

## Confirmation of a natural hybrid species in *Petrocosmea* (Gesneriaceae) based on molecular and morphological evidence

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**Abstract** A natural hybrid species in *Petrocosmea* named *Longianthera* in Yanshan County, Yunnan Province is confirmed for the first time based on molecular and morphological evidence. The character count procedure of the variable characters show that *Longianthera* populations are characteristic of the intermediate morphological traits between its putative parents *Yanshan* and *Petrocosmea martinii*. The nuclear ribosomal internal transcribed spacer region and three chloroplast regions of *matK*, *trnL-F*, and *trnT-L* are sequenced in the putative hybrid and the related species. Both alignment of DNA sequences and the phylogenetic trees could exclude all the other species in *Petrocosmea* as the parental species except for *Yanshan* and *P. martinii*. Eight haplotypes in the 31 internal transcribed spacer sequences and six haplotypes in 42 cpDNA sequences were found from 14 individuals of *Longianthera* populations. The analyses of DNA sequences, haplotypes, and phylogenetic trees indicate that *Longianthera* is likely a hybrid species between its putative parents *Yanshan* and *P. martinii*, in which *Yanshan* might be the most possible maternal parent. Several factors may contribute to the natural hybridization between these two parental species in *Petrocosmea*, such as the overlapped geographic distribution, habitats, flowering periods, and shared pollinators. Finally, the new species of *Yanshan* and the natural hybrid species of *Longianthera* are described.

**Key words** chloroplast DNA, Gesneriaceae, ITS, morphology, natural hybridization, *Petrocosmea*.

Hybridization, occurring frequently in plants and animals, is a natural phenomenon (Harrison, 1993). Natural hybridization is omnipresent in flowering plants and plays a significant role in plant evolution and diversification (Arnold, 1997). In Gesneriaceae, several interspecific and intergeneric natural hybridizations have been reported (Byrne & Morley, 1976; Wagner et al., 1990; Wiehler, 1994; Smith et al., 1996).

*Petrocosmea* Oliv. comprises 27 species and 4 varieties mostly distributed in China (Wang, 1985, 1990, 1998; Burt, 1998, 2001; Li & Wang, 2004). The plants of *Petrocosmea* are perennial and acaulescent herbs, terrestrial or epipetric, and usually rhizomatous with cymose inflorescences and zygomorphic flowers (Wang, 1985, 1990, 1998; Li & Wang, 2004). Most species of *Petrocosmea* are distributed in the Yunnan plateau where there are the most diverse variants of *Petrocosmea* (Wang, 1985). This region is well known for its high level of endemism and species diversity in plant and animal groups originated through the climatic changes and continuous uplift of the Himalayas in the Eocene (Wu, 1980; Wu & Zhu, 1987). In the field investiga-

tion of genus *Petrocosmea*, two possibly new species were found in Yanshan County, Yunnan Province. One was the two *Longianthera* populations and the other was the *Yanshan* population. However, detailed observations led us to find that a series of morphological characters in flowers and leaves of *Longianthera* show the status of intermediate between *Yanshan* and *P. martinii*, which is also distributed in Yanshan County. Therefore, we hypothesize that *Longianthera* populations might be a hybrid of the putative parents *Yanshan* and *Petrocosmea martinii*.

A number of methods have been used to investigate putative hybrids based on the morphological data (Heiser, 1949; Wagner et al., 1990). One of the most widely used approaches is the “character count procedure” proposed by Wilson (1992). Character count procedure is a successful approach used to examine hybridity (Wilson, 1992; Albaladejo et al., 2004; Tovar-Sanchez & Oyama, 2004). In this study, we used the character count procedure to examine the possible hybrid and the putative parents and to test whether the observed morphological intermediacy in *Longianthera* populations is due to natural hybridization or not by comparison with the assigned putative parental groups.

During recent years, single- or low-copy nuclear genes have been successfully used to identify hybrids in plants (Sang & Zhang, 1999; Ferguson & Sang, 2001;

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Gaskin & Schaal, 2002; Pan et al., 2007; Qiu et al., 2008). In addition, chloroplast DNA maternally transmitted in angiosperms (Morgensen, 1996) is usually used to determine the maternal parent of hybrids (e.g., Ferris et al., 1997; Moody & Les, 2002). In the present study, the nuclear ribosomal DNA internal transcribed spacer (ITS) sequences from *Yanshan* and *P. martinii* and the putative hybrid *Longianthera* were sequenced to determine the hybrid status of *Longianthera*, a morphologically intermediate taxon. The maternally inherited chloroplast *matK* gene and two other intergenic spacers of chloroplast DNA (*trnL-F* and *trnT-L*) were further sequenced to determine the direction of hybridization when its hybrid status was confirmed. These multiple experimental approaches have been used to find and test putative hybrid and parents, which would help to improve our understanding of natural hybridization as well as the origin and evolution of some key morphological traits in Gesneriaceae.

## 1 Material and methods

### 1.1 Plant materials

Fourteen accessions from two populations of *Longianthera*, five accessions from the only population of *Yanshan*, and one accession from the only population of *P. martinii* were sampled from Yanshan County, Yunnan Province. Five accessions from the only population of *Petrocosmea sericea*, three species (*P. nervosa*, *P. oblata*, and *P. sinensis*) from sect. *Petrocosmea*, three species (*P. grandifolia*, *P. kerrii*, and *P. menglianensis*) from sect. *Deinanthera* and two other species and one variety (*P. minor*, *P. iodoides*, and *P. martinii* var. *leiantra*) from sect. *Anisochilus* were sampled as the ingroup of the phylogenetic analysis. We chose *Raphiocarpus begoniifolius* and *Raphiocarpus petelotii* as outgroups for the analyses of molecular datasets. The voucher information of all sampled taxa and GenBank accession numbers are listed in Appendix 1.

### 1.2 Morphological data collection

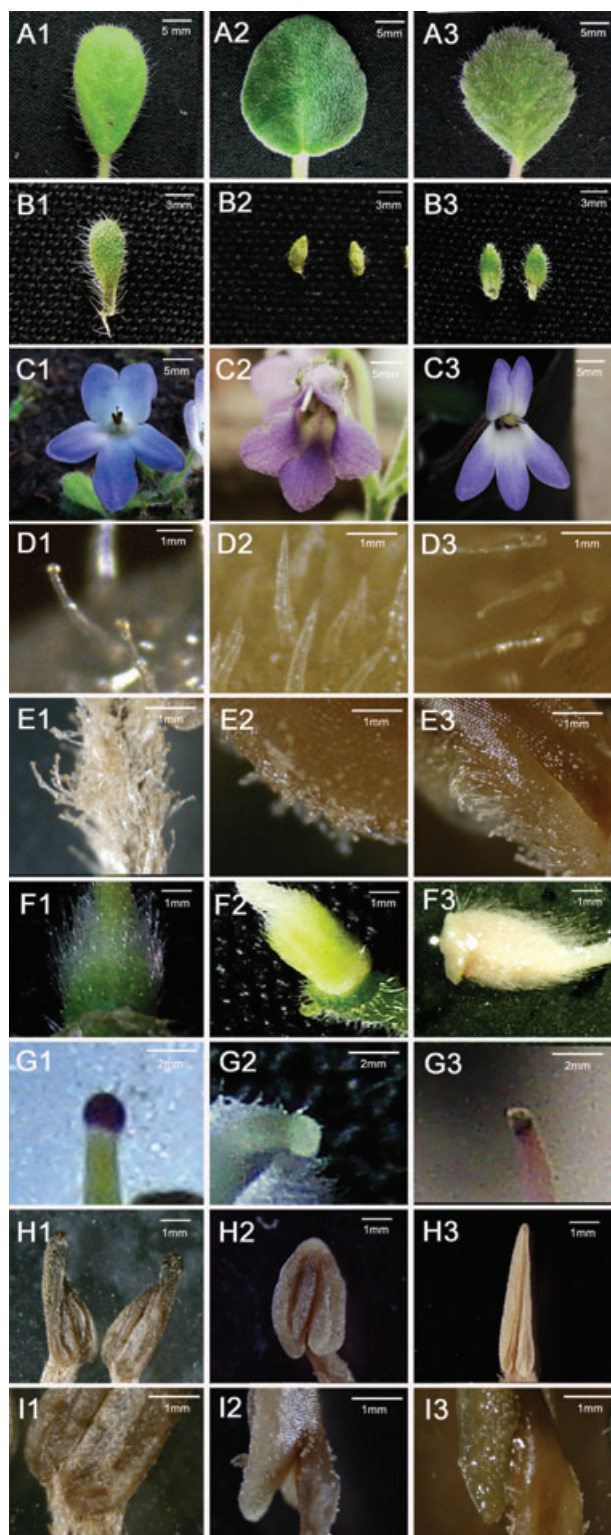
In total, 20 individual plants of *Longianthera*, 15 individual plants of *Yanshan*, and 15 individual plants of *P. martinii* collected in Yanshan County were identified based mainly on leaf (shape, margin, hairs), bract (length, shape), corolla (length ratio of upper to lower lips, hairs, inflation at the bottom of the tube), anthers (length, shape, hairs), and pistil (ovary shape, stigma color).

In this study, nine quantitative and 17 qualitative variables were measured for every specimen (Table 1, Fig. 1). Values for quantitative variables were the aver-

**Table 1** Set of quantitative and qualitative variables assessed for morphometric analyses

Variable	Description
1. LHL	Length of hairs on the abaxial surface of leaves (mm)
2. BL	Bract length (mm)
3. LR/LL	Length ratio of upper / lower corolla lip
4. FL	Filament length (mm)
5. CTL	Corolla tube length (mm)
6. AL	Anther length (mm)
7. LHF	Length of hairs on the filament (mm)
8. LHA	Length of hairs on the back side of anther (mm)
9. LHC	Length of hairs on the abaxial surface of corolla (mm)
10. LS	Leaf shape; ovate (0), elliptical (1), obovate (2)
11. LM	Leaf margin; shallowly crenate (0), repand (1), entire (2)
12. BS	Bract shape; linear (0), lanceolate (1), broadly lanceolate (2)
13. BLP	Bract location on pedicel; below middle (0), middle (1), upper middle (2)
14. WSO	The way the style arises out of the ovary; gradually (0), slightly suddenly (1), suddenly (2)
15. OS	Ovary shape; ellipsoidal (0), ovoid (1), spheroidal (2)
16. FC	Filament curvature; no curve (0), curved a little (1), geniculation (2)
17. HDF	Hair density on the filament; thin (0), slightly thick (1), thick (2)
18. HDA	Hair density on the back side of anthers; thin (0), slightly thick (1), thick (2)
19. HDC	Hair density on the abaxial surface of corolla; thin (0), slightly thick (1), thick (2)
20. AA	Anther apex; convergent (0), split slightly (1), split (2)
21. AF	Anther attached on the filament; dorsifixed (0), slight dorsifixed (1), basifixed (2)
22. SC	Stigma color; white (0), pink (1), purple (2)
23. CTB	Shape of corolla tube bottom; not inflated (0), slightly inflated (1), inflated (2)
24. AS	Anther shape; ovate (0), rostriform (1), long-rostriform (2)
25. SUC	Shape of the upper corolla lip; porrect and carinate (0), ascendent and patulous (1)
26. DTA	Dehiscence type of the anther; longitudinally dehiscent (0), poricidal (1)

ages of three measurements. Both vegetative and floral characters were scored in the qualitative variables. In the quantitative characters, we observed the length of hairs on the abaxial surface of leaf (LHL), on the filament (LHF), on the backside of anther (LHA), and on the abaxial surface of corolla (LHC). The bract length (BL), filament length (FL), corolla tube length (CTL), anther length (AL), and the length ratio of upper:lower corolla lips (LR/LL) were also investigated. In the qualitative characters, four of them were vegetative, leaf shape (LS), leaf margin (LM), bract shape (BS), and the bract location on pedicel (BLP). The remaining 13 characters were the most important floral traits. Three of them were about the corolla, including the hair density on the abaxial surface of corolla (HDC), the corolla tube bottom inflation (CTB), and the shape of the upper corolla lip (SUC). Six of them were about the stamen, including the filament curvature (FC), the hair density on the filament (HDF), the hair density on the backside of



**Fig. 1.** Photographs of the major morphological characters in *Yanshan*, *Petrocosmea martinii* and the hybrid *Longianthera*. **1**, *Yanshan*; **2**, *P. martinii*; **3**, *Longianthera*. **A**, Leaf. **B**, Bract. **C**, Corolla. **D**, Hairs on abaxial surface of corolla. **E**, Hairs on filament. **F**, Ovary. **G**, Stigma color. **H**, Anther. **I**, Anther attached to filament.

anther (HDA), the anther apex (AA), the way the anther is attached on the filament (AF), the anther shape (AS), and the dehiscence type of the anther (DTA). Three of them were about the pistil, including the way the style arises out of the ovary (WSO), the ovary shape (OS), and the stigma color (SC).

### 1.3 DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted from silica-gel dried and fresh leaf materials using the CTAB method of Rogers & Bendich (1988) and used as the templates in the polymerase chain reaction (PCR). The primers used for amplification were ITS1 and ITS4 (White et al., 1990) for the nuclear ribosomal ITS region, c and f (Taberlet et al., 1991) for the chloroplast intergenic region *trnL-F*, a and b (Taberlet et al., 1991) for chloroplast *trnT-L*, and matK-AF and trnK-2R (Johnson & Soltis, 1994; Ooi et al., 1995) for chloroplast *matK* gene. Polymerase chain reaction was carried out under the following conditions: 94 °C for 4 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; and a nal extension at 72 °C for 10 min. The PCR products were purified using Tian Quick Midi Purification Kit (Tiangen Biotech, Beijing, China) following the manufacturer's protocol and were directly sequenced. The sequencing primers were the same as for amplification primers except the *matK* region. A reverse primer matK-8R (Ooi et al., 1995) was added in the *matK* region sequencing. Direct sequencing was successful except the ITS regions of several individuals of *Longianthera*, which produced chimeric or unreadable peaks in the chromatogram. For these individuals, cloning and sequencing was carried out subsequently using PGEM-T Easy Vector (Promega, Madison, WI, USA) following the manufacturer's instructions. About eight clones were sequenced from each polymorphic accession using the universal T7 and SP6 primers.

### 1.4 DNA sequence alignment and phylogenetic analysis

Sequences were aligned using ClustalX 1.83 (Thompson et al., 1997) and adjusted manually using BioEdit 5.0.9 (Hall, 1999). All the DNA data were analyzed with maximum parsimony (MP) and Bayesian inference methods, which were implemented in PAUP\* 4.0b10 (Swofford, 2003) and MrBayes version 3.0b4 (Ronquist & Huelsenbeck, 2003), respectively.

For MP analysis, all characters were given equal weights and character states were unordered. Heuristic searches were carried out with 1000 replicates of random addition, one tree held at each step during

stepwise addition, tree bisection reconnection branch swapping, Multrees in effect, and steepest descent off. Bootstrap support (Felsenstein, 1985) for each clade was estimated from 1000 heuristic search replicates as described above.

In Bayesian inference analysis, Modeltest 3.06 (Posada & Crandall, 1998) was used to select an appropriate model of sequence evolution for each DNA dataset from a comparison of 56 models. Four chains of the Markov Chain Monte Carlo were run each for 10 000 000 generations and were sampled every 10 000 generations. For each run, the first 200 samples were discarded as burn-in to sure that the chains reached stationary. In the majority rule consensus from Bayesian analysis, posterior probability (PP) was used to estimate robustness.

## 2 Results

### 2.1 Morphological analysis for hybrid and putative parental plants

The results of the character count procedure are shown in Table 2. As expected, *Longianthera* populations presented a series of intermediate morphological traits between *Yanshan* and *P. martinii*. Twenty-two in-

termediate characters including both quantitative and qualitative traits were found out of 26 examined characters in the sampled materials from Yanshan County (Table 2). They are the LHL, BL, LR/LL, FL, CTL, LHF, LHC, LS, LM, BS, BLP, WSO, OS, FC, HDF, HDA, HDC, AA, AF, SC, CTB, and AS. Two characters (AL, LHA), in *Longianthera* were different from those of both putative parental species, but were not intermediate between them. Two other characters (SUC, DTA) in *Longianthera* were very similar to those of one putative parental species *Yanshan* but were remarkably different from the other putative parental species *P. martinii* (Table 1, Fig. 1).

The plants of *Longianthera* can flower normally in the wild. Fruits and seeds of *Longianthera* were not found in the field when we investigated the hybrid. However, seedlings and young plants were frequently observed in the field, which indicated that *Longianthera* can normally fruit with fertile seeds. In order to investigate the morphology and viability of the hybrid pollen, we observed the pollen under the microscope and tested the pollen vitality of *Longianthera* using the hanging drop method (Khatum & Flowers, 1995). The pollens of *Longianthera* are nearly round or blunt triangle, like the morphology of the pollen of the other species in *Petrocosmea*, under the optical microscope. In the

**Table 2** Character count procedure from four populations of *Longianthera*, *Yanshan*, and *Petrocosmea martinii*

Character	<i>Yanshan</i> (mean ± SD)	<i>Longianthera</i> (mean ± SD)	<i>P. martinii</i> (mean ± SD)	<i>Longianthera</i> is different from	<i>Longianthera</i> is intermediate ?
1.	3.35 ± 0.31	2.22 ± 0.45	0.98 ± 0.24	Both	+
2.	8.62 ± 1.60	4.45 ± 1.05	2.80 ± 0.95	Both	+
3.	0.71 ± 0.07	0.55 ± 0.05	0.47 ± 0.06	Both	+
4.	1.50 ± 0.11	2.20 ± 0.13	3.21 ± 0.10	Both	+
5.	5.20 ± 0.21	3.81 ± 0.20	2.70 ± 0.18	Both	+
6.	3.21 ± 0.20	7.52 ± 0.42	2.90 ± 0.19	Both	-
7.	1.72 ± 0.10	0.90 ± 0.06	0.50 ± 0.04	Both	+
8.	0.12 ± 0.01	0.70 ± 0.03	0.15 ± 0.02	Both	-
9.	2.52 ± 0.15	1.81 ± 0.12	1.20 ± 0.10	Both	+
10.	2	1	0	Both	+
11.	2	1	0	Both	+
12.	2	1	0	Both	+
13.	2	1	0	Both	+
14.	2	1	0	Both	+
15.	2	1	0	Both	+
16.	0	1	2	Both	+
17.	2	1	0	Both	+
18.	2	1	0	Both	+
19.	2	1	0	Both	+
20.	2	1	0	Both	+
21.	2	1	0	Both	+
22.	2	1	0	Both	+
23.	0	1	2	Both	+
24.	1	2	0	Both	+
25.	1	1	0	<i>P. martinii</i>	-
26.	1	1	0	<i>P. martinii</i>	-
Scores of intermediate characters					22: 4

+, Hybrid is intermediate between the parental species; -, hybrid is not intermediate; SD, standard deviation.

experiment of pollen germination, almost all of the pollen of *Longianthera* can germinate in triphenyltetrazolium chloride medium (data not shown).

### 2.2 Internal transcribed spacer sequence characteristics of *Longianthera* and its putative parents

We aligned and compared the ITS sequences of *Longianthera* and all the possible parental species including *P. martinii*, *Yanshan*, *P. sericea*, *P. minor*, *P. iodoides*, *P. martinii* var. *leiandra*, *P. oblata*, *P. sinensis*, and *P. nervosa* based on the phylogenetic study of *Petrocosmea* (Table 3). The result showed that only *Yanshan* and *P. martinii* had the most similar ITS sequences to haplotype Lon-I and Lon-VIII of *Longianthera*, respectively. The sequences variations of *Longianthera* and its putative parents are summarized in Fig. 2.

Thirty-seven ITS sequences were obtained from 14 individuals of *Longianthera* and six individuals of its putative parents. The total length of ITS sequences of *Longianthera* varied from 635 bp to 638 bp. The ITS1 region ranged from 230 bp to 233 bp, the 5.8 S subunit region ranged from 171 bp to 172 bp, and the ITS2 region ranged from 241 bp to 243 bp. Thirty-four variation sites, including 30 substitutions and four indels, were found in the ITS sequences of *Longianthera*. These 34 variation sites divided the ITS sequences of *Longianthera* into eight distinct haplotypes (defined as haplotypes Lon-I–VIII) (Fig. 2). Haplotype Lon-I was the main haplotype of *Longianthera*, which was found in 16 accessions. It showed 29 substitutions and one indel (from position of 110 to 112) compared with the other haplotypes. Haplotype Lon-II–VI were found in only one accession. Haplotype Lon-VII and Lon-VIII were found in seven and three accessions, respectively, which were similar to each other with only four nucleotides differences.

Both *Yanshan* and *P. martinii* had only one type of ITS sequence. Twenty-five variations including 23 substitutions and two indels were found between *Yanshan* and *P. martinii* in the ITS sequence (Fig. 2). The

ITS sequence of putative parent *Yanshan* was similar to the haplotype Lon-I of *Longianthera* with only five nucleotides differences. The other putative parent *P. martinii* had a similar ITS sequence with the haplotypes Lon-VIII of *Longianthera* with three nucleotides and two indels of differences (positions 196, 197, and 407).

### 2.3 Chloroplast sequences of *matK*, *trnL-F*, and *trnT-L* characteristics of *Longianthera* and its putative parents

The *matK* matrix comprised 14 individuals of two *Longianthera* populations, five individuals of one *Yanshan* population, and one individual of one *P. martinii* population. It had aligned sequences of 1627 bp with 21 substitutions and two indels (from position 230 to 235 and 1474) in the *matK* matrix (Fig. 3: A). There were two genotypes divided by three variations on positions 503, 863, and 1093. Individuals of Lon13 and Lon14 from *Longianthera* had T, A, and T on these three positions, whereas the other individuals of *Longianthera* had C, T, and G on these positions. The putative parents of *Yanshan* and *P. martinii* had only one *matK* genotype at 503, 863, and 1093 positions. The *trnL-F* matrix had aligned sequences of 821 bp with only five substitutions and five indels (positions from 91 to 98, 160 to 165, 185 to 191, 604 to 608, and 630 to 634) (Fig. 3: B). Lon13 had one nucleotide and five indels different from the other individuals of *Longianthera*. The putative parents *Yanshan* and *P. martinii* had only one *trnL-F* genotype. We were surprised to find that the Lon13 accession of *Longianthera* and all of the five accessions of *Yanshan* shared the same haplotype of *trnL-F* (Fig. 3: B). The *trnT-L* matrix had aligned sequences of 620 bp with nine substitutions and three indels (positions 252, from 262 to 266, and 558 to 565) (Fig. 3: C). At position 214, Lon13 and Lon14 had nucleotide A instead of the T characteristic of the other individuals of *Longianthera*, which divided the *trnT-L* sequence of *Longianthera* into two genotypes. The putative parents of *Yanshan* and

**Table 3** Similarities of internal transcribed spacer sequences between the eight haplotypes in *Longianthera* and all of its possible parents in *Petrocosmea*, expressed as percentages

Haplotypes	Yan01–05	<i>P. martinii</i>	Ser01–03	Ser04	Ser05	<i>P. minor</i>	<i>P. iodoides</i>	<i>P. martinii</i> var. <i>leiandra</i>	<i>P. oblata</i>	<i>P. sinensis</i>	<i>P. nervosa</i>
Lon-I	99.28	94.44	97.84	97.69	97.49	94.85	96.17	96.61	94.13	93.84	94.43
Lon-II	97.42	96.49	97.42	97.27	97.06	96.19	96.33	96.77	93.58	93.43	93.72
Lon-III	97.13	95.91	96.98	96.84	96.62	95.74	95.88	96.32	93.27	93.13	93.42
Lon-IV	97.70	96.49	97.27	97.13	96.92	96.04	96.18	96.62	93.43	93.28	93.72
Lon-V	97.99	95.61	97.42	97.27	97.06	95.31	96.04	96.48	93.72	93.43	93.87
Lon-VI	95.41	97.95	96.98	96.84	96.62	97.65	96.91	97.35	93.86	93.71	94.15
Lon-VII	95.70	97.95	96.84	96.70	96.47	97.80	97.06	97.50	94.01	93.86	94.15
Lon-VIII	95.70	98.10	96.84	96.70	96.47	97.80	97.06	97.50	94.01	93.86	94.15

Lon, *Longianthera*; Ser, *Petrocosmea sericea*; Yan, *Yanshan*. The numbers in accession names are sampling numbers of populations.

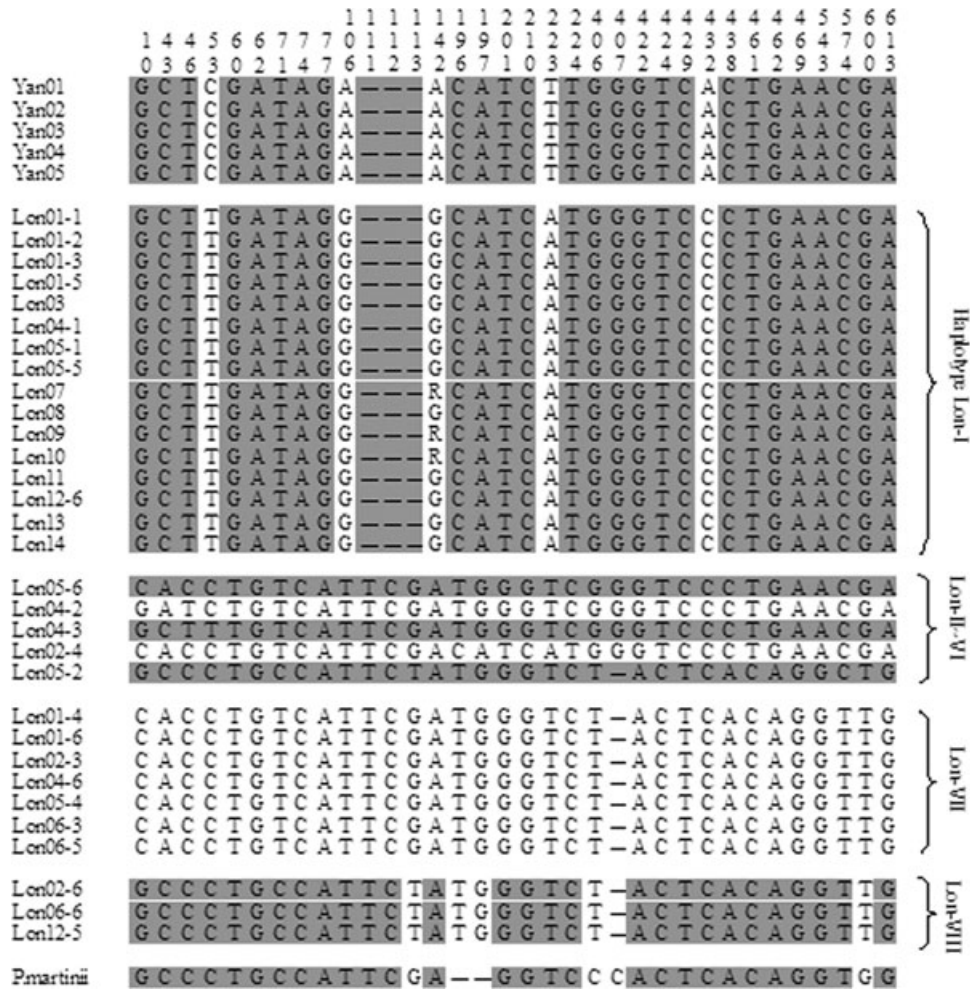


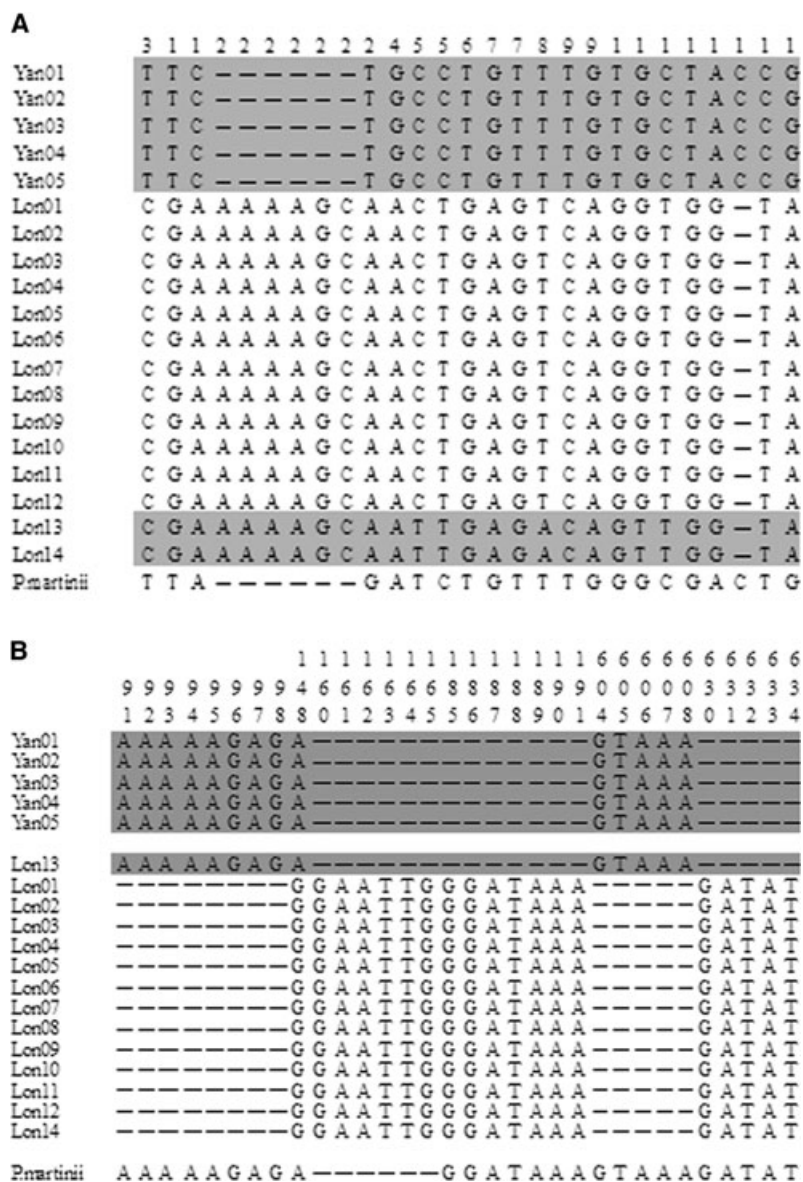
Fig. 2. Variable nucleotide sites in internal transcribed spacer sequences of *Yanshan* (Yan), *Petrocosmea martinii*, and the hybrid *Longianthera* (Lon). Numbers in front of “-” are sampling numbers of populations; numbers following “-” are the clone numbers of each accession.

*P. martinii* had only one *trnT-L* genotype. As outlined above, the putative parents of *Yanshan* and *P. martinii* had only one genotype, whereas *Longianthera* had two haplotypes in the three cpDNA sequences of *matK*, *trnL-F*, and *trnT-L*, respectively. Accession Lon13 of *Longianthera* and *Yanshan* shared the same haplotype of *trnL-F* sequence.

**2.4 Phylogenetic analysis of *Longianthera* and its putative parental species based on nuclear ribosomal ITS sequences**

The aligned ITS dataset consisted of 737 bp, of which 72 (9.77%) were parsimony informative characters. The strict consensus of 13 530 trees yielded by MP analysis (L = 203, CI = 0.892, RI = 0.963) was generally congruent with the majority rule consensus Bayesian tree in the topology (Fig. 4).

The ITS tree comprised four clades, designated A–D. Species of *P. grandifolia*, *P. kerrii*, and *P. menglianensis* from sect. *Deinanthera* formed clade A with the support of 73% bootstrap (BS) values and 97% PP. Species of *P. nervosa*, *P. oblata*, and *P. sinensis* from sect. *Petrocosmea* were grouped together in clade B with maximum support (BS = PP = 100%). Four accessions of *Longianthera* (Lon02–6, Lon05–2, Lon06–6, and Lon12–5) were gathered together with support of BS = 67% and PP = 100%, and were further sister to *P. martinii* with support of BS = 52% and PP = 100%. Together with seven other accessions of *Longianthera*, Lon01–4, Lon01–6, Lon02–3, Lon04–6, Lon05–4, Lon06–3, and Lon06–5, they formed a lineage with strong support (BS = 92%, PP = 100%). This lineage was sister to *P. minor* (BS = 94%, PP = 100%) and together were sister to *P. iodoides* and *P.*



**Fig. 3.** Variable nucleotide sites in *matK* (A), *trnL-F* (B), and *trnT-L* (C) sequences of *Yanshan* (Yan), *Petrocosmea martinii*, and the hybrid *Longianthera* (Lon). Numbers in accession names are sampling numbers of populations.

*martinii* var. *leiantra* with strong support (BS = 88%, PP = 100%) to form the clade C.

In clade C, *P. martinii* was nested in the 11 accessions of *Longianthera*, in which four of them formed a lineage and had the haplotypes Lon-II–VI, whereas the remaining seven accessions had the haplotype Lon-VII. The phylogenetic branches in clade C showed close relationships of *P. martinii* and the haplotypes Lon-VI, Lon-VII, and Lon-VIII of *Longianthera*.

Clade D was separated into four lineages, designated I–IV. Sixteen accessions of *Longianthera* formed lineage I with strong support (BS = 92%, PP = 99%).

All five accessions (Yan01, Yan02, Yan03, Yan04, and Yan05) of *Yanshan* were gathered together in lineage II with support of BS = 76% and PP = 95%, which was sister to lineage I (BS = 99%, PP = 100%). Four other accessions (Lon04–2, Lon04–3, Lon05–6, and Lon02–4) of *Longianthera* formed lineage III with support of BS = 70% and PP = 100% as the sister to lineage I + II (BS = 76%, PP = 100%). All five accessions (Ser01, Ser02, Ser03, Ser04, and Ser05) of *P. sericea* formed lineage IV with strong support of BS = 97% and PP = 100%, which was further grouped together with the above lineages to form clade D with strong

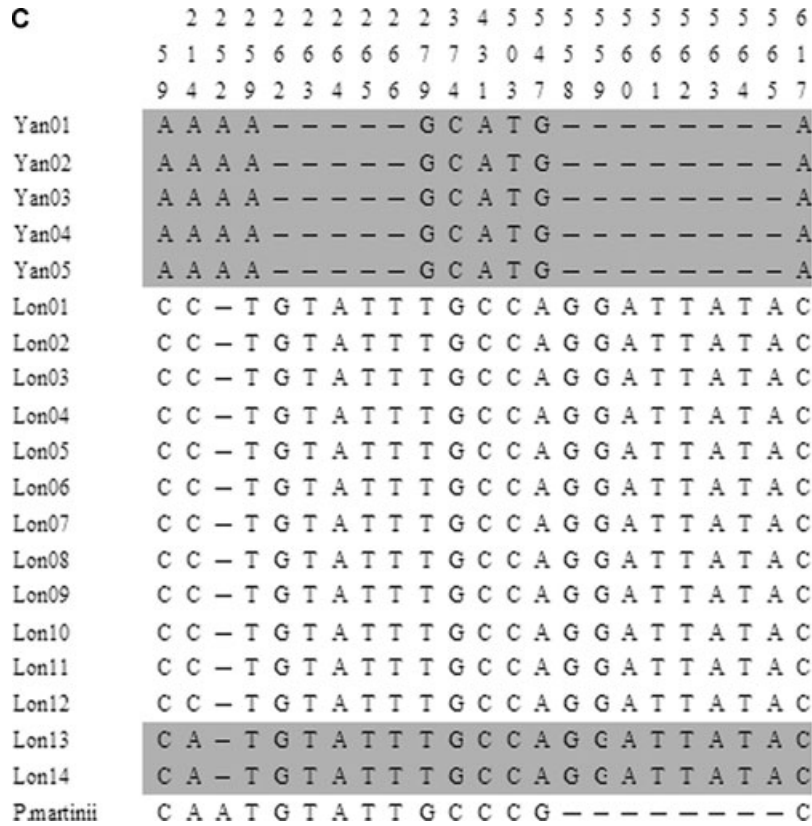


Fig. 3. Continued.

support (BS = 88%, PP = 100%). In this clade, all five accessions of *Yanshan* (Lineage II) were nested in 20 accessions of *Longianthera*, of which 16 in lineage I had the haplotype Lon-I, and the remaining four accessions in lineage III had the haplotypes Lon-II–V. The phylogenetic branches in clade D showed close relationships of *Yanshan* and the haplotype Lon-I of *Longianthera*. It was evident that *Longianthera* was not monophyletic. The individual cloned sequences of *Longianthera* were grouped with either *Yanshan* or *P. martinii*.

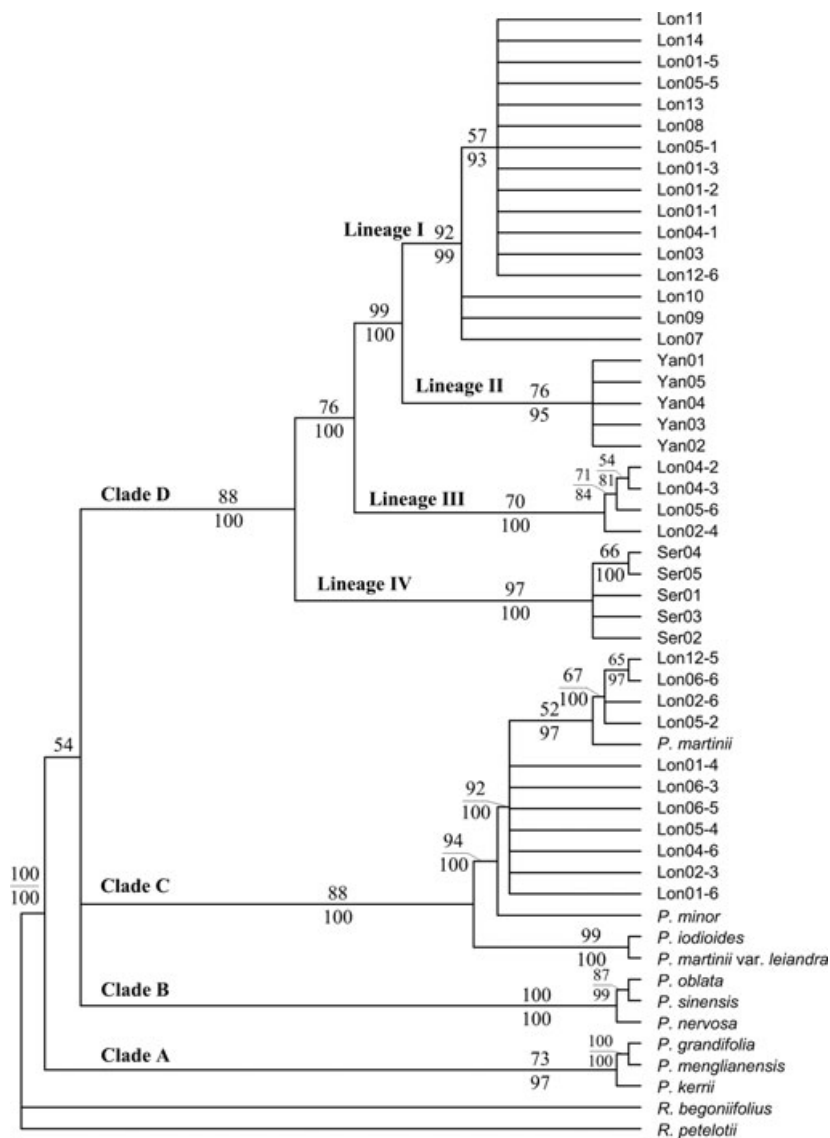
**2.5 Phylogenetic analysis based on combined chloroplast sequences of *matK*, *trnL-F*, and *trnT-L***

The combined cpDNA matrix with three chloroplast regions, *matK*, *trnL-F*, and *trnT-L*, had aligned sequences of 3293 bp, of which 93 (2.82%) were parsimony informative. The strict consensus of three trees yielded by MP analysis (L = 231, CI = 0.931, RI = 0.964) was generally congruent with the majority rule consensus Bayesian tree in the topology (Fig. 5).

In the cpDNA tree, the species of *P. grandifolia*, *P. kerrii*, and *P. menglianensis* from sect. *Deinanthera*

formed a clade with maximum support (BS = PP = 100%) which was sister to the remaining groups with maximum support (BS = PP = 100%) as in the ITS tree. The remaining groups were in a polytomy of three branches with maximum support (BS = PP = 100%). *Petrocosmea minor* was in a branch itself and *P. oblata*, *P. sinensis*, and *P. nervosa* together with *P. iodoides* and *P. martinii* var. *leiandra* formed the second branch with support of BS = 74% and PP = 81%. The remaining 24 accessions formed the third branch with two sister lineages with support of BS = 80% and PP = 100%. In the first lineage, all five accessions of *P. sericea* were gathered together and were further sister to the five accessions of *Yanshan* with maximum support (BS = PP = 100%). They were sister to *P. martinii* with strong support (BS = 96%, PP = 100%). The second lineage, comprised of all 14 accessions of *Longianthera* with maximum support (BS = PP = 100%), in which 12 of them grouped together were sister to the remaining two accessions (Lon13 and Lon14). The branches of accessions of *Longianthera* corresponded to the two genotypes of the sequences in *matK*, *trnL-F*, and *trnT-L* of *Longianthera* and showed





**Fig. 4.** Strict consensus of 13 530 maximum parsimony trees generated from analysis of internal transcribed spacer sequence data. Maximum parsimony bootstrap values ( $\geq 50\%$ ) are above the branches, Bayesian posterior probabilities ( $\geq 90\%$ ) are below the branches. Lon, *Longianthera*; Ser, *Petrocosmea sericea*; Yan, *Yanshan*. Numbers in front of “-” are sampling numbers of populations; numbers following “-” are the clone numbers of each accession.

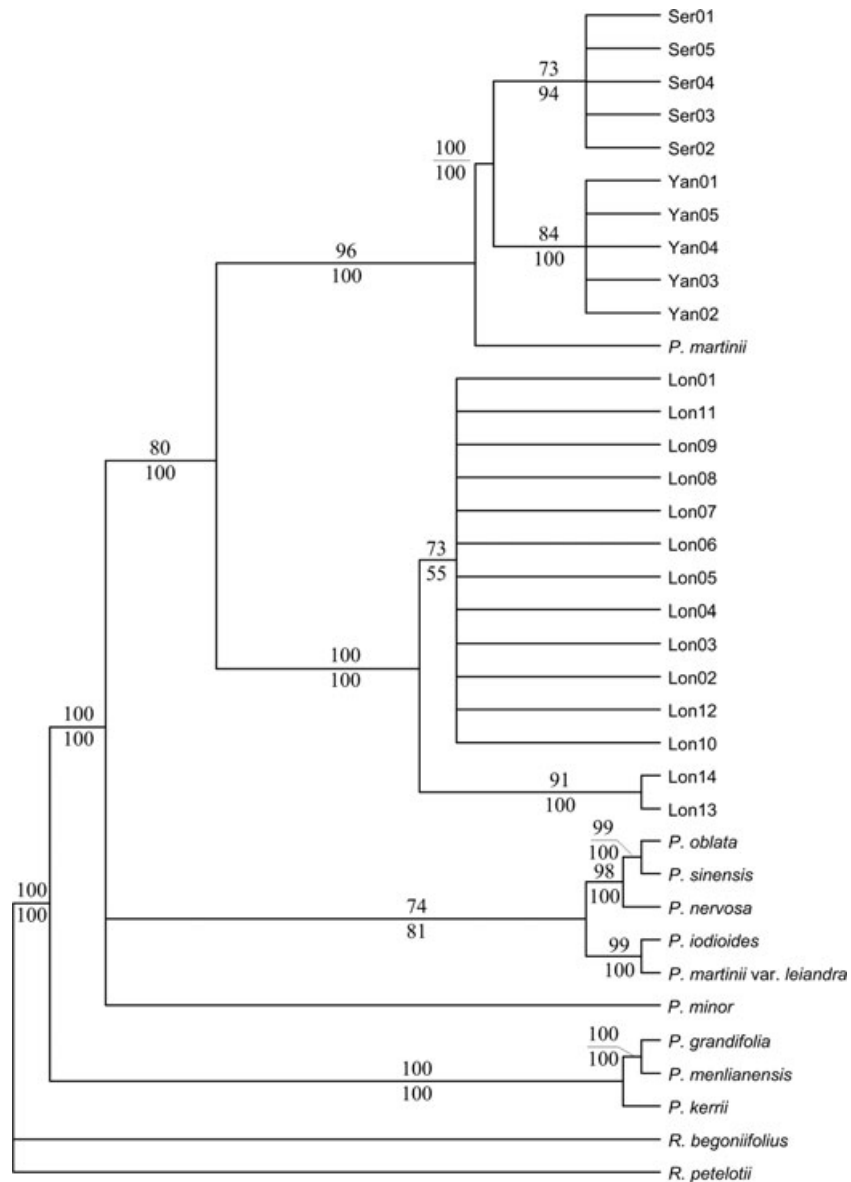
a close relationship with *P. martinii*, *Yanshan*, and *P. sericea*.

### 3 Discussion

#### 3.1 *Longianthera* a hybrid between *Yanshan* and *P. martinii*

The morphological intermediates are usually suggested to originate from direct hybridization or introgressive hybridization of two distinguished species (Grant, 1981). In the present study, we investigated 26

morphological characters in a possible hybrid and two putative parents. Seven of nine quantitative characters in *Longianthera* are intermediate between *Yanshan* and *P. martinii*. For example, LHL is ca. 3.3 mm in *Yanshan* and ca. 0.98 mm in *P. martinii*, whereas it is ca. 2.2 mm in *Longianthera* (Table 2, Fig. 1). Similar situations are also observed in LHC and LHF. Dense glandular hairs are observed on the abaxial surface of corolla of *Yanshan*. Meanwhile, the abaxial surface of corolla is sparsely pubescent in *P. martinii*. In *Longianthera*, as expected, there are both pubescent and glandular hairs on the abaxial surface of corolla. The lengths of the



**Fig. 5.** Strict consensus of three maximum parsimony trees generated from analysis of the combined chloroplast regions of *matK*, *trnL-T*, and *trnT-L* sequence data. Maximum parsimony bootstrap values ( $\geq 50\%$ ) are above the branches, Bayesian posterior probabilities ( $\geq 50\%$ ) are below the branches. Lon, *Longianthera*; Ser, *Petrocosmea sericea*; Yan, *Yanshan*. Numbers in accession names are sampling numbers of populations.

hairs on leaves, filaments, anthers, and corolla are very important taxonomic characters in the species identification in *Petrocosmea* (Table 2, Fig. 1). *Longianthera* is also characteristic of intermediate lengths of bracts (ca. 4.45 mm), filaments (ca. 2.2 mm), and the corolla tube (ca. 3.81 mm) between *Yanshan* (ca. 7.62 mm, 1.5 mm, and 5.2 mm, respectively) and *P. martinii* (ca. 2.8 mm, 3.2 mm, and 2.7 mm, respectively).

In addition, many qualitative characters demonstrate intermediate statuses in *Longianthera* compared with its putative parents, such as LS, BS, AS, and OS,

which are usually considered the important morphological characters in the systematics of *Petrocosmea*. Furthermore, *Yanshan* always has thicker hairs on the filament, anther, and the corolla than *P. martinii*. *Longianthera* usually has an intermediate number of hairs on such organs. However, in some characters such as SUC and DTA, *Longianthera* is similar to *Yanshan*. Both *Yanshan* and *Longianthera* have poricidal anthers and ascendent and patulous bi-lobed upper corolla lips; *P. martinii* has a longitudinally dehiscent anther and porrect and carinate bi-lobed upper lip (Fig. 1).

As outlined above, the character count procedure analyses show that 22 out of 26 examined quantitative or qualitative morphological characters from both vegetative and reproductive organs in the wild populations of *Longianthera* vary between *Yanshan* and *P. martinii*, with some characters similar to one putative parent, but others exclusive to *Longianthera* (Table 1, Fig. 1).

It has been suggested for many hybrids and hybrid derivatives that the putative hybrid species is usually intermediate between the two putative parental species in morphological features with some additional deviations (Rieseberg, 1995). In addition, if two different genomes of putative parents are recombined into a new genetic background after hybridization, the additive inheritance of nuclear sequence that is otherwise unique in the putative parental species is convincing evidence of hybridity (Rieseberg, 1997; Rieseberg & Carney, 1998; Hegarty & Hiscock, 2005).

In the present study, sequence data further showed that the ITS sequences from *Yanshan* and *P. martinii* are distinctively different from each other in 29 nucleotide sites (Fig. 2), and both haplotypes of ITS sequences from the two putative parents are found in some accessions (haplotypes Lon-I and Lon-VIII) of *Longianthera* with just several substitutions or indels differences. Such tiny differences of ITS sequences between *Yanshan* and haplotype Lon-I of *Longianthera*, and between *P. martinii* and haplotype Lon-VIII, might have been caused by random mutations in ITS loci in the long-term process of evolution after the hybrid origin of *Longianthera* because of the fast evolutionary rate of ITS sequences (Hamby & Zimmer, 1992; Sang et al., 1995). In addition to Lon-I and Lon-VIII, the DNA sequences of haplotypes of Lon-II–VII from *Longianthera* bear stronger similarities to *Yanshan* or *P. martinii* than to any other species in *Petrocosmea* (Table 3). Correlatively, *Yanshan* and *P. martinii* are nested in the accessions of *Longianthera* in the phylogenetic tree based on the ITS sequences (Fig. 4). As analyzed above, *Longianthera* contains similar ITS paralogous loci to its putative parents, and the additivity of ITS sequences from its putative parents can be observed in *Longianthera* populations. Putting both morphological and molecular evidence into consideration, we suggest that *Longianthera* is of hybrid origin with *Yanshan* and *P. martinii* as putative parental species.

In the cpDNA tree, all of the accessions of *Longianthera* are grouped together in a clade that is sister to another clade containing *Yanshan*, *P. martinii*, and *P. sericea* (Fig. 5). This suggests that the three species may be the possible maternal parents because of maternal inheritance of these cpDNA regions. However, all five accessions of *P. sericea* are gathered together, which

are separate from rather than merged into the accessions of *Longianthera* as *Yanshan* and *P. martinii* in the ITS phylogenetic tree (Fig. 4). Both the evidence from the ITS phylogenetic tree and the ITS sequence similarity comparison also support the exclusion of *P. sericea* as the putative parental species of *Longianthera*. In addition, the accession Lon13 of *Longianthera* and *Yanshan* share exactly the same haplotype of *trnL-F* sequence. *Yanshan* is the only putative parent which has the same haplotype as *Longianthera* in one of the three cpDNA sequences. Besides the molecular evidence, a series of morphological traits demonstrate strong similarity between *Longianthera* and *Yanshan*, such as the cuneate leaf base, the ascendent and patulous upper corolla lip, and the similar anther shape with the same dehiscence type (poricidal anther) (Fig. 1). Both molecular and morphological evidence suggests that *Yanshan* should be the most possible maternal parent of *Longianthera*.

With regard to the two different haplotypes in each of the three cpDNA sequences, one possible reason may be the back-cross with *P. martinii* and the reciprocal hybridizations between *Longianthera* and the putative parents in the long-term process of evolution of the hybrid species, especially at the early stage of hybrid origin of *Longianthera* (Feng et al., 2007; Li et al., 2008). There is no strong evidence to exclude *P. martinii* from the putative maternal parents of *Longianthera*. In addition, the variation and mutants in the cpDNA loci of *Longianthera* in the long-term reproductive process after hybrid origin might be another explanation for the incomplete consistence in the cpDNA sequences between *Longianthera* and its putative maternal parent. Further artificial hybridization and reciprocal hybridization with molecular genetic research of related genes would provide insight into the evolutionary mechanism of the hybrid origin and speciation of *Longianthera*.

### 3.2 Possible factors contributing to hybridization between *Yanshan* and *P. martinii* and hybrid origin of *Longianthera*

So far there has been no report of natural hybridization in *Petrocosmea*. The putative hybrid of *Longianthera* studied here presents a series of morphological intermediates between *Yanshan* and *P. martinii* with some additional deviations. Combined with the molecular sequence analysis, the results provide compelling evidence for the natural hybridization between *Yanshan* and *P. martinii*.

*Yanshan* and *P. martinii* have partially overlapped geographic distribution and habitats. The *Yanshan* population is located at 1500–1600 m altitude in Baga village, Yanshan County, Yunnan Province. Abundant individuals of *P. martinii* have been found at 1500–1545 m

altitude in the Lantern Mountain of Dawaige village in the same County. The linear distance between them is approximately 20 km. The hybrid *Longianthera* populations live in the cliff of Luokezhe village, Yanshan County, at an altitude of 1500–1600 m. The distance between the two populations of *Longianthera* is ca. 20 m, and the linear distances between *Longianthera* and *Yanshan* and *P. martinii* are 15 km and 30 km, respectively. They all grow in dark and damp limestone habitat at ca. 1400–1600 m elevation. Yanshan County is of the karst topography in Southeastern Yunnan with altitudes of 1080–2263 m. There are many small limestone hills in this county with complex terrain, in which there are many dark and moist niches. The specimen records show wide distribution of *P. martinii* in Yanshan County, whereas the *Yanshan* population is only known at present from the collecting locality herein. They are close to, but remarkably distinctive from, *P. sericea* both in morphology and molecular phylogeny. There is considerable overlap in geographic distribution and habitats between *Yanshan* and *P. martinii*, providing spatial chances for hybridization.

Another factor that might contribute to the natural hybridization between *Yanshan* and *P. martinii* is the long and partially overlapping flowering periods and possibly shared pollinators. *Yanshan* flowers from June to September and *P. martinii* from May to August. Bees were observed as the pollinators in the field of some species of *Petrocosmea*, such as *P. forrestii* and *P. barbata*. In *P. martinii*, bees and some kinds of flies were observed to visit their flowers as well as in *Yanshan*. Plants of *Petrocosmea* are usually pollinated in the field by bee or flies non-specifically. It is possible that they share the same or similar pollinators, which could strongly enhance the possibility of hybridization between them. The successful artificial hybridizations between several pairs of species in *Petrocosmea* carried out in the greenhouse further confirm the possibility of natural hybridization in *Petrocosmea* plants. The artificial hybridization proves that most species of *Petrocosmea* can accept the pollens from other species of the same genus. Successful hybridization is usually achieved between two closely related species (Qiu et al., 2010, unpublished data). In the present study, the phylogenetic trees show a close relationship between *Yanshan* and *P. martinii*. Therefore, it would be easy for each to successfully receive pollen from the other and to achieve natural hybridization.

Hybridization between species can lead to introgression of genes from one species to another, which provides a potential mechanism for preserving and recombining key traits during evolution. The gene exchange, especially in regulatory genes, caused by in-

terspecific hybridization in evolution could allow the traits lost under short-term selective pressures to be regained at a later stage (Kim et al., 2008). *Longianthera* shows a series of intermediate morphological characters between its putative parents with some morphological traits similar to one parent, which may be caused by the genetic heterozygosity in the hybrid transferred from the parents. Two or more than two haplotypes of the regulatory genes may exist in the hybrid, in which the gene interactions could lead to a series of morphological variation and the gain of some important traits such as long anther. During natural hybridization, gene transfers between the parents play an important role in the evolution of the key morphological and ecological traits. Detailed investigations of the genes responsible for the intermediate and deviate morphological traits with functional analyses would shed new light on the mechanisms underlying gene introgression during natural hybridization.

## 4 Taxonomic treatments

### 4.1 Description of new species of *Yanshan*

***Petrocosmea yanshanensis*** Z. J. Qiu & Y. Z. Wang, sp. nov.

**Type:** China. Yunnan Province: Yanshan County, Baga village, 2007-09-06. Z. J. Qiu QZJ2007077 (holotype, PE).

Species *P. sericeae* affinis, sed foliis villosis, corollis extus glandulosis, labio postico ascendente et applanato, labio antico lobis oblongis, antheris triangule ovatis, filamentis dense glandulosis differt.

Perennial acaulescent herb, with short rhizome and crowded fibrous roots. Rosette leaves 8 to 20, the inner leaves have short petioles or petioles absent, the outer leaves have longer petioles; leaf blade obovate or oblong, 0.5–2 × 0.5–1.5 cm, herbaceous, apex round, base truncate, cuneiform or rounded, margin entire, both surfaces with densely pilose, lateral veins 3 on each side, not distinct; petioles 0.5–3 cm, densely pilose. Cymes 2 to 10, 1-flowered; peduncle 4–8 cm, densely pilose; bracts 2 at the upper middle of peduncle, broadly lanceolate, 8–9 mm, pubescent; sepals 5-divided to the base, lanceolate, ca. 5 mm, densely pilose externally. Corolla royal purple, glandular pubescent externally; tube ca. 5 mm, adaxial lip ca. 6–7 mm, bi-lobed near to middle, lobes semicircular, abaxial lip ca. 9–10 mm, tri-lobed almost at base, lobes oblong; stamens 2, ca. 5 mm; filaments adnate to ca. 1 mm above base of corolla tube, ca. 1.5 mm long, densely glandular pubescent; anthers triangular-ovate, ca. 3.2 mm long, with short mahogany glandular trichome at the back side and connectivum;

staminodes 3, adnate to ca. 0.2–0.4 mm above base of corolla tube, ca. 0.2–0.5 mm long, glabrous; pistil ca. 4.5 mm; ovary spheroidal, densely villous, ca. 1.5 mm long; style sparsely pubescent at the base, ca. 3 mm long; stigma purple.

**Habitat and distribution:** *Petrocosmea yanshanensis* grows on shady cliffs of limestone, at 1500–1600 m altitude in Baga village, Yanshan County, Yunnan Province, China.

**Phenology:** The new species flowers from June to September; fruits not seen.

**Additional discussion:** According to the floral structure, especially the anthers not constricted near apex and abaxial corolla lip about two times longer than the adaxial, *Petrocosmea yanshanensis* belongs to sect. *Anisochilus*. The new species is similar to *P. sericea*, but differs mainly in having expanded villus leaves, triangular-ovate anthers, densely glandular hairs on filaments, glandular hairs on the abaxial surface of the corolla, and the ascendant and patulous upper corolla lip.

#### 4.2 Description of new hybrid species of *Longianthera*

*Petrocosmea* × *longianthera* Z. J. Qiu & Y. Z. Wang, nothosp. nov.

**Type:** China. Yunnan Province: Yanshan County, Luokezhe village, 2007-10-07. Z. J. Qiu QZJ2007079 (holotype, PE).

Species *P. yanshanensis* similis, sed foliis villosis, infra purpuratis, petiolis et pedicellis purpuratis, corollis extus pubescentibus et glandulosis, antheris longitriangularibus, filamentis triplo longioribus differt; etiam affinis *P. martinii*, sed foliis villosis, infra purpuratis, et pedicellis purpuratis, corollis extus pubescentibus et glandulosis, labio postico ascendente et applanato, antheris longitriangularibus, filamentis triplo longioribus differt.

Perennial acaulescent herb with short rhizome and crowded fibrous roots. Rosette leaves 6 to 30, the inner leaves have short petioles or petioles absent, the outer leaves have longer petioles; leaf blade rhombic, 0.5–3 × 0.5–3 cm, herbaceous, apex round, base truncate or cuneiform, margin repand, both surfaces with densely russet villus, the abaxial surface russet, lateral veins 3 on each side, somewhat distinct; petioles russet, 0.5–5 cm, densely reddish-brown pilose. Cymes 3 to 15, 1–3 flowers per cyme; peduncle russet, 5–10 cm, densely reddish-brown pilose; bracts 2 at mid-peduncle, lanceolate, ca. 4.5 mm, pubescent, pedicel russet, 3–5 cm, densely reddish-brown hairy; sepals russet, 5- (occasionally 6-) divided to the base, narrow-lanceolate, ca. 5 mm, densely reddish-brown pilose externally. Corolla

purple, puberulent and glandular pubescent externally, 2 dark purple spots inside the tube throat; tube ca. 3.7–4 mm, adaxial lip ca. 5.5–7 mm, bi-lobed near to middle, lobes oblong, abaxial lip ca. 10–12 mm, tri-lobed deeply, lobes oblong; stamens 2, ca. 8–9 mm; filaments adnate to ca. 2 mm above base of corolla tube, ca. 2.2 mm long, short glandular pubescent; anthers long-triangular, taper from base to top, ca. 7–8 mm long, with very short glandular pubescence at the back side; staminodes 3, adnate to ca. 0.2–0.4 mm above base of corolla tube, ca. 0.5–2 mm long, glabrous; pistil ca. 10 mm; ovary densely villous, ovoid, ca. 2 mm long; style ca. 7 mm long, glabrous; stigma pink.

Some variation of flowers can be observed in the field occasionally with 3-lobed adaxial corolla lip or 4-lobed abaxial corolla lip or both of them, and actinomorphic flowers rarely.

**Habitat and distribution:** *Petrocosmea* × *longianthera* grows on shady cliffs of limestone, at 1500–1600 m altitude in Luokezhe village, Yanshan County, Yunnan Province, China.

**Phenology:** The new hybrid species flowers from August to October; fruits not seen.

**Additional discussion:** According to the floral structure, especially the anthers not constricted near apex and abaxial corolla lip two times longer than adaxial, *Petrocosmea* × *longianthera* belongs to sect. *Anisochilus*. The new hybrid species is similar to *P. yanshanensis* but differs mainly in having russet abaxial surface of leaves, petioles, and pedicels with russet hairs, long-triangular anthers, whose length is more than four times longer than width, anther length more than three times longer than filament's, inflated filaments in the middle, and both puberulent and glandular hairs on the abaxial surface of the corolla. This new hybrid species is also similar to *P. martinii* but differs mainly in having russet hairs on leaves, petioles, and pedicels, long-triangular anthers, whose length is more than four times longer than width, anther length more than three times longer than filament's, both puberulent and glandular hairs on the abaxial surface of the corolla, and the ascendant and patulous upper corolla lip.

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**Appendix I** Species, voucher with collection locality, and GenBank accession number for taxa included in this study.

Taxon	Voucher, collection locality and citation	GenBank Accession No.			
		<i>trnL-F</i>	<i>matK</i>	<i>trnT-L</i>	ITS
<i>Petrocosmea grandifolia</i> W. T. Wang	QZJ-2007-037, Yunnan, China (PE)	JN092472	JN092541	JN092505	JN092439
<i>P. iodoides</i> Hemsl.	QZJ-2007-074, Yunnan, China (PE)	JN092473	JN092542	JN092506	JN092440
<i>P. kerrii</i> Craib	04603, Yunnan, China (KUN)	JN092474	JN092543	JN092507	JN092441
<i>P. martinii</i> H. Lévl.	QZJ-2007-074, Yunnan, China (PE)	JN092475	JN092544	JN092508	JN092442
<i>P. martinii</i> var. <i>leiandra</i> W. T. Wang	QZJ-2008-33, Guizhou, China (PE)	JN092476	JN092545	JN092509	JN092443
<i>P. menglianensis</i> H. W. Li	QZJ-2007-026, Yunnan, China (PE)	JN092477	JN092546	JN092510	JN092444
<i>P. minor</i> Hemsl. Hook.	QZJ-2008-54, Yunnan, China (PE)	JN092478	JN092547	JN092511	JN092445
<i>P. nervosa</i> Craib	QZJ-2008-45, Sichuan, China (PE)	JN092479	JN092548	JN092512	JN092446
<i>P. oblata</i> Craib	Q060923-1, Sichuan, China (PE)	GU350692	JN092549	JN092513	GU350661
<i>P. sericea</i> C. Y. Wu ex H. W. Li	991104(1-5), Yunnan, China (KUN)	JN092467– JN092471	JN092536– JN092540	JN092500– JN092504	JN092434– JN092438
<i>P. sinensis</i> Oliver	QZJ-2008-41, Sichuan, China (PE)	GU350691	JN092550	JN092514	GU350660
<i>Yanshan</i>	QZJ-2007-077(1-5), Yunnan, China (PE)	JN092462– JN092466	JN092531– JN092535	JN092495– JN092499	JN092429– JN092433
<i>Longianthera</i>	QZJ-2007-079(1-14), Yunnan, China (PE)	JN092448– JN092461	JN092517– JN092530	JN092481– JN092494	JN092398– JN092428
<b>Outgroups</b>					
<i>Raphiocarpus begoniifolius</i> (Lévl) Burt	QZJ-2008-026, Guizhou, China (PE)	GU350680	JN092551	JN092515	GU350648
<i>R. petelotii</i> (Pellegr) Burt	GX_NP_1, Guangxi, China (PE)	JN092480	JN092552	JN092516	JN092447

ITS, Internal transcribed spacer; KUN, Herbarium, Kunming Institute of Botany, Chinese Academy of Sciences; PE, Herbarium, Institute of Botany, Chinese Academy of Sciences.