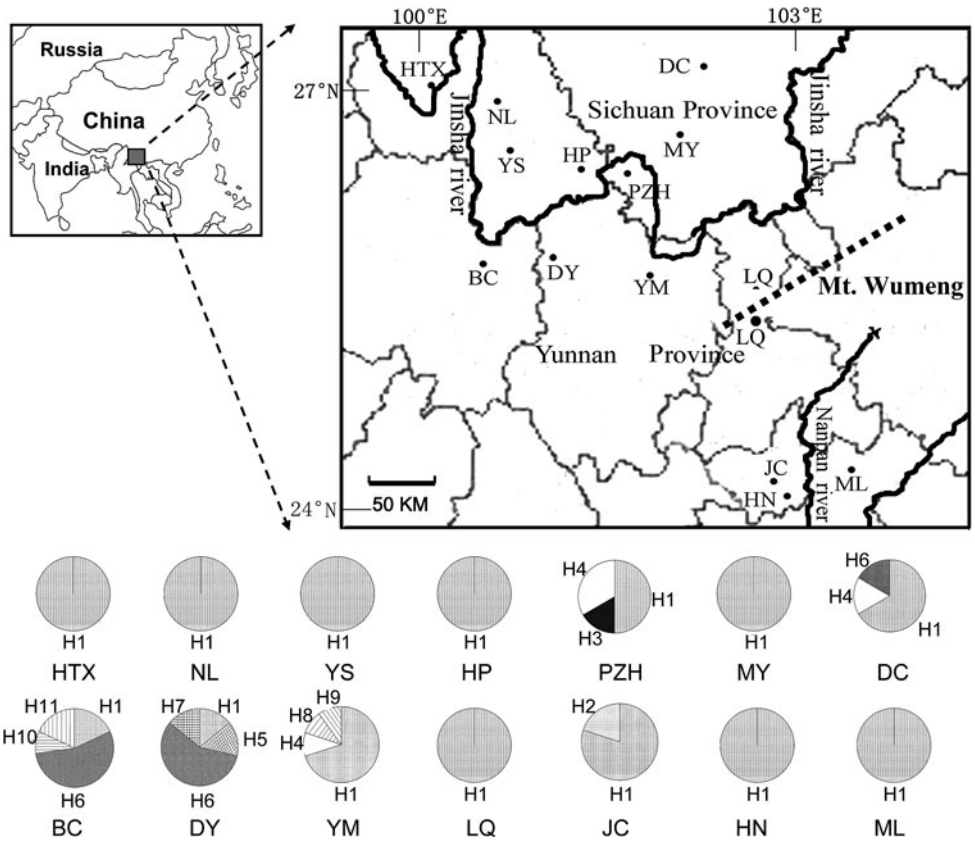

Fig. 1 Map showing the spatial distribution of genetic polymorphisms of cpDNA in populations of *Nouelia insignis*

Table 1 Sites, sample sizes, and the distribution of cpDNA haplotypes in 14 populations of *Nouelia insignis* examined for cpDNA variation

Population	Code	Sample size	Coordinates		cpDNA haplotypes											Nucleotide diversity	
					H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	θ (bp)	π (bp)
Jinsha River drainage																	
Bingchuan	BC	22	25°50′N	100°36′E	4					12				2	4	0.00150	0.00166
Dechang	DC	12	27°38′N	102°17′E	8			2		2						0.00144	0.00132
Dayao	DY	14	25°51′N	101°06′E	2				2	8	2					0.00171	0.00182
Huaping	HP	12	26°35′N	101°21′E	12											0	0
Hutiaoxia	HTX	12	27°19′N	100°08′E	12											0	0
Luquan	LQ	8	25°26′N	102°29′E	8											0	0
Miyi	MY	10	26°54′N	102°13′E	10											0	0
Ninglang	NL	10	27°21′N	100°51′E	10											0	0
Panzhihua	PZH	12	26°24′N	101°46′E	6		2	4								0.00072	0.00073
Yuanmou	YM	20	25°46′N	101°50′E	14			2				2	2			0.00215	0.00209
Yongsheng	YS	8	26°34′N	100°48′E	8											0	0
Nanpan River drainage																	
Jiangchuan	JC	10	24°21′N	102°43′E	8	2										0.00039	0.00039
Mile	ML	14	24°41′N	103°40′E	14											0	0
Huaning	HN	12	24°17′N	102°51′E	12											0	0
Total		176			128	2	2	8	2	22	2	2	2	2	4	0.00335	0.00393

Nucleotide diversity, both θ and π per base pair, for each population is indicated

Nucleotide sequencing

The noncoding *rp*L16 intron of cpDNA was amplified and sequenced. A reaction of, 20 μL was carried out, consisting of, 20 ng template DNA, 2.0 μL of 10× reaction buffer, 1.2 μL of $MgCl_2$ (25 mM), 1.4 μL of dNTP mix (10 mM), 1 μL of DMSO, 3.5 pmol of each primer [P1: 5′GCT ATG CTT AGT GTG TGA CTC GTT G 3′ and P2: 5′ CCC TTC ATT CTT CCT CTA TGT TG 3′], 1.5 U of *Taq* polymerase (Takara) and double-distilled water. The PCR were carried out on the ABI GeneAmp PCR System 9700 (Applied Biosystems/Perkin Elmer) as one cycle of denaturation at 94°C for 3 min, 30 cycles of 45 s denaturation at 94°C, 1 min 10 s annealing at 53°C, and 1 min 30 s extension at 72°C, followed by a 7 min extension at 72°C. PCR products were electrophoresed on agarose gels and the desired fragments were excised from the gel and purified (Glenn and Glenn 1994). Eluted PCR products were sequenced directly in both directions by standard methods using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA). Products of the cycle sequencing reactions were run on an ABI 377XL automated sequencer (Applied Biosystem, Foster City, CA, USA).

Sequence alignment and phylogenetic analyses

Nucleotide sequences were aligned with the program, CLUSTAL V (Higgins et al. 1992). Phylogenetic trees were reconstructed using maximum likelihood (ML) analyses of the nucleotide sequences with software PHYML v2.4.5 (Guindon and Gascuel 2003) and bootstrap consensus values calculated using 1,000 replicates. The general time reversible HKY model was determined to be the most suitable model by Modeltest v3.6 (Posada and Crandall 1998) and was used for all subsequent nucleotide analyses.

Population genetic analysis

In the study, the number of mutations between haplotypes in pairwise comparisons estimated from the data using the MEGA V3.1 (Kumar et al. 2004), and were used to construct a minimum spanning network (cf. Chiang et al. 2006) with the aid of program MINSPNET (Excoffier and Smouse 1994).

Levels of intra-population genetic diversity were quantified by indices of haplotype diversity (h) (Nei and Tajima 1983) and by pairwise estimates of nucleotide divergence, π (Jukes and Cantor 1969) and θ (Watterson 1975). The parameters were calculated by using DnaSP V4.0 (Rozas et al. 2003).

We also used AMOVA version 1.55 (Excoffier 1993) to deduce the significance of geographical divisions both among populations and regions, i.e. Jinsha vs. Nanpan River drainages. The statistics of molecular variances Φ_{ct} (among regions) Φ_{st} (among populations), and Φ_{sc} (among populations within regions) were estimated. The significance of these F-statistic analogues was evaluated by 1,000 random permutations of sequences among populations.

Estimation of coalescence times of cpDNA lineages

For estimating coalescent time within populations or species based on the cpDNA, a well-documented evolutionary rate is needed. In plants, the rates were estimated to be 1.4–1.6×10^{-9} substitutions per site per year for the cpDNA (Wolfe et al. 1987). Bayesian estimates of the ages of the most recent common ancestor (TMRCA) of the Nouelia sequences were obtained using BEAST v. 1.4, available from http://beast.bio.ed.ac.uk (Drummond et al. 2006). We used the HKY model and a strict molecular clock with uncorrelated log normal distribution of branch lengths. Posterior estimates of the mutation rate and age of the TMRCA were obtained by Markov Chain Monte Carlo (MCMC) analysis, with samples drawn every 500 steps over a total of 25,000,000 steps. Convergence of parameters and mixing of chains were followed by visual inspection of parameter trend lines and checking of effective sampling size (ESS) values by three pre-runs. The ESS parameter was found to exceed 200, which suggests acceptable mixing and sufficient sampling. Adequate sampling and convergence to the stationary distribution were checked using TRACER v. 1.3 (Rambaut and Drummond 2004). Posterior estimates of parameters were all distinctly unimodal (although with wide 95% highest posterior densities), and all parameters were identifiable, despite the relatively low information content in the sequences and the small age range of the sequences.

Demographic inferences

In order to make inferences about demographic changes of *N. insignis*, we employed both mismatch distributions and statistical tests of neutrality. Population expansion in the recent past usually results in a unimodal mismatch distribution, while a multimodal distribution is encountered in populations at demographic equilibrium or constituted by several units. In addition, we calculated Tajima's D (Tajima 1989) in the untranslated DNA fragments as indicators of demographic expansion (de Gelas and de Meester 2005). Tajima's D, which examines the conformity of DNA sequence evolution to neutrality, was performed using DnaSP vs. 3.95 (Rozas and Rozas 1999). Tajima's (1989) D based on the difference between θ and π values is informative about the effects of selective events on the patterns of cpDNA variation, or historical demographic

events on a sample of sequences (Rand 1996). Positive D values ($\pi > \theta$) suggest either a recent population bottleneck or some form of balancing selection, and negative D values ($\pi < \theta$) suggest either population expansion or purifying selection.

Results

Genetic diversity and gene genealogy of cpDNA in *Nouelia insignis*

The *rp*L16 intron of cpDNA in *N. insignis* were aligned with a consensus length of 917 bp. No indels or rearrangement was detected. Most of sequences were single base mutations scattering along sequences. A total of eight sites (0.872%) were variable (Fig. 2). Haplotype sequences of cpDNA were deposited in the GenBank database under the accession numbers HM208349−HM208358, and HM208360. Among the 176 samples examined, 11 cpDNA haplotypes, H1 to H11, were identified. Among them, haplotypes H1 (128/176 individuals) and H6 (22/176 individuals) were most dominant in the genetic composition of the species. Of the 14 populations, populations BC, DY, and YM possessed the highest number of haplotypes (Table 2), while the cpDNA was fixed in many other populations (Table 1) at the haplotype H1. Higher levels of nucleotide diversity were detected in the above three populations, with θ varying from 0.00039 (JC) to 0.00215 (YM) (Table 1). The geographical distribution of cpDNA haplotypes is illustrated in Fig. 1. Notably, the three populations plus another population PZH having high genetic diversities are located south of the Jinsha River. In addition, the cpDNA was also polymorphic in a marginal population (DC). Of the Nanpan River Drainage, no variation of the cpDNA gene occurred in most populations, except for JC.

	1	3	10	18	27	57	64	69
	T	T	A	C	G	G	G	C
H1	-	-	G	T	-	A	A	-
H2	-	G	G	T	-	A	A	-
H3	G	-	G	T	-	A	A	-
H4	G	-	G	T	-	A	-	-
H5	A	-	G	T	-	A	A	-
H6	-	-	G	-	-	-	-	-
H7	-	-	G	-	-	-	-	T
H8	-	-	-	-	A	-	-	T
H9	-	-	-	-	A	-	A	-
H10	-	G	-	-	-	-	-	-
H11	-	-	-	-	-	-	-	-

Fig. 2 Polymorphic sites of the haplotypes of the *rp*L 16 intron of cpDNA in *Nouelia insignis*

Population structuring and geographical subdivision in *N. insignis* were estimated based on pairwise F_{st} (Table 3). Since most populations were fixed at haplotype H1, they remained undistinguished. Nevertheless, most populations with polymorphic DNAs were differentiated from each other genetically, except for population YM, based on the *Nm* values mostly less than one. In addition, AMOVA analyses revealed a trend toward genetic differentiation at population level, as 31.17% of the genetic variance was distributed among populations ($\Phi_{st} = 0.320$, $P < 0.001$), while only 0.83% of variants were distributed among geographical regions ($\Phi_{ct} = 0.008$, $P = 0.360$).

Phylogenetic relationships were reconstructed among cpDNA haplotypes of *N. insignis*. Rooted at *Leucomeris decora* sequences (accession number of HM208359), maximum likelihood tree was obtained based on the genetic variation of the cpDNA intron sequences (Fig. 3). Two clusters I and II, with three mutational differences, were identified in the ML tree and supported with 75 and 76 bootstrap values. Five (H1–H5) and six (H6–H11) haplotypes existed in cluster I and II, respectively.

A minimum-spanning network (Fig. 4) was reconstructed based on mutational changes between cpDNA haplotypes. In the study, haplotype H1 that was nested in the cluster I as the interior node was widespread in all populations geographically, while most exterior haplotypes were restricted to single populations. In contrast, the cluster II with haplotype H6 as the interior node was mostly restricted to the region south of the Jinsha River. Within clusters, most haplotypes were one mutation away from the most interior node, making the gene genealogy star-like. The two major clusters were differentiated with three mutations. In addition, the outgroup haplotype L3 was linked to the haplotype H1 with three mutations (Fig. 4).

Molecular dating and population demography

In this study, Bayesian estimates of the mutation rates and the age of the most recent common ancestor (TMRCA) of the cpDNA sequences of *N. insignis* were obtained using BEAST v. 1.3. Using 1.4–1.6 $\times$ 10^{-9} substitutions per site per year as the rates for the evolution of cpDNA, the divergence between lineages I and II of cpDNA tree can be dated to about 3.224 (95% CI 1.070–6.089) million years ago. Using a coalescence-based approach, times coalesced back to TMRCA were dated to 1.062 (95% CI 0.226–2.180) and 0.934 (95% CI 0.226–1.848) MYA for lineages I and II, respectively (Fig. 3).

Tajima's D was positive for the cpDNA sequences, but not differing significantly from zero, in the overall dataset ($D = 0.72109$; $P > 0.10$). At the population level, most

Table 2 Haplotype number, polymorphic sites of the cpDNA fragment in populations with polymorphic DNA and major lineages of *Nouelia insignis*

Populations, lineages	No. of haplotypes	No. of polymorphic sites	Singletons	Fu and Li's *F*	Tajima's *D*
Overall	11	8	0	1.05097	0.72109
BC	4	5	1	1.08209	0.32982
DC	3	2	2	0.92337	−0.29855
DY	4	3	3	1.10227	0.21595
PZH	3	1	0	0.82867	0.01936
YM	4	7	3	1.04561	−0.09548
JC	2	1	1	0.68403	0.01499

Table 3 Pairwise comparisons of F_{st} estimates (above diagonal) and deduced *Nm* values (below diagonal) between populations of *Nouelia insignis*

	BC	DC	DY	HN	HP	HTX	JC	LQ	ML	MY	NL	PZH	YM	YS
BC		**0.351**	0.023	**0.600**	**0.600**	**0.600**	**0.500**	**0.600**	**0.600**	**0.600**	**0.600**	**0.500**	**0.305**	**0.600**
DC	0.46		**0.347**	0	0	0	0	0	0	0	0	0.008	0.031	0
DY	10.84	0.47		**0.625**	**0.625**	**0.625**	**0.532**	**0.625**	**0.625**	**0.625**	**0.625**	**0.486**	**0.328**	**0.625**
HN	0.17	–	0.15		0	0	0	0	0	0	0	**0.267**	0.063	0
HP	0.17	–	0.15	–		0	0	0	0	0	0	**0.267**	0.063	0
HTX	0.17	–	0.15	–	–		0	0	0	0	0	**0.267**	0.063	0
JC	0.25	–	0.22	–	–	–		0	0	0	0	0.190	0.049	0
LQ	0.17	–	0.15	–	–	–	–		0	0	0	**0.267**	0.063	0
ML	0.17	–	0.15	–	–	–	–	–		0	0	**0.267**	0.063	0
MY	0.17	–	0.15	–	–	–	–	–	–		0	**0.267**	0.063	0
NL	0.17	–	0.15	–	–	–	–	–	–	–		**0.267**	0.063	0
PZH	0.25	31.5	0.26	0.69	0.69	0.69	1.06	0.69	0.69	0.69	0.69		0.039	**0.267**
YM	0.57	8.38	0.52	3.75	3.75	3.75	4.85	3.75	3.75	3.75	3.75	6.18		0.063
YS	0.17	–	0.15	–	–	–	–	–	–	–	–	0.69	3.75	

F_{st} values showing significant genetic differentiation are in bold. '–' indicates estimate unavailable due to the monomorphism within populations

estimates were positive, especially those with two lineages intermixed, such as populations BC (Fu and Li's $F = 1.082$, $P < 0.10$) and YM (Fu and Li's $F = 1.046$, $P < 0.10$). We also used mismatch distribution analyses to infer the long-term demographic history of populations. Mismatch analyses revealed bimodal distributions in populations BC, DC, DY, and in the species (Fig. 5). A scenario of recent demographic expansion therefore can be recognized in *N. insignis*.

Discussion

Low levels of cpDNA genetic diversity within populations

In this study, we examined the genetic diversity of *rp*L16 intron of cpDNA among populations of *N. insignis*. Usually, narrowly distributed and rare species, such as *Dunnia sinensis* (Ge et al. 2002), are expected to exhibit lower levels of genetic variations (Hamrick and Godt 1989). Nevertheless, *rp*L16 intron of cpDNA displays relatively high levels of genetic diversity in this endangered species (11 haplotypes), which is higher than that in other plants (such as *Leman minor* with 2 haplotypes, Jordan et al. 1996; *Spirodela punctata* with 1 haplotype, Jordan et al. 1996; *Pinus tabulaeformis* with 4 haplotypes, Chen et al. 2008). In contrast, the levels of genetic diversity and haplotype diversity were low within populations (Table 1). Of the 14 populations, eight populations (57.1%) were fixed at the haplotype H1, with no genetic variation herein. The reduced genetic diversity in noncoding spacers of the chloroplast genome is often associated with the demographic history of a species, such as genetic bottlenecks (Chiang et al. 2001; Huang et al. 2001).

Nevertheless, it is also possible that high genetic diversity in rare plants has been attributed to evolutionary factors including insufficient length of time for reducing genetic diversity following reduction in population size (Coates 1988), adaptation of genetic system to small

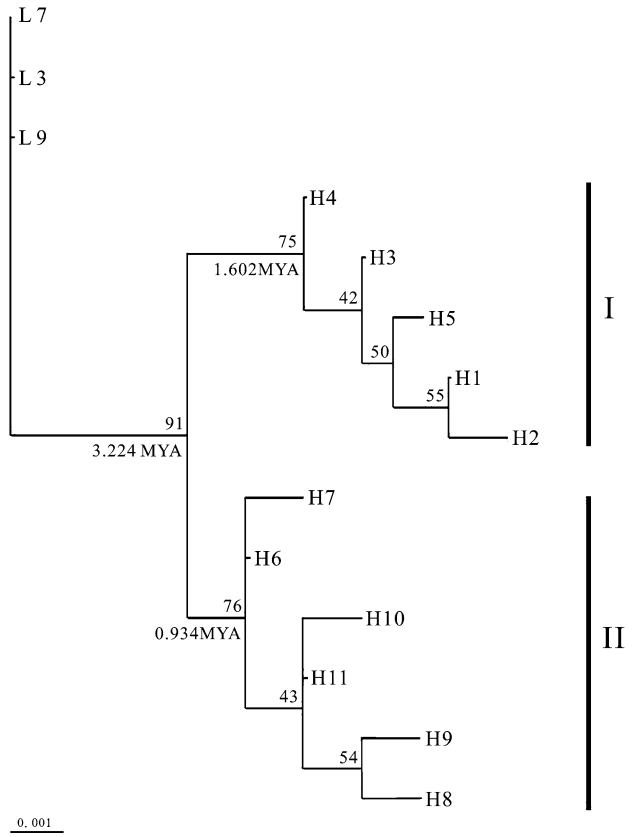

Fig. 3 Maximum likelihood (ML) tree of cpDNA haplotypes. Bootstrap values were indicated at nodes. Major cpDNA lineages are indicated. The divergence time between the two major lineages, and the coalescence time for each lineage are indicated at nodes

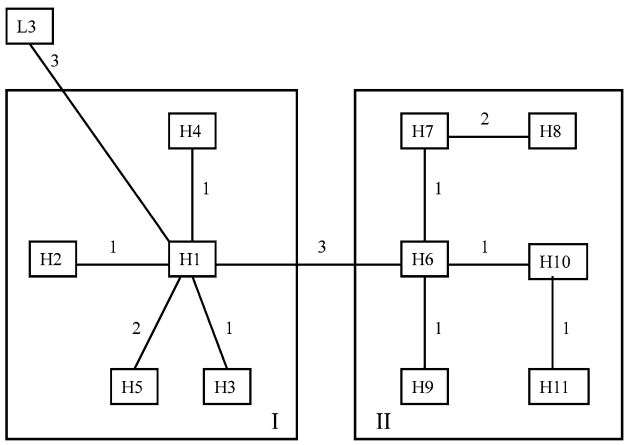

Fig. 4 Minimum spanning network generated for cpDNA haplotypes (H1–H11) of *Nouelia insignis* and the outgroup haplotype (L3). The number of mutational changes between haplotypes is indicated

population size (Coates 1988; Rossetto et al. 1995; James 2000), and recent population fragmentation (human disturbance) of a continuous genetic system (Rossetto et al. 1995). Given a short history of disturbance, many polymorphisms can still be maintained within species by chance, like many endangered plants such as *Acacia anomala* (Coates 1988), *Grevillea scapigera* (Rossetto et al. 1995), *Banksia cuneata* (Maguire and Sedgley 1997), and *Leucopogon obtectus* (Zawko et al. 2001).

This result, however, contradicts previous studies based on allozymes and ISSR fingerprinting, both of which detected high levels of genetic diversity within populations of *N. insignis* (Peng et al. 2003; Luan et al. 2006). Since all genes are carried by individuals and exist in populations, the extrinsic evolutionary force would have similar or identical impacts on these unlinked organelle and nuclear genomes (Chiang et al. 2003; Chen et al. 2004); that is, the footprint of genetic bottlenecks should also be detected in the nuclear fingerprints and allozymes. It has been known that the reduced effective population size of haploid genomes makes maternally inherited organelle markers more likely to record the effects of population history in present-day genetic patterns than nuclear markers (Schaal et al. 1998; Ennos et al. 1999). The influences of demographic bottleneck would be stronger on plastid than on nuclear markers because the effective population size for the chloroplast genome is half of that of the nuclear genome (Birky 1988). The effects of random genetic drift that lead to the loss of genetic diversity seemingly have been weaker in the nuclear genome. Besides, a system of mating of likely outcrossing in the perennial species, and highly structured populations, plus genetic recombination, could have compensated or deterred the diversity loss from populations (Luan et al. 2006). However, genetic drift alone cannot explain the fixation of the identical cpDNA haplotype, i.e. H1, across Nouelia populations, implying some other demographic forces prior to the recent bottlenecks.

Population genetic theory predicts that large populations tend to maintain high genetic diversity. High levels of genetic diversity based on allozyme and ISSR fingerprinting evidence indicate a large effective population size of the species (cf. Ellstrand and Elam 1993). Such an inference, apparently, contradicts the fact that populations of *N. insignis* remain fragmented in the wild. It has been known that the rarity of this long-life perennial species is likely to be attributable to the recent large-scale habitat destruction caused by human over-exploitation (Luan et al. 2006). The present isolated and fragmented populations were therefore likely to be derived from a previously large population. Apparently, the period since the population decline, fewer than 10 generations or so, may not be ample for the loss of nuclear genetic diversity. In this study, demographic bottlenecks seem to have relatively stronger impacts on the chloroplast genome, resulting in the depletion of genetic diversity in the cpDNA intron. In addition to the stochastic effects of genetic drift, low evolutionary rates of the cpDNA intron (Houliston and Olsen 2006), and lack of

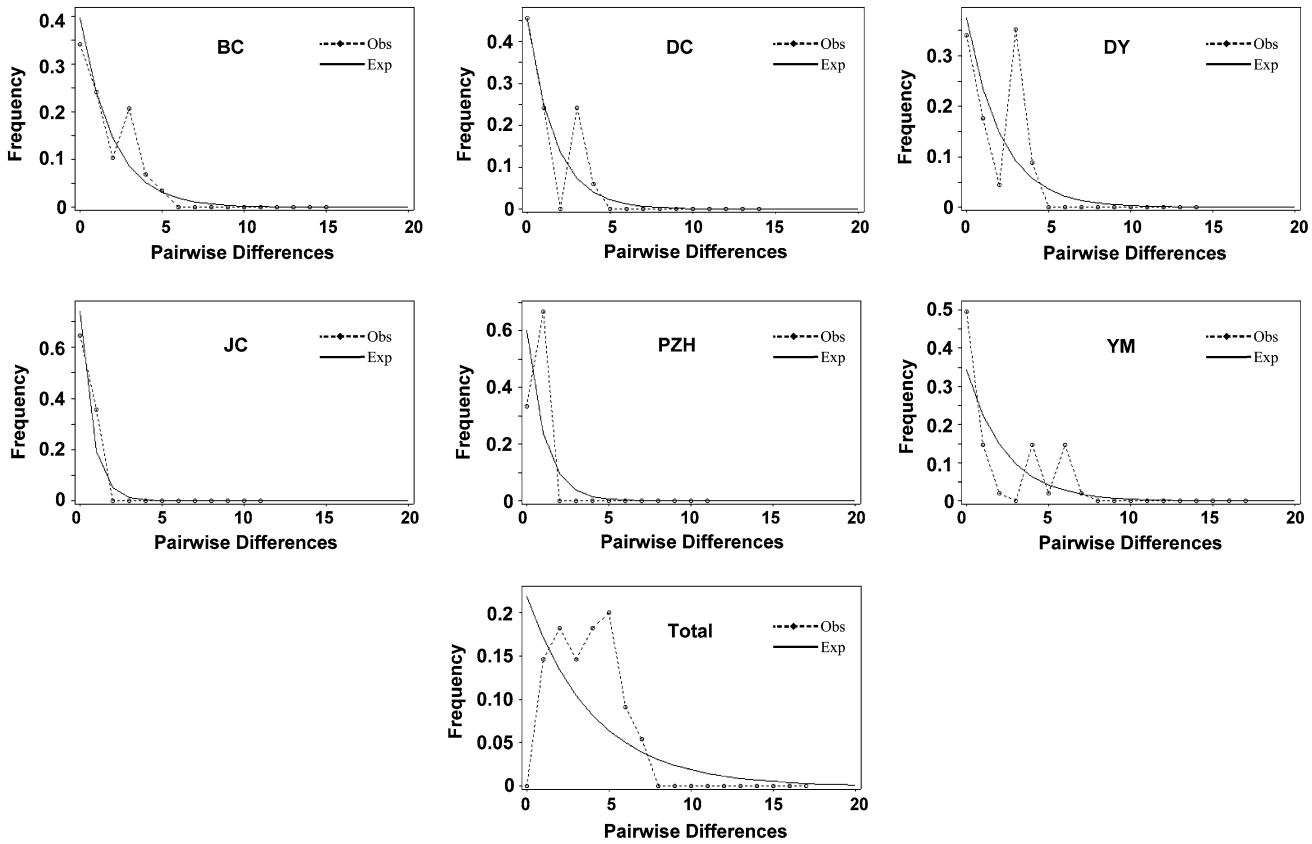

Fig. 5 Mismatch distributions of cpDNA sequences in populations of *Nouelia insignis*

genetic crossover (Graur and Li 1999), may have also contributed to the low genetic diversity.

Current population structure of *N. insignis*

The inconsistency between the nuclear and organelle DNA markers may primarily stem from low mutation rates in the cpDNA, which inevitably provided low resolution for differentiating populations. Even so, populations that possessed polymorphic cpDNA tend to be differentiated from others (Table 3), resulting in overall geographic structuring across populations as indicated by AMOVA, with 31.17% of the genetic variance distributed among populations ($\Phi_{st} = 0.320$). Nevertheless, like ISSR fingerprinting (Luan et al. 2006), cpDNA displayed very low differentiation between drainage along the two rivers ($\Phi_{ct} = 0.008$) (Fig. 1), suggesting that populations of the geographical regions may have long been connected with migration. That is, both seed and pollen dispersal could have taken place across the Mt. Wumeng.

Of *N. insignis* populations, BC, DY, and YM, all southern of the Jinsha River, were characterized by high levels of cpDNA polymorphisms. A contrasting pattern showed that YM was dominated by the cpDNA haplotype H1 (70.0%), while populations BC and DY possessed H6 with high frequencies, 54.5 and 57.1%, respectively (Table 1). Based on the coalescent theory, tip nodes of a network are likely to represent descendents derived from ancestral, interior nodes (Donnelly and Tavare 1986; Crandall and Templeton 1993). Accordingly, the cpDNA haplotypes H1 and H6 would represent some old polymorphisms that have been long maintained via random processes associated with its large population size. In addition, given the absence of haplotype H6 in population YM, the low frequency of haplotype H1 vs. dominance of haplotype H6 in populations BC and DY, i.e. 18.2 and 14.3% respectively, indicates a unidirectional, westward migration. The possession of high private polymorphisms, as also indicated by high levels of nucleotide variability, characterized populations DY and YM as hot spots of genetic diversity. The scenario of secondary mergence of haplotypes H1 and H6, as further supported by a bimodal distribution of mismatch analyses, in turn, recognizes populations BC and DY as melting spots (cf. Petit et al. 2003). Nonetheless, admixture of H1 and H6 haplotypes in populations of BC and DY could also be a result of direct mutations from one to the other, as revealed by the pattern showing a limited distribution of the newly arisen mutant, i.e. H6 in this study, vs. a widespread ancestral type, i.e. H1. However, the coalescence-based dating revealed

similar ages of the two lineages, 1.062 versus 0.934 MYA. Geographic subdivision prior to secondary contacts, in turn, necessarily accounts for the apportionment pattern of genetic polymorphisms. It has been known that the Quaternary glaciers largely shaped the distribution of plant species and the ranges of forests (Hewitt 2000). During glacial maxima, the majority of animals and plants of the temperate were forced to migrate into the refuges, often resulting in high diversities in south (Chiang and Schaal 2006). Floristic characteristics of these hotspots include adaptive radiation with a large number of plant groups, and the occurrence of many relict plant lineages (cf. Luan et al. 2006). In the study, the molecular dating revealed that the cpDNA polymorphisms may have long been maintained since the Pleistocene, as revealed by the molecular dating showing a coalescence time about 3.224 million years ago, some time between Quaternary and Tertiary (Pliocene). Palaeoecological biome reconstructions indicate that at the Last Glacial Maximum (LGM), approximately 18,000–20,000 years ago, steppe and desert vegetation extended to the modern coastline of eastern China, in place of temperate deciduous forest currently found over the same area at the present time (Wang and Sun 1994). It is possible that the populations of *N. insignis* experienced demographic expansion before the last glacial maximum (LGM), as occurring in *Pedicularis longiflora*, a species distributed in the Qinghai-Tibetan Plateau (Yang et al. 2008). According to geological evidence, since the uplifting of the Yunnan-Guizhou Plateau in the late Tertiary, the prevalent climate in the Jinsha drainage area has remained relatively steady and no great change has occurred across the extensive vegetation (Yan 1984). The region thus provided sheltering for the surviving species, including *N. insignis*, over the periodical glaciations. In the study, as populations that represent hotspots or melting pots may have long survived over the periodical glaciations, as supported by the existence of private haplotypes within populations (Table 1), populations fixed at the haplotype H1, in contrast, were possibly founded, following the glacier retreats, via a demographic expansion from the hotspot population. Based on its genetic composition dominated by haplotype H1 (Table 1) and the location approximating the center of the geographical distribution (Fig. 1), the population YM was most likely to be the source population for such colonizing events. The population PZH was another possible source population. Evidence of star-like genealogies of cpDNA, and the unimodal distribution of mismatch analyses all suggested demographic expansion following glacial retreats. Postglacial, long-distance recolonization and population expansions have also been well documented in European and East Asian plants (Petit et al. 2003; Chiang and Schaal 2006). Apparently, the process not only explained the low genetic diversity in most Nouelia populations, but also satisfied the scenario of the secondary mergence in populations BC and DY.

Conclusions

In the study, cpDNA exhibited low levels of genetic diversity in populations of the rare species *N. insignis*. Genetic analyses revealed that low evolutionary rates of the organelle DNA intron, plus postglacial recolonization and the recent demographic bottlenecks, which led to fixation of the dominant alleles within many populations, contributed to the depletion of genetic diversity in most populations and the lacking of genetic differentiation between populations. Hotspots and melting pots for maintaining genetic diversity were thereby identified. Although the low levels of genetic variation often found within species or populations seem unable to resolve the phylogeographical patterns, the recently developed coalescence-based analyses enable one to recover the demographic history of a species that evolved through bottlenecks.

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