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ORIGINAL ARTICLE

Evolution of the *Euphrasia transmorrisonensis* complex (Orobanchaceae) in alpine areas of Taiwan

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ABSTRACT

Aims To unravel isolation and differentiation of the genetic structure of the *Euphrasia transmorrisonensis* complex, a showy herb, among alpine regions of mountain peaks in subtropical Taiwan and to infer its evolutionary history.

Location Alpine ecosystems of high-montane regions of Taiwan.

Methods Phylogenetic analyses of the *trn*L intron and the *trn*L–*trn*F intergenic spacer of chloroplast (cp) DNA, and the intertranscribed spacer (ITS) of nuclear ribosomal (nr) DNA between 18S and 26S were carried out on 18 populations of the *E. transmorrisonensis* complex in Taiwan.

Results In total, 10 haplotypes for cpDNA and 14 haplotypes for nrDNA were detected. Three population groups located in the northern, north-eastern, and south-central regions of the Central Mountain Range (CMR) were revealed according to the frequencies of haplotypes and haplotype lineages of nrDNA. Balancing selection might have played a role in the evolution of *Euphrasia* in Taiwan.

Main conclusions By integrating the spatial-genetic patterns of cpDNA and nrDNA, two possible evolutionary histories of *Euphrasia* in Taiwan were inferred. The favourable hypotheses for interpreting the data suggest at least three origins of the *E. transmorrisonensis* complex in Taiwan, corresponding to each nuclear lineage in the northern (II), northern/north-eastern (I), and central/southern regions (III) with subsequent hybridization between lineages I and II and lineages II and III. These lineage boundaries are strengthened by the finding that haplotypes of C derived from cpDNA were found in the geographical region of lineage II of nrDNA, while haplotypes of A derived from cpDNA were found in the region of lineage III of nrDNA. Thus, the origin of chloroplasts exclusive to lineages II and III supports their long-term isolation from one another.

Keywords
Balancing selection, chloroplastic DNA, *Euphrasia*, intertranscribed spacer, phylogeography, Taiwan.

INTRODUCTION

The genus *Euphrasia* is conventionally classified under the tribe Rhinantheae of the Scrophulariaceae. In recent years, Rhinantheae was moved to the Orobanchaceae due to similarities in the chloroplast (cp) DNA although the molecular data do not support the monophyly of Rhinantheae (Olmstead *et al.*, 2001). Globally, *Euphrasia* contains *c.* 170 species (Barker, 1982). However, due to frequent autogamy, patterns of ecological convergence, and wide-ranging variations in morphological characteristics within species (Barker, 1982; Vitek, 1998), species delineations within this genus are still a challenge. The study of phylogeography attempts to decipher the genetic structure of extant populations and provides information for inferring contemporary biogeographical patterns of genetic variations (Avise, 2000). A phylogeographical study might offer insights

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into the evolutionary history of a species and a species complex as it provides information on the genealogical relationship and spatial distribution of populations (Templeton, 1998).

Plants of *Euphrasia* are herbaceous, hemiparasitic, and mainly distributed in temperate zones, but extend somewhat into tropical zones in both hemispheres. Three species and one variety are recognized in Taiwan according to morphological characteristics, i.e. *E. nankotaizanensis*, *E. tarokoana*, *E. transmorrisonensis* var. *transmorrisonensis*, and *E. transmorrisonensis* var. *durietziana* (Wu & Huang, 1998, 2004). *Euphrasia transmorrisonensis* var. *transmorrisonensis* is the most common variety, while *E. tarokoana* is very rare and restricted. The variety *durietziana* is densely covered with glandular hairs, whereas the variety *transmorrisonensis* has scarce hairs or is glabrous. The variety *durietziana* is found in drier areas, while the variety *transmorrisonensis* is found throughout the entire species range. A clear description of the *E. transmorrisonensis* complex in Taiwan was recently published (Wu & Huang, 2004). This species complex is endemic to Taiwan and is mainly distributed in open rocky fields between 2000 and 3900 m in elevation with a few extending to below 2000 m. It is classified under the section Malesianae associated with species from the Philippines, Borneo, and Ceram (Indonesia) according to their morphological characteristics (Barker, 1982). Malesianae is closely related to the section *Euphrasia*, which is distributed in the northern temperate zone, and differs from it mainly by its habits, i.e. perennial vs. annual. Thus, *Euphrasia* in Taiwan is related to species from temperate parts of Asia and Malesia, but is closer to Malesia according to Barker (1982). However, the temperate flora of Taiwan is strongly influenced by that of temperate Asia (Hsieh, 2002). A phylogeographical study of Taiwan's *Euphrasia* may provide an example for uncovering possible diverse origins of the temperate flora of Taiwan.

The extant spatial-genetic variation patterns of a taxon are a result of its evolutionary history. Demographic and historical details can be revealed by geological patterns of genealogical relationships of molecular haplotypes, and these provide a basis for understanding the evolutionary history of a species. Usually, sequences of partial DNA, especially non-coding regions, are used as markers to detect the spatial-genetic structure including nuclear ribosomal (nr) DNA and cpDNA (Grivet & Petit, 2002; Huang *et al.*, 2002; Holderegger & Abbott, 2003). Herein, the results of cpDNA and nrDNA data are combined to examine the spatial-genetic structure of the *E. transmorrisonensis* complex that led to the elucidation of two possible biogeographical histories for Taiwan.

MATERIALS AND METHODS

Sampling and DNA sequencing

In total, 18 populations were sampled (Fig. 1; Table 1). Each population was represented by between 3 and 10 individuals whenever samples were available. Fresh leaves for each individual were collected and immediately deposited in a

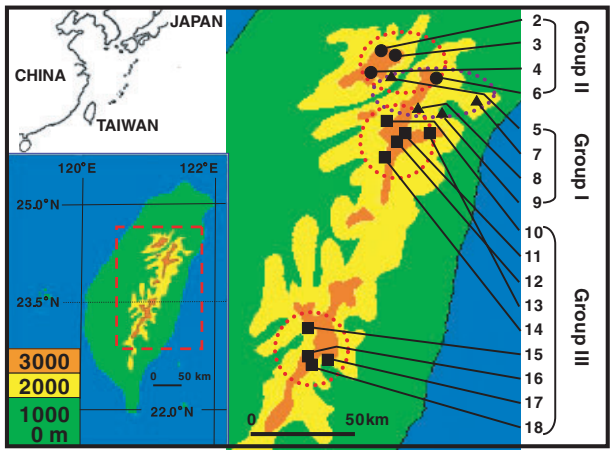

Figure 1 Populations sampled for the phylogeographical study of *Euphrasia* in Taiwan. According to the variation in the inter-transcribed spacer (ITS) of nuclear ribosomal (nr)DNA, three populations groups are recognized (see Fig. 4 for details). Solid circles are for group II, solid triangles for group I, and solid squares for group III. See Table 1 for population numbers.

freezer at −70 °C. *Euphrasia petiolaris* and *E. hirtella*, close relatives to the section *Euphrasia*, were obtained from the herbarium of Rancho Santa Ana Botanical Garden (RSA), Santa Ana, TX, USA.

The two kinds of DNA were extracted from sample leaves using the protocol of Doyle & Doyle (1990). The DNA extraction solution was then used to amplify the markers for detecting variations in the polymerase chain reaction (PCR). For sequences of cpDNA, two markers were used in this study, i.e. the intron, *trn*L, and the intergenic spacer, *trn*L–*trn*F (Taberlet *et al.*, 1991). The forward and reverse primers for *trn*L were 5′-CGA AAT CGG TAG ACG CTA GC-3′ and 5′-GGG GAT AGA GGG ACT TGA AC-3′, and those for *trn*L–*trn*F were 5′-GGT TCA AGT CCC TCT ATC CC-3′ and 5′-ATT TGA ACT GGT GAC ACG AG-3′, respectively. Thirty-five thermal cycles were carried out for amplification, with an annealing temperature of 50 °C for 30 s, and an extension temperature of 72 °C for 60 s. The PCR products were then purified using a commercial kit (cat. no. PF1001; Viogene-Biotek, Taipei, Taiwan) and sequenced with an ABI PRISM® 3100 Genetic Analyzer using a Big Dye version 3.0 terminator (Applied Biosystems, Foster City, CA). The fragment was sequenced from both ends to ensure that the sequences agreed.

Sequence analysis

DNA sequences were aligned by eye. Construction of the haplotypes of cpDNA and estimation of the genetic genealogy were performed using TCS version 1.06, as described in Templeton *et al.* (1992). For all populations, nrDNA sequences were obtained from a previous publication (Wu & Huang, 2004; GenBank accession nos. AY165600–AY165624, AY264947–AY265060, AY513682–AY513689, AY596814, and

Table 1 Location of populations of *Euphrasia* in Taiwan sampled for the phylogeographic study and occurrence of haplotypes of chloroplast (cp)DNA and nuclear ribosomal (nr)DNA

Local population and population no.	Latitude–longitude	Elevation (m)	Haplotypes of cpDNA*	Haplotypes of nrDNA†
1. Itsershan	24°28′15″ N, 121°13′50″ E	3000–3300	A(8)	–
2. Tapachienshan	24°27′35″ N, 121°14′55″ E	3200–3300	H(4), C(3)	G(20)
3. Taoshan	24°25′49″ N, 121°17′44″ E	3100–3200	A(3), C(2)	A(2), C(2), E(3), F(3), G(2)
4. Hsueshan	24°23′27″ N, 121°15′14″ E	3100–3885	C(9)	F(10), G(12)
5. Wuling	24°23′18″ N, 121°17′30″ E	2000–2200	A(3), C(1)	C(6)
6. Nanhutashan	24°22′50″ N, 121°23′32″ E	2600–2750	A(1), C(5), I(2), J(1)	A(6), E(26)
7. Chingshuishan	24°14′10″ N, 121°38′54″ E	1600–2400	C(7)	A(18), B(6)
8. Kuanglu	24°12′00″ N, 121°20′26″ E	2200–2300	C(3)	A(3), B(3)
9. Yenhai	24°10′10″N, 121°30′39″ E	1100–1200	G(4)	B(6)
10. Shihmenshan	24°09′15″ N, 121°16′34″ E	2950–3200	A(4), C(3)	A(1), D(4), I(13),
11. Chilaishan North	24°07′12″ N, 121°19′31″ E	3550–3600	C(6)	D(10), I(4), N(6)
12. Chilaishan	24°05′47″ N, 121°18′39″ E	3350–3550	A(6)	I(2), N(14)
13. Tarokotashan	24°04′48″ N, 121°25′22″ E	3000–3050	A(1), C(1), F(1)	N(6)
14. Nengkaoshan	24°03′02″ N, 121°13′53″ E	2300–2400	A(4), B(1), C(1)	I(9), N(5)
15. Shangtungpu	23°29′30″ N, 120°52′44″ E	2500–2700	C(3), D(2)	L(4), H(2), I(2), K(12)
16. Kuhanoshan	23°15′47″ N, 120°53′36″ E	2950–3100	A(3), E(2)	J(6), K(4)
17. Yakou	23°15′36″ N, 120°58′06″ E	2400–2500	C(3)	L(10)
18. Kuanshan	23°14′24″ N, 120°54′27″ E	3200–3700	A(4)	J(10), M(2)

*Numbers in parenthesis indicate the number of haplotypes.
†Numbers in parenthesis indicate the gamete number of haplotypes.

AY596817). Construction of the haplotypes of nrDNA and estimation of the genetic genealogy were performed using PAUP 4.10 (Swofford, 2000). Nucleotide diversity, haplotype diversity, tests of neutrality, and determination of their associated significance were performed using the DnaSP program (Rozas & Rozas, 2000). Measures of genetic diversity in relation to population differentiation followed Pons & Petit (1996).

Analyses of the population substructure

To test whether there is a geographical variation in the genetic structure in *Euphrasia* in Taiwan, the exact contingency test as implemented in GeoDis 2.0 (Posada *et al.*, 2000) was employed. A tree showing similarities among local populations was constructed using PAUP 4.10 (Swofford, 2000) with all characteristics being set in order for the maximum parsimony criterion. The data matrix used to construct the tree was composed of local populations as the operational unit, the haplotype and its lineage as the characters, and their frequencies as the character states. Frequencies were separated into 11 classes that represented 0%, ≤ 10%, ≤ 20%, …, ≤ 100%, respectively. The neighbour-joining trees based on the criterion of distance were then generated with character states being set in order.

RESULTS

Sequence analysis

For cpDNA, 903 nucleotide base pairs of the *trn*L intron and the *trn*L–*trn*F spacer (GenBank accession nos. AY512681–AY512778) were sequenced. Among the sequences, nine substitutions were detected (Table 2). Indels (insertion-deletion) were also detected at position 257 for polyT, and at positions 505–521 for replicates of 5′-CCCCCAAGAACC-TATTT-3′ in several individuals. These indels were excluded from the polymorphic sites to avoid possible homoplasy. For nrDNA, 735 nucleotide base pairs were found for the intertranscribed spacer between 18S and 26S (Wu & Huang, 2004). These included ITS1, 5.8S, and ITS2. Among sequences of the *E. transmorrisonensis* species complex, 28 substitutions were detected, and no indels were observed for nrDNA. *Euphrasia petiolaris* and *E. hirtella* were detected as haplotypes X and Y, respectively (accession nos. AY831431 and AY831432 for cpDNA, and AY596814 and AY596817 for nrITS, respectively).

Haplotypes and their distribution

For cpDNA, 10 haplotypes were detected (Table 2). Relationships among these haplotypes are shown in Fig. 2. Haplotypes A and C were widely distributed in high montane regions of Taiwan. Type A was considered more ancestral as is implied by the outgroup. The other haplotypes were each restricted to a single population only. For example, type B, an intermediate between types A and C, was restricted to population 14; types D–F, derived from type A, were restricted to populations 13, 15, and 16, respectively, and were spread out over the central to southern Central Mountain Range (CMR). The remainder, types G–J, were all derived from type C, and were spread across the northern CMR. Types G and H were restricted to populations 9 and 2, respectively, while types I and J were restricted to population 6 (Table 2).

Table 2 Haplotypes of *Euphrasia* in Taiwan according to variations in the sequence of chloroplast DNA including the *trn*L intron and the *trn*L–*trn*F spacer

	Polymorphic sites*										
	2	3	4	5	5	5	6	7	8		
	6	4	6	3	4	9	2	7	5		
Haplotype	5	1	5	4	6	0	5	6	8	*n*†	Populations with such a haplotype‡
A	C	T	T	A	T	A	T	G	C	37	1, 3, 5, 6, 10, 12, 13, 14, 16, 18
B	C	T	T	A	T	**G**	T	G	C	1	14
C	C	T	T	A	T	**C**	T	G	C	47	3, 4, 5, 6, 7, 8, 10, 11, 13, 14, 15, 17
D	C	T	T	A	T	A	**G**	G	C	2	15
E	C	T	T	A	T	A	T	**A**	C	2	16
F	C	T	T	A	**C**	A	T	G	C	1	13
G	**A**	T	T	A	T	**C**	T	G	C	4	9
H	C	T	T	**C**	T	**C**	T	G	C	4	2
I	C	**C**	**G**	A	T	**C**	T	G	C	2	6
J	C	T	T	A	T	**C**	T	G	**G**	1	6

*Polymorphic sites are numbered by beginning at the primer for the *trn*L intron. The nucleotide variation is indicated in bold.
†Number of individuals.
‡Population numbers follow those given in Table 1.

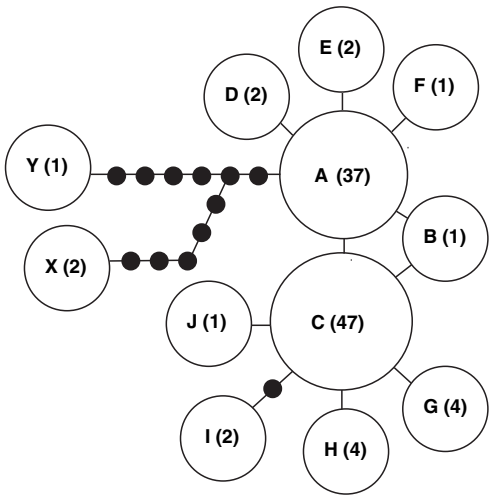

Figure 2 Relationship of haplotypes of chloroplast DNA of *Euphrasia* in Taiwan on the basis of the *trn*L intron and the *trn*L–*trn*F intergenic spacer. This gene tree was constructed using the TCS program (Templeton *et al.*, 1992). Each bar represents one substitution. Dots refer to missing haplotypes. Numbers of individuals are in parentheses. *Euphrasia petiolaris* (haplotype X) and *E. hirtella* (haplotype Y) from Georgia, Eastern Europe were used as the outgroup.

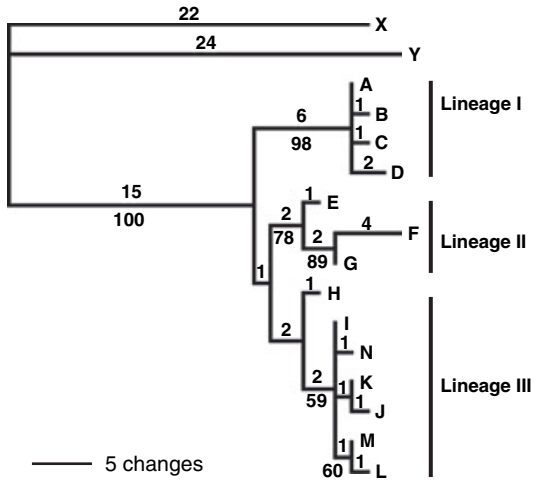

Figure 3 Rooted consensus tree, constructed by PAUP 4.10 (Swofford, 2000), from six maximum-parsimonious trees showing the relationship of haplotypes of *Euphrasia* in Taiwan on the basis of the intertranscribed spacer (ITS) of nuclear ribosomal (nr)DNA between 16S and 28S. Numbers above the branches indicate base changes. Numbers below the branches are bootstrap values. *Euphrasia petiolaris* (haplotype X) and *E. hirtella* (haplotype Y) of Eastern Europe were used as the outgroup. This figure was modified from Wu & Huang (2004).

For nrDNA, among the 127 samples, 14 haplotypes were recognized (Wu & Huang, 2004). Relationships among these types are shown in Fig. 3. Three haplotype lineages were recognized. The first lineage included haplotypes A–D, the second lineage included haplotypes E–G, and the third lineage included haplotypes H–N. Lineage I was found in the northern and north-eastern areas of the CMR and contained populations 3 and 5–11; lineage II was found in the northern part of the CMR and consisted of populations 2–4 and 6; and lineage III was found in the central and southern parts of the CMR and contained populations 10–18 (Fig. 4).

According to the frequencies of the haplotypes and their lineages, three population groups could be recognized, even though their bootstrap values were low (Fig. 4). The first group included populations 5 and 7–9, the second group included populations 2–4 and 6, and the third group included populations 10–18. These three groups are appar-

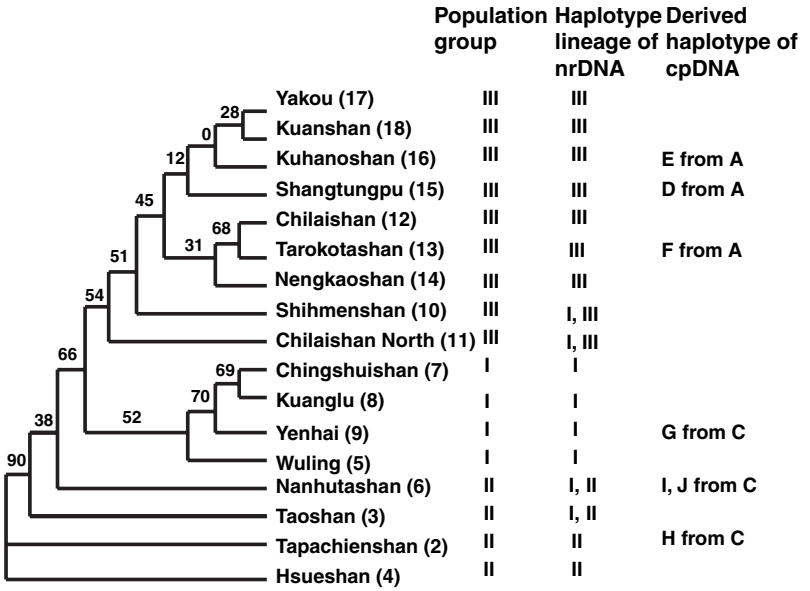

Figure 4 Grouping of local populations of *Euphrasia* in Taiwan according to the frequency of haplotypes and haplotype lineages on the basis of variations in the intertranscribed spacer (ITS) nuclear ribosomal (nr)DNA. The program PAUP 4.10 (Swofford, 2000) was used to construct this tree. Numbers on the branches are bootstrap values. Population numbers are in parentheses and follow Table 1. For derived haplotypes of cpDNA and haplotype lineages see Figs 2 and 3.

ently associated with the geographical distribution, i.e. the first is in north to north-eastern Taiwan, the second is in northern Taiwan, and the third is in central and southern areas of Taiwan.

Regarding genetic distances, the longest distance between haplotypes was five substitutions between types I and F in cpDNA (Fig. 2) and 17 substitutions between types D and F in nrDNA (Fig. 3). As the length of nucleotides is 903 in cpDNA and 735 in nrDNA, the mutation rate of nrDNA is about five times faster than that of cpDNA, which is within the range of 2–5 times estimated by synonymous mutations between nrDNA and cpDNA (Wolfe *et al.*, 1987).

Haplotype and nucleotide diversity

For cpDNA, the haplotype diversity was 0.612, and the nucleotide diversity per site (π) was 0.0009 for all populations (Table 3). Regarding local populations, half of the local populations contained only one haplotype, the remainder contained two types of haplotype except for population 6 which contained four types and populations 13 and 14 had three types (Table 1). Selection was not detected to play a role in shaping the genetic structure when tests of neutrality were performed using Tajima's D test ($\chi^2 = -1.424$, $P > 0.1$), Fu and Li's D^* test ($\chi^2 = -0.749$, $P > 0.1$) and Fu and Li's F tests ($\chi^2 = -1.170$, $P > 0.1$). Among these populations, Tarokotashan (13) had the highest haplotype diversity and nucleotide diversity, followed by Nanhutashan (6).

For nrDNA, the haplotype diversity was 0.913, and the nucleotide diversity per site (π) was 0.0112 for all populations (Table 3). Regarding local populations, five local populations of 2, 5, 9, 13, and 17 contained only one haplotype. Population 3 contained five haplotypes, followed by population 15 with four types, populations 10 and 11 with three types, and the remaining populations with two types (Table 1). When tests of neutrality were performed using Tajima's D test and Fu and Li's D^* and F tests, P values significantly deviated from the expectation according to Fu and Li's D^* and F tests (Table 3) indicating balanced selection. Among multiple tests of neutrality, at least one of these tests was significant in populations 3, 4, 6, 11, and 18 (Table 3), which mainly contained lineages II and III. Among the investigated populations, Taoshan (3) had the highest haplotype diversity and Chilaishan North (11) had the highest nucleotide diversity followed by Shihmenshan (10).

Population structure

The spatial-genetic pattern significantly deviated from random according to the exact contingency test for both cpDNA ($\chi^2 = 70.45$, $P < 0.0001$, for the total cladogram) and nrDNA ($\chi^2 = 401.85$, $P < 0.0001$, for the total cladogram). Population differentiation values were 0.529 and 0.547 for G_{st} and N_{st}, respectively, for cpDNA, while population differentiation values were 0.629 and 0.784 for G_{st} and N_{st}, respectively, for nrDNA. In cpDNA, the difference between G_{st} and N_{st} was insignificant, while in nrDNA, N_{st} was significantly larger than G_{st} (Table 4).

DISCUSSION

Congruent phylogenetic relationships among nrDNA lineages, cpDNA lineages, and population groups of the *E. transmorrisonensis* complex in Taiwan

The cpDNA haplotypes of *Euphrasia* in Taiwan could be separated into two main lineages: haplotype A and its derivatives, and haplotype C and its derivatives. However, both lineages contained admixtures of populations from the north and south, suggesting that these two lineages were widely distributed at least during the most recent glaciations. In contrast, the nrDNA haplotypes of *Euphrasia* could be separated into three lineages (Fig. 3). On the basis of the

Table 3 Haplotype and nucleotide diversity and neutrality tests of populations of *Euphrasia* in Taiwan on the basis of 903 base pairs of chloroplast (cp)DNA including the *trn*L intron and the *trn*L–*trn*F spacer, and 735 base pairs of the intertranscribed spacer (ITS) of nuclear ribosomal (nr)DNA between 18S and 26S (Wu & Huang, 2004)

	cpDNA		ITS of nrDNA				
Local populations	Haplotype diversity (standard error)	Nucleotide diversity, π	Haplotype diversity (standard error)	Nucleotide diversity, π	Tajima's *D*	Fu & Li's *D*	Fu & Li's *F*
1. Itsershan	0.000 (0.000)	0.0000	–	–	–	–	–
2. Tapachienshan	0.571 (0.119)	0.0006	0.000 (0.000)	0.0000	–	–	–
3. Taoshan	0.600 (0.030)	0.0006	0.864 (0.055)	0.0014	1.679	1.536**	.803**
4. Hsueshan	0.000 (0.000)	0.0000	0.519 (0.038)	0.0028	2.509*	1.095	1.722*
5. Wuling	0.500 (0.070)	0.0005	0.000 (0.000)	0.0000	–	–	–
6. Nanhutashan	0.733 (0.014)	0.0014	0.315 (0.087)	0.0047	0.846	1.439 *	1.470
7. Chingshuishan	0.000 (0.000)	0.0000	0.391 (0.091)	0.0005	0.776	0.622	0.762
8. Kuanglu	0.000 (0.000)	0.0000	0.600 (0.129)	0.0008	1.445	1.052	1.157
9. Yenhai	0.000 (0.000)	0.0000	0.000 (0.000)	0.0000	–	–	–
10. Shihmenshan	0.571 (0.014)	0.0006	0.549 (0.127)	0.0079	1.283	1.185	1.403
11. Chilaishan North	0.000 (0.000)	0.0000	0.653 (0.065)	0.0106	3.126***	1.518**	2.306*
12. Chilaishan	0.000 (0.000)	0.0000	0.233 (0.126)	0.0003	−0.448	0.688	0.450
13. Tarokotashan	1.000 (0.074)	0.0015	0.000 (0.000)	0.0000	–	–	–
14. Nengkaoshan	0.600 (0.046)	0.0006	0.495 (0.088)	0.0006	1.212	0.715	0.952
15. Shangtungpu	0.600 (0.030)	0.0013	0.611 (0.103)	0.0026	−0.060	1.299	1.057
16. Kuhanoshan	0.600 (0.030)	0.0006	0.533 (0.095)	0.0007	1302	0.804	1.026
17. Yakou	0.000 (0.000)	0.0000	0.000 (0.000)	0.0000	–	–	–
18. Kuanshan	0.000 (0.000)	0.0000	0.545 (0.062)	0.0022	2.123*	1.104	1.538
Total	0.612 (0.031)	0.0009	0.913 (0.005)	0.0112	2.038	1.992**	2.425**

*$P < 0.05$; **$P < 0.02$; ***$P < 0.001$.

Table 4 Measures of the genetic diversity of *Euphrasia* in Taiwan on the basis of sequences of chloroplast (cp)DNA and nuclear ribosomal (nr)DNA with standard errors in parentheses. In the analysis of H_s, H_t, and G_{st}, each haplotype was treated as a separate allele at a single haploid locus. In the analysis of V_s, V_t, and N_{st}, in addition to the number of alleles, sequence distances among haplotypes were also included in the calculation

Diversity parameter	Values of cpDNA	Values of nrDNA
H_s*	0.319 (0.080)	0.351 (0.069)
H_t†	0.676 (0.050)	0.945 (0.011)
G_{st}‡	0.529 (0.116)	0.629 (0.069)
V_s*	0.307 (0.085)	0.206 (0.079)
V_t†	0.677 (0.094)	0.953 (0.052)
N_{st}‡	0.547 (0.115)	0.784 (0.080)
N_{st}–G_{st}	0.018 ($P > 0.1$)	2.740 ($P < 0.01$)

*H_s and V_s, intrapopulation diversity.
†H_t and V_t, total diversity.
‡G_{st} and N_{st}, degree of substructure.

frequencies of the haplotypes and their lineages, three population groups were also recognized (Fig. 4). Relationships among the nrDNA haplotype lineages and population groups, therefore, agree well. Populations 3 and 6 contained both lineages I and II, which are situated in areas where lineages I and II overlap, populations 10 and 11 contained both lineages I and III and were localized in areas where lineages I and III overlap, while each of the other populations belonged to one lineage only. Haplotypes of A derived from cpDNA, i.e. D, E, and F were located in population group III and lineage III of nrDNA, whereas the haplotypes of C derived from cpDNA, i.e. H, I, and J were located mainly in population group II and lineage II of nrDNA, and type G was located in the first group. Thus, the geographical distributions of lineages of cpDNA coincided with those of lineages of nrDNA. In comparison with other populations, the extant populations of lineage I are mainly distributed below 2400 m. For example, Wuling (5) is at 2000–2200 m, Chingshuishan (7) is at 1600–2400 m, Kuanglu (8) is at 2200–2300 m, and Yenhai (9) is at 1100–1200 m.

Delineating boundaries between species is beyond the range of this discussion; however, in the light of the molecular data presented here, the species boundary cannot be upheld. For example, in terms of differentiation between varieties *transmorrisonensis* and *durietziana*, both share the same haplotypes of nrITS and cpDNA. Thus these markers cannot be used to differentiate these two varieties even though slight morphological variations exist.

Genetic diversity and population differentiation between cpDNA and nrDNA

Regarding population differentiation in nrDNA, N_{st} was significantly larger than G_{st} (Table 4) implying that the genetic

distance between haplotypes within local populations was much less than that among local populations (Pons & Petit, 1996). This indicates that the recently evolved nrDNA haplotypes are restricted to local populations. In contrast, the difference between G_{st} and N_{st} in cpDNA was insignificant, suggesting that the genetic distance between haplotypes within local populations is more or less equal among local populations. This may be due to the wide distribution of the common types A and C and/or there being too few of the derived types because of the slower mutation rate in cpDNA. This implication explains that haplotypes of nrDNA were recently geographically differentiated and the admixture of haplotypes has not proceeded to the extent that was observed in cpDNA.

The most-significant discrepancy probably comes from differences in the highest haplotype diversity and nucleotide diversity among populations of cpDNA and nrDNA results. The Tarokotashan (13) population had the highest nucleotide diversity of cpDNA among populations, followed by the populations at Nanhutashan (6) and Shangtungpu (15). However, Chilaishan North (11) and Shihmenshan (10) had the highest values of nrDNA among all populations. Such a discrepancy was also observed in the argon tree (El Mousadik & Petit, 1996a,b) and in *Trochodendron aralioides* (Huang *et al.*, 2004). The reason for disagreement between cpDNA and nrDNA data remains unclear, but it may be related to male-driven evolution. Pollen may bring in genetic diversity to increase nrDNA diversity in the zygote. Pollen tends to carry a greater number of mutations that can lead to gender-specific mutation rates (Whittle & Johnston, 2002). Pollen is also more susceptible to damage-induced mutations because it is released from the parent plant upon maturity and is therefore exposed to UV radiation, chemical mutagens, and dehydration. These induced deleterious mutations are transmitted to the progeny by pollen (Whittle & Johnston, 2003) and might reduce the genetic diversity of the population.

Possible evolutionary history of *Euphrasia* in Taiwan

The nrDNA haplotype lineages of Taoshan-Nanhutashan-Hsueshan (lineage II) and Nengkaoshan-Kuanshan (lineage III) are disjunctive in distribution. An obvious potential explanation of the genotype structure is a recent or ancient population subdivision of the *Euphrasia* species complex because these two lineages (groups II and III) show evidence of significant differentiation. Another potential explanation for the observed polymorphism is that little or no gene flow has occurred between the two lineages. The populations of group I have a much lower elevational distribution than the adjacent populations in groups II and III (Fig. 1 and Table 1). The significant results of a neutrality test (Table 3) show that the observed haplotype structure is highly improbable by chance alone under a neutral model. The force according to the neutrality test must be due to balancing selection. A genealogy of this structure is also called over-dominant selection, which tends to maintain population subdivisions or to preserve alleles for longer than expected under genetic drift (Page & Holmes, 1998). Given that selfing is common in *Euphrasia*, the effect of the mating system on ITS variations among populations may have influenced the haplotype patterns. Samples of clonal populations of one lineage differing from those of another lineage may lead to a pattern similar to balancing selection. However, the polymorphism in nrDNA found in *Euphrasia* was not due to sampling of clonal populations of two lineages.

Balanced polymorphism refers to mutations being maintained in populations by natural selection through heterozygote advantages, frequency-dependent selection, or spatial–temporal selection of alternative alleles (Tian *et al.*, 2002). There are no known cases of a heterozygote advantage in the non-coding sequence. Herein we were unable to find a target for balancing selection because ITS1 and ITS2 are non-coding sequences. Balancing selection can be checked by using gene sequences coding for a protein as was shown in *Leavenworthis* (Filatov & Charlesworth, 1999). In conclusion, population subdivision and/or balancing selection working together may be acting to maintain the haplotype structure.

In cpDNA of *Euphrasia*, haplotype A differs from haplotype C by a substitution at position 590 (Table 2). Compared with the sequences of *Melampyrum lineare* (GenBank accession no. AF48608) and *Pedicularis chamissonis* (GenBank accession no. D88051), which belong to the same tribe Rhinantheae as does *Euphrasia*, haplotype A is considered to be ancestral as its nucleotides at position 590 were shown to be of haplotype A. Of course, this primitiveness of haplotype A was also evident with the two outgroup species of *Euphrasia petiolaris* and *E. hirtella*. Although haplotype A is considered more ancestral than haplotype C, there was no sign showing that haplotype C in Taiwan was derived directly from haplotype A, as these two haplotypes show similar distribution (Table 2) and mutation patterns (Fig. 2). Rather, they could have coexisted in Taiwan for a long time and they may have evolved independently during climate fluctuations. It is surprising to find that derived cpDNA haplotypes such as haplotypes D, E, and F (derived from A) and nrDNA haplotype lineage III both evolved in the same geographical region, while derived cpDNA haplotypes H, I, and J (derived from C) and nrDNA haplotype lineage II both developed in the same region (Fig. 4).

The scenario of the evolutionary history of the *E. transmorrisonensis* complex in Taiwan could be as follows. The data suggest at least three origins of the *E. transmorrisonensis* complex in Taiwan, corresponding to each nuclear lineage in the northern (II), northern/north-eastern (I), and central/southern regions (III) with subsequent hybridization between lineages I and II (populations 3 and 6) and lineages II and III (populations 10 and 11). Alternate hypotheses suggest that lineage I of nrDNA was the first colonizing area of *Euphrasia*. Once the ancestors of *Euphrasia* colonized Taiwan, they contained both cpDNA haplotypes A and C (Fig. 1). They then gradually spread throughout the alpine regions of the island. Eventually, two major areas were colonized, i.e. the Taoshan-Nanhutashan-Hsueshan area (group II of Fig. 1), and

the Nengkaoshan–Kuanshan area (group III). In these two regions, derived cpDNA and nrDNA mutations accumulated and developed into the present status. The origin of chloroplasts that are exclusive to each lineage supports the conclusion that each lineage has had long-term isolation from the other, and does not appear to be the result of more recent migrations from the northern/north-eastern region. Also, because lineages II and III are related relatively closely to lineage I (Fig. 3), the first hypothesis' interpretation of the origin of *Euphrasia* in Taiwan is more favourable.

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