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Phylogenetic relationships in the *Pterygiella* complex (Orobanchaceae) inferred from molecular and morphological evidence

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The *Pterygiella* complex, i.e. *Pterygiella*, *Phtheirospermum*, *Pseudobartsia* and *Xizangia*, is controversial in terms of both its relationships and taxonomic status. The genera of the complex are all species-poor and endemic to eastern Asia. In this study, we sampled all taxa in the complex for seven genic regions [*atpB-rbcL*, *atpH-I*, *psbA-trnH*, *rpl16* intron, *trnL-F*, *trnS-G* and internal transcribed spacer (ITS)], which were analysed separately and in combination. We examined capsule and seed morphology of these genera using scanning electron microscopy (SEM). Our results indicate that the complex is polyphyletic and comprises two clades. The first, designated '*Pterygiella* complex I', includes only *Phtheirospermum japonicum*. This clade is uniquely diagnosed by its papery yellow capsule and reticulate secondary thickening on the seed coat. The second clade, '*Pterygiella* complex II', includes *Phtheirospermum tenuisectum*, *P. parishii*, *P. muliense*, *Pseudobartsia*, *Xizangia* and *Pterygiella*. These taxa share a coriaceous capsule. *Pterygiella* is monophyletic and is characterized by eglandular, unicellular, pilose hairs on the capsule and hook-like protuberances on the horizontal ridges of the striate or reticulate seed coat. Excluding *P. japonicum*, the remaining taxa of *Phtheirospermum* cluster together and are sister to *Pterygiella*. *Xizangia bartschioides* and *Pseudobartsia yunnanensis* are separated from *Pterygiella* and *Phtheirospermum*, respectively, and are supported as genera by molecular and morphological evidence. *Botanical Journal of the Linnean Society*, 2013, **171**, 491–507.

ADDITIONAL KEYWORDS: Eastern Asia – fruit – genic regions – hemiparasite – phylogenetic analysis – *Phtheirospermum* – *Pseudobartsia* – *Xizangia*.

INTRODUCTION

The *Pterygiella* complex, first proposed by Li (2002), was defined to include *Pterygiella* Oliv., *Bartsia* L., *Pseudobartsia* D.Y. Hong and *Xizangia* D.Y. Hong. However, this complex is the subject of considerable uncertainty with respect to its circumscription, genus and species delimitation. *Pseudobartsia* has been treated as a synonym of *Phtheirospermum glandulosum* Benth. (Tao, 1993). Thus, *Phtheirospermum* Bunge ex Fisch. & C.A. Mey. should be used in place of *Bartsia*, giving a revised complex comprising *Pterygiella*, *Phtheirospermum*, *Pseudobartsia* and *Xizangia*. In fact, the relationships and taxonomy of the group are so confused that Fischer (2004) mistakenly treated *Xizangia* and *Pseudobartsia* as synonyms of *Pterygiella* and *Parentucellia* Viv., respectively. The

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four genera in the complex are species-poor and endemic to eastern Asia, and were formerly placed in tribe Rhinantheae of Scrophulariaceae (Bentham & Hooker, 1876; Oliver, 1896). Morphologically, the complex shares many characters in common with Rhinantheae, including a herbaceous habit, bilabiate corolla, five-lobed calyx, capsule with numerous seeds and parasitic habit.

Recent results of molecular phylogenetics have shown that Rhinantheae are polyphyletic (Young, Steiner & dePamphilis, 1999; Bennett & Mathews, 2006; Tank *et al*., 2006) within Orobanchaceae (Olmstead & Reeves, 1995; Olmstead *et al*., 2001). Based on internal transcribed spacer (ITS) data, Wolfe *et al*. (2005) supported a redefined classification of Orobanchaceae and proposed a hypothesis of Laurasian origin for the family. The Himalayas are known to have been a hotspot of diversification in Laurasia for some genera of Orobanchaceae, including *Lindenbergia* Lehm. ex Link & Otto (Hjertson, 1995), *Pedicularis* L. (Hong, 1983) and *Phtheirospermum* (Tao, 1996). Furthermore, eastern Asia is home to more genera and endemic genera, of Rhinantheae than anywhere else (Hong, 1983). Despite this, phylogenetic analyses seldom focus on this area.

Xizangia was first described as a monotypic genus from Tibet, with the type species *X. serrata* D.Y. Hong (Hong, 1986). However Tao (1999) disputed its generic status and considered *X. serrata* to be the same as *Pterygiella bartschioides* Hand.-Mazz. *Pterygiella bartschioides* is variable and differs from other members of *Pterygiella* in having orbicular–ovate leaves with serrate margins and seeds with reticulate hyaline appendages (Hong, 2001). Wu (1999) and Hong (2001) stated that *P. bartschioides* should be separated from *Pterygiella* and that the legitimate name for the taxon was *X. bartschioides* (Hand.-Mazz.) Z.Y. Wu & D.D. Tao. Recently, the generic status of *Xizangia* has been confirmed, and a revision of *Pterygiella* is required (Dong, Li & Wang, 2011a; Dong *et al*., 2011b).

In *Phtheirospermum*, new taxa have frequently been proposed. For instance, Bonati (1921) proposed a new species, *P. auratum* Bonati, endemic to Yunnan, China. This species differs greatly from others in *Phtheirospermum* in terms of stem, leaf, flower and seed morphology. Li (1949) found that the species should be merged into *Pedicularis* under the new combination, *P. aurata* (Bonati) Li, and subsequent authors (e.g. Tao, 1996; Mill, 2001) accepted Li's treatment. Hong (1979) and Hong *et al*. (1998) proposed the new genus *Pseudobartsia* with type *P. yunnanensis* D.Y. Hong, but Tao (1993) treated *P. yunnanensis* as conspecific with *Phtheirospermum glandulosum* Benth. The generic rank of *Pseudobartsia* is supported by pollen data (Lu *et al*., 2007), but the placement of *Phtheirospermum glandulosum* is still disputed. Tao (1996) proposed another new species, *Phtheirospermum muliense* C.Y. Wu & D.D. Tao, based on its dimorphic leaf, glandular pilose pubescence and distribution (endemic to the Hengduan Mountains, China). Since that time, *Phtheirospermum* has been defined to include *P. japonicum* (Thunb.) Kanitz, *P. tenuisectum* Bureau & Franch., *P. parishii* Hook.f and *P. muliense* according to the treatment of Tao (1996). Molecular studies have shown *Phtheirospermum* to be close to *Pedicularis* (Bennett & Mathews, 2006), but only one species, *P. japonicum*, was sampled in that analysis.

Seed morphology potentially contains a great deal of systematic information (Thieret, 1955; Kuijt, 1969), but has been insufficiently studied in systematics. Chuang & Heckard (1972) relied heavily on seed coat characters for classification of *Cordylanthus* Nutt. ex Benth. at sectional and subsectional levels; Musselman & Mann (1976), studying 23 species in 11 genera, showed that seed surface characters have systematic value in Scrophulariaceae *s.l.* and Orobanchaceae; Chuang & Heckard (1983) discovered that seed coat characters also exhibit differences at species level in *Orthocarpus* Nutt. In addition to seed coat characters, Juan, Pastor & Fernández (2000) determined that indumentum and dehiscence of the capsule also provided useful data in Scrophulariaceae *s.l*. Hong (2001) showed that the seed coat provides useful information for classification of *Xizangia* and *Pterygiella*, but this study did not extend to additional variation in capsule and seed characters.

In this study, we selected spacers and introns from the plastid genome, *atpB-rbcL*, *atpH-I*, *psbA-trnH*, *rpl16* intron, *trnL-F* and *trnS-G*, showing medium to high variability, and combined these with the nuclear ITS region, to reconstruct a phylogenetic tree for the *Pterygiella* complex. Using these seven DNA regions with capsule and seed morphology, we aim to clarify the phylogenetic relationships in the complex, evaluate the utility of micromorphological capsule and seed characters for classification and provide new morphological data for capsules and seeds in this poorly studied complex.

MATERIAL AND METHODS

Taxon sampling and outgroup selection

All taxa in the *Pterygiella* complex were sampled (see Table 1). For *Phtheirospermum*, the classification of which is controversial, we based our sampling on the treatment of Tao (1996), including *P. japonicum*, *P. tenuisectum*, *P. parishii* and *P. muliense*. *Pterygiella cylindrica* Tsoong, *P. duclouxii* Franch., *P. nigrescens*

Table 1. Materials and voucher specimens used in molecular study

Taxon	Locality	Voucher specimen (herbarium code)	ITS	*atpB-rbcL*	*atpH-I*	*psbA-trnH*	*rpl16*	*trnL-F*	*trnS-G*
Phtheirospermum japonicum (Thunb.) Kanitz	–	Institute of Forestry of Sangzhi, Hunan, 1621 (KUN)	JQ910089	JQ910066	JQ910080	JQ910098	JQ910112	JQ910128	JQ910142
Phtheirospermum japonicum	Shiping, Yunnan	Dong LN, SP2 (KUN)	JQ910090	JQ910067	JQ910081	JQ910099	JQ910113	JQ910129	JQ910143
Phtheirospermum tenuisectum Bureau & Franch.	Lijiang, Yunnan	Lu L, LJ377 (KUN)	FJ746383**	JN416509*	JQ910082	JN416416*	JQ910114	JQ910130	JN416478*
Phtheirospermum muliense C.Y. Wu & D.D. Tao	Muli, Sichuan	Chuanjinxi, 3582 (KUN)	JQ910091	–	–	JQ910100	JQ910115	JQ910131	–
Ph. parishii Hook.f.	Kangbei, Sichuan	Ying JS, 4144 (KUN)	JQ910092	–	–	–	–	JQ910132	–
Pseudobartsia yunnanensis D.Y. Hong	Songming, Yunnan	Zhang YB, 4 (KUN)	JQ910093	JQ910068	–	–	JQ910116	–	JQ910144
Pterygiella cylindrica Tsoong	Muli, Sichuan	Dong LN, CML13 (KUN)	JF746386**	JN416481*	JQ910076	JN416388*	JQ910108	JQ910124	JN416450*
Ptterygiella cylindrica var. suffruticosa (D.Y. Hong) L.N. Dong & H. Wang	Shiping, Yunnan,	Dong LN, SML11 (KUN)	JF746400**	JN416508*	JQ910079	JN416415*	JQ910111	JQ910127	JN416477*
Pterygiella duclouxii Franch.	Dali, Yunnan	Dong LN, DML6 (KUN)	JQ910087	JQ910064	JQ910077	JQ910096	JQ910109	JQ910125	JQ910140
Pterygiella nigrescens Oliv.	Nansha, Yunnan	Dong LN, NNS9 (KUN)	JQ910088	JQ910065	JQ910078	JQ910097	JQ910110	JQ910126	JQ910141
Xizangia bartschioides (Hand.-Mazz.) C.Y. Wu & D.D. Tao	Fugong, Yunnan	Dong LN, GS6-8 (KUN)	JF746405**	JQ910075	JQ910086	JQ910107	JQ910123	JQ910139	JQ910151
Melampyrum carstiense Fritsch	–	Krajsek *s.n.* (LJU)	GU445314**	JQ910069	–	JQ910101	JQ910117	JQ910133	JQ910145
M. klebelsbergianum Soó	Wuding, Yunnan	Dong LN, WD1 (KUN)	GU445315**	JQ910070	JQ910083	JQ910102	JQ910118	JQ910134	JQ910146
Pedicularis rex C.B. Clarke ex Maxim.	Fuming, Yunnan	Yu WB, LIDZ-1300 (KUN)	JQ910095	JQ910072	JQ910084	JQ910104	JQ910120	JQ910136	JQ910148
Pedicularis thamnophila (Hand.-Mazz.) H.L. Li	Daocheng, Sichuan	Yu WB, LIDZ-1243 (KUN)	JQ910094	JQ910071	–	JQ910103	JQ910119	JQ910135	JQ910147
Rhinanthus alectorolophus (Scop.) Pollich	–	Nyffler *s.n.* (GH)	GU445318**	JQ910073	–	JQ910105	JQ910121	JQ910137	JQ910149
R. freynii Fiori	–	Bennett 88 (GH)	GU445319**	JQ910074	JQ910085	JQ910106	JQ910122	JQ910138	JQ910150

*For details see Dong *et al.* (2011a); **for details see Dong *et al.* (2011b).
–, missing data.

Oliv. and *P. cylindrica* var. *suffruticosa* (D.Y. Hong) L.N. Dong & H. Wang (Dong *et al*., 2011a), *Pseudobartsia yunnanensis* and *Xizangia bartschioides* (Hand.-Mazz.) D.Y. Hong were also sampled.

Six species were included as outgroups: *Pedicularis rex* C.B. Clarke ex Maxim. and *P. thamnophila* (Hand.-Mazz.) H.L. Li (Bennett & Mathews, 2006), *Melampyrum carstiense* Fritsch, *M. klebelsbergianum* Soó, *Rhinanthus alectorolophus* (Scop.) Pollich and *R. freynii* Fiori (S. Mathews, pers. comm.). Of these, *P. rex* and *P. thamnophila* were used to root the trees, based on existing phylogenetic analyses of Orobanchaceae (Bennett & Mathews, 2006).

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from silica-gel-dried and herbarium materials using the 2 × cetyl trimethylammonium bromide (CTAB) procedure (Doyle & Doyle, 1987). The ITS region was amplified using primers ITS-5 and ITS-4 (White *et al*., 1990), the *atpB-rbcL* region with primers atpBr and atpBf (Barbrook *et al*., 2001), the *atpH-I* region with primers atpI and atpH (Shaw *et al*., 2007), the *psbA-trnH* region with primers psbA and trnH^{GUG} (Hamilton, 1999), the *rpl16* intron with primers rpl16-F71 and rpl16-R1516 (Jordan, Courtney & Neigel, 1996), the *trnL-F* region with primers c and f (Taberlet *et al*., 1991) and the *trnS-G* region with primers trnS^{GCU} and trnG^{UUC} (Shaw *et al*., 2005).

Sequences were amplified using two alternative protocols for the different sources of material. For silica-gel-dried materials, amplification procedures were carried out according to Shaw *et al*. (2005, 2007). For herbarium materials, reactions were performed with 2 × Taq PCR MasterMix (Tiangen Biotech, Beijing, China), 0.2 μM of each primer and 50–100 ng of template DNA per 25 μL reaction volume. PCR parameters were as follows: initial denaturation at 94 °C for 3 min, followed by 30 cycles of 45 s at 94 °C for denaturation, ramped down to 72 °C at maximum rate, holding for 1 s at 72 °C, then ramped down to an annealing temperature at 0.1 °C s^{-1}, primer annealing at 50 °C for 45 s, ramped up to an extension temperature at 0.2 °C s^{-1} and primer extension at 68 °C for 1 min, with a final extension step consisting of 10 min at 68 °C. Where amplification was weak, PCR products were ligated and transformed using a pEASY-T3 vector (TransGen Biotech, Beijing, China). Ten clones were screened in each case, amplified with corresponding primers, and at least three positive clones sequenced. Reaction products were purified using Waterson's purification kit (Sangon, Shanghai, China) prior to sequencing. Cycle sequencing products were subjected to electrophoresis on an ABI 3730 automated sequencer.

Sequence alignment and phylogenetic analyses

Base determination was complete and unambiguous in all cases. DNA sequences were edited with SeqMan (DNASTAR Package), aligned using the MUSCLE online server (http://www.ebi.ac.uk/Tools/msa/muscle) and adjusted manually where necessary. Indels were coded using the software GapCoder (Young & Healy, 2003). Indels situated in polynucleotide tract regions were excluded from coding because they often provide homoplasious characters (Small *et al*., 1998). Indels in the ITS and *psbA-trnH* regions were also excluded because of high sequence variation. The remaining, coded indels were added to the matrix as binary presence/absence characters. Congruence between data sets was assessed using the incongruence length difference (ILD) test (Farris *et al*., 1994) implemented in PAUP* ver. 4.0b10 (Swofford, 2003). The ILD value for the entire data set was $P = 0.043$, suggesting congruence among the data sets. Therefore, analyses were conducted on the following data partitions: (1) individual region; (2) the six plastid regions combined; and (3) the nuclear ITS and plastid regions combined.

Nucleotide substitution saturation and tree-length distributions were used to assess the phylogenetic information contained in the studied regions. Each of the data subsets was separately subjected to a test of nucleotide substitution saturation using index of substitution saturation (I_{ss}) of Xia *et al*. (2003), implemented in DAMBE ver. 5.2.13 (Xia & Xie, 2001; Xia & Lemey, 2009). The skewness of tree length distribution was assessed according to Hillis & Huelsenbeck (1992) using PAUP*4.0b10 (Swofford, 2003).

Maximum parsimony (MP) analysis was performed with PAUP* 4.0b10 (Swofford, 2003). Heuristic tree searches were conducted with 1000 random addition sequence replicates and tree–bisection–reconnection (TBR) branch swapping, with multrees option in effect. All characters in the data matrix were unordered and equally weighted. All most parsimonious trees were summarized into a strict consensus tree. Node support was evaluated using the bootstrap (BS) method with 1000 replicates, TBR branch swapping and multrees option in effect.

Bayesian inference (BI) analyses were performed with MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Optimal substitution models for all data sets were determined using the Akaike information criterion (AIC) executed in the program jModeltest 0.1.1

(Posada, 2008); the model was then set as nst = 6, rates = gamma. Bayesian analyses were started from random trees, sampling one tree every 1000th generation, with four incrementally heated chains. The Markov chain Monte Carlo (MCMC) algorithm was run for 10 000 000 generations for each data set. Stationarity of the Markov chain was ascertained by the online application AWTY (Wilgenbusch, Warren & Swofford, 2004). The first 1000 trees corresponding to the 'burn-in' period were discarded, and the remaining trees were used to construct a majority-rule consensus tree. Posterior probability (PP) was used to estimate robustness.

Maximum-likelihood tree and bootstrap analyses were performed using RAxML Blackbox (http://phylobench.vital-it.ch/raxml-bb/) with the default specified model (Stamatakis, Hoover & Rougemont, 2008). Node support was evaluated using bootstrap (BS) methods with 100 replicates.

Capsule and seed morphology

Mature fruits were selected from herbarium collections for ten taxa (see Table 4). Impurity deposits in seeds were removed using the method of Martínez-Ortega & Rico (2001): dried seeds were soaked in a 1:1 solution of chloroform and methanol for 48 h, dehydrated through an ethanol series (70, 90 and 100%) and finally treated with xylene for 3 days. Treated seeds and capsules were mounted on stubs, coated with gold in a sputter coater, and observed with a KYKY-1000B scanning electron microscope (SEM; Science Instrument Company, Beijing, China) at 5 kV.

The shape and appearance of capsules and seeds were recorded descriptively. Size measurements of capsules and seeds were taken from multiple herbarium sheets to obtain a standardized measurement across variable specimens. Measurements were taken using Vernier calipers. Terminology for capsule and seed morphology is taken from Musselman & Mann (1976), Chuang & Heckard (1972, 1983), Juan *et al*. (2000) and Zhang *et al*. (2005).

RESULTS

Assessing phylogenetic information content

Each of the sequenced regions is characterized in Table 2. Divergence values between all taxa (including outgroups) ranged from 15.92 to 27.98%, and divergence in the *Pterygiella* complex ranged from 5.85 to 8.29% (see also Supporting Information, Fig. S1A). There were 176 potentially parsimony-informative characters (PIC) in the *trnS-G* spacer across all taxa, but this decreased to 42 in the complex (see also Supporting Information, Fig. S1B).

Table 2. Data matrix and tree statistics from phylogenetic analyses

Results		*atpB-rbcL*	*atpH-I*	*psbA-trnH*	*rpl16* intron	*trnL-F*	*trnS-G*	ITS	Combined plastid	Combined all
Variation in sequence length (bp)		727–796	724–1055	284–598	457–844	720–844	621–679	588–618	835–4624	1499–5185
Average A content (%)		32.5	38.5	38.4	39.8	35.8	36.8	19.6	36.9	34.4
Average T content (%)		38.0	31.8	34.9	28.6	29.1	33.8	20.3	32.2	30.6
Average C content (%)		14.0	12.9	15.8	14.6	18.2	13.0	30.6	14.8	17
Average G content (%)		15.5	16.9	10.9	17.1	16.9	16.4	29.5	16.0	17.9
GC%		29.5	29.8	26.6	31.7	35.1	29.4	60.1	30.8	34.9
Aligned length (bp)		837	1171	772	917	899	821	653	5417	6070
Number of incomplete taxa (%)		2 (11.8%)	6 (35.3%)	2 (11.8%)	1 (5.9%)	1 (5.9%)	2 (11.8%)	0 (0%)	0 (0%)	0 (0%)
Number of incomplete characters (%)		94 (0.7%)	584 (4.5%)	299 (2.6%)	457 (6.3%)	199 (1.4%)	57 (0.5%)	24 (0.2%)	15 328 (16.6%)	15 322 (14.8%)
MP	Number of trees	1	1	1	1	1	1	1	1	1
	Length of most-parsimonious tree	151	225	254	180	168	265	489	1553	2045
	Consistency index (CI)	0.947	0.951	0.937	0.928	0.940	0.891	0.753	0.903	0.866
	Retention index (RI)	0.963	0.926	0.929	0.953	0.963	0.920	0.830	0.920	0.894
BI	AIC selected model	GTR	TVM+G	TPM1uf+G	TPM1uf+G	TVM+G	TVM1+G	GTR+G	GTR+G	GTR+G

MP, maximum parsimony analysis; BI, Bayesian inference analysis.

Table 3. Results of substitution saturation tests (using DAMBE) and g_1 value of tree-length distribution (using PAUP*)

	Substitution saturation					Random tree distribution		
Generic regions	I_{ss}	$I_{ss.cSym}$*	P_{Sym}†	$I_{ss.cAsym}$‡	P_{Asym}§	Mean	Std.	g_1
atpB-rbcL	0.1890	0.7541	0.0000	0.5811	0.0000	330.9067	18.8324	–1.2597
atpH-I	0.3870	0.7774	0.0000	0.6348	0.0000	338.6237	19.6426	–1.0958
psbA-trnH	0.7295	0.7478	0.0000	0.5527	0.0000	428.4677	22.6853	–1.0957
rpl16 intron	0.2934	0.7596	0.0000	0.5888	0.0000	406.5358	23.4013	–1.1412
trnL-F	0.3030	0.7573	0.0000	0.5664	0.0000	391.6733	24.4520	–1.1111
trnS-G	0.3956	0.7529	0.0000	0.5794	0.0000	551.5194	30.9178	–1.2217
ITS	0.2680	0.7437	0.0000	0.5339	0.0000	962.4957	49.9013	–0.6986
Combined plastid regions	0.5767	0.8239	0.0000	0.6671	0.0000	3009.4565	143.1064	–1.0217
Combined all regions	0.5395	0.8270	0.0000	0.6679	0.0000	3973.0317	188.8673	–1.0001

*Index of substitution saturation assuming a symmetrical true tree.
†Probability of significant difference between I_{ss} and $I_{ss.cSym}$ (two-tailed test).
‡Index of substitution saturation assuming an asymmetrical true tree.
§Probability of significant difference between I_{ss} and $I_{ss.cAsym}$ (two-tailed test).

Divergence and PIC were higher in nuclear ITS than in the plastid regions and were mainly concentrated between the outgroups and the complex (see also Supporting Information, Fig. S1).

The results of the substitution saturation test were analysed using DAMBE (see Table 3). For six of the regions (*atpB-rbcL*, *atpH-I*, *rpl16* intron, *trnL-F*, *trnS-G* and ITS), the value of the substitution saturation index (I_{ss}) was significantly smaller than the critical value, under the assumption of either a symmetrical ($I_{ss.cSym}$) or a very asymmetrical true tree ($I_{ss.cAsym}$). This indicates that these data subsets are unlikely to have experienced saturation. For the *psbA-trnH* region, little saturation was detected under the assumption of a symmetrical topology of the true tree. Under the assumption of a very asymmetrical tree topology, however, the I_{ss} values were significantly larger than the critical values ($I_{ss.cAsym}$), indicating a poor signal for phylogenetic analysis in the *psbA-trnH* data subsets. However, the value of I_{ss} showed that it was unlikely the sequences had experienced saturation in both the plastid and all region combined data sets.

The g_1 statistic displayed a left-skewed distribution, with $g_1 < 0$ in all studied regions (Table 3). Distributions of tree lengths also had a strong skewedness, implying that relatively few solutions existed near the optimal solution compared with elsewhere in the distribution (see also Supporting Information, Fig. S2). This indicates correlation among characters beyond that expected at random, implying that the data are significantly structured for the reconstruction of phylogenetic trees.

The value of g_1 was similar in *atpB-rbcL* and *trnS-G*, which contained the strongest signals. The *rpl16* intron and *trnL-F* displayed a highly structured signal, with g_1 values –1.1412 and –1.1111, respectively. Although *atpH-I* and *psbA-trnH* showed rather low signals compared with the other studied plastid regions, the values for these regions were clearly higher than for the nuclear ITS region. For the combined data sets, the value of g_1 was lower for the combination of all plastid regions (–1.0217) than the average value for separate analyses (–1.1542). When all regions were combined, the value of g_1 (–1.0001) was lower than for each plastid data set, but greater than for the nuclear ITS data set (–0.6986). This indicates that the value of g_1 is not necessarily increased by combining regions of high structure: the signal may be increased in some parts and decreased in others.

Relationships in the *Pterygiella* complex

We obtained 17 ITS, 15 *atpB-rbcL*, 11 *atpH-I*, 15 *psbA-trnH*, 16 *rpl16* intron, 16 *trnL-F* and 15 *trnS-G* sequences from the 17 taxa sampled. The total number of new sequences generated in this study was 88 (see Table 1). For the plastid regions, missing data (mainly attributable to failed sequencing; excluding gaps created during alignment) existed in each region (Table 2). *Phtheirospermum muliense* failed to amplify for the *atpB-rbcL*, *atpH-I* and *trnS-G* regions, which resulted in 2829 bp of missing data in the plastid data set. *Phtheirospermum parishii* was only successfully sequenced for *trnL-F*, resulting in 4518 bp of missing data. *Pseudobartsia yunnanensis* failed to amplify for the *atpH-I* and *trnL-F* regions, resulting in 3347 bp of missing data. The remaining species were successfully sequenced for all six regions.

Each plastid region was incomplete for some taxa because of missing data (Table 1; Fig. 1). Maximum parsimony analyses resulted in a single tree (see Table 2). The results of the parsimony analyses were congruent with Bayesian and maximum likelihood analyses for every studied region (Fig. 1). The division of the *Pterygiella* complex into two clades was well supported by Bayesian posterior probability (PP), maximum parsimony bootstrap (MPBS) and maximum likelihood bootstrap (MLBS) values. Trees based on *psbA-trnH* displayed different relationships in the complex than those based on other regions: *Phtheirospermum japonicum* clusters with the *Pterygiella* complex II clade (0.78 PP, 77 MPBS, 61 MLBS), whereas *Xizangia bartschioides* clusters with *Melampyrum* and *Rhinanthus* (1.00 PP, 97 MLBS). Within *Phtheirospermum*, relationships were reconstructed differently with ITS and *trnL-F*. Both regions found *P. parishii*, *P. muliense* and *P. tenuisectum* to be a grade, but based on the *trnL-F* data set, *P. muliense* was sister to *P. tenuisectum* (1.00 PP, 87 MPBS, 99 MLBS), whereas based on the ITS data set, *P. parishii* was sister to *P. tenuisectum* (with lower support).

The combined plastid alignment was 5417 bp long and included 247 coded indels. Analysis of the data set resulted in a single most parsimonious tree with a length of 1553 steps [consistency index (CI) = 0.903, retention index (RI) = 0.920]. The phylogenetic relationships were slightly different from those obtained using Bayesian inference (Fig. 2A), but congruent with the maximum likelihood analysis (Fig. 2B). In likelihood and parsimony analyses, *P. parishii*, *P. muliense* and *P. tenuisectum* formed a monophyletic group (89 MPBS, 98 MLBS); based on Bayesian inference these species were paraphyletic (Fig. 2A). Compared with the separate analyses, this paraphyletic relationship was found consistently with two regions (*trnL-F* and ITS; Fig. 1).

The complete combined alignment was 6070 bp long, plus 247 coded indels. Analysis of this data set produced a single most-parsimonious tree of 2045 steps (CI = 0.866, RI = 0.894). Phylogenetic relationships were congruent with the Bayesian and maximum likelihood analyses (Fig. 2C). Compared with the separate and combined plastid regions, the topology was also highly consistent across methods (Figs 1, 2).

Because of the high degree of congruence found among phylogenetic trees, we discuss relationships within the *Pterygiella* complex based on resulted from the combination of all regions (Fig. 2C). Overall, the *Pterygiella* complex is clearly not monophyletic and can be divided into two groups found in all analyses (Figs 1, 2). One group, designated the '*Pterygiella* complex I', comprises only *Phtheirospermum japonicum* (1.00 PP, 100 MPBS, 100 MLBS). The second, '*Pterygiella* complex II', is monophyletic (1.00 PP, 100 MPBS, 100 MLBS) and includes the remaining taxa of the complex. This group was found to be sister to a clade comprising *Melampyrum* and *Rhinanthus* (1.00 PP, 100 MLBS). In *Pterygiella* complex II, the first taxon to diverge from the group is *Xizangia* (1.00 PP, 100 MPBS, 100 MLBS). The next diverging taxon, *Pseudobartsia*, lies on a branch that was supported in all analyses (1.00 PP, 100 MPBS, 100 MLBS). The remaining *Phtheirospermum* spp. also form a clade (81 MPBS, 96 MLBS). *Phtheirospermum tenuisectum* is sister to *P. muliense* (1.00 PP, 87 MLBS). *Pterygiella* was resolved as monophyletic (1.00 PP, 100 MPBS, 100 MLBS) and as sister to *Phtheirospermum* (1.00 PP, 100 MPBS, 100 MLBS). In *Pterygiella*, *Pterygiella duclouxii* was found to be sister to *P. nigrescens* (1.00 PP, 100 MPBS, 100 MLBS) and *P. cylindrica* sister to *Pterygiella cylindrica* var. *suffruticosa* (1.00 PP, 100 MPBS, 100 MLBS).

Description of capsule and seed morphology

On the basis of the light microscope and SEM observations that follow, a key to capsule and seed morphology of genera in the *Pterygiella* complex is provided (see below).

Phtheirospermum Bunge ex Fisch. & C.A. Mey. (Figs 3A, E, 4A–F)

Capsule 5–10 mm in length, 3–5 mm in width, ovoid, laterally symmetrical, loculicidal, yellow or brown, papery or coriaceous. Capsule surface covered with eglandular multicellular pilose hairs with enlarged nodes and bases.

Seeds numerous, 0.5–1.0 mm in length, 0.3–0.5 mm in width, length to width ratio *c*. 1.6–2.0, elliptic, yellow or black. Hilum terminal, small, obscurely protruding. Seed coat reticulate, tight-fitting, cell wall granular or punctate or with fibrous protuberances. Tangential wall of seed coat with irregularly elongated epidermal cells with reticulate secondary thickening in *P. japonicum* (Fig. 4A, B).

Pseudobartsia D.Y. Hong (Figs 3C, G, 4G, H)

Capsule *c*. 3 × 2 mm, oblong, laterally symmetrical, loculicidal, brown, coriaceous, with persistent style. Capsule surface covered with eglandular unicellular strigose hairs, the hairs with enlarged bases.

Seeds numerous, *c*. 1.0 × 0.2 mm, length to width ratio *c*. 1.75, elliptic, black. Hilum terminal, small, obscurely protruding. Seed coat irregular, tight-fitting, possessing hook-like protuberances.

Pterygiella Oliv. (Figs 3D, H, 4I–N)

Capsule 7–13 mm in length, 3–6 mm in width, ovoid to narrowly ovoid, laterally symmetrical, coriaceous,

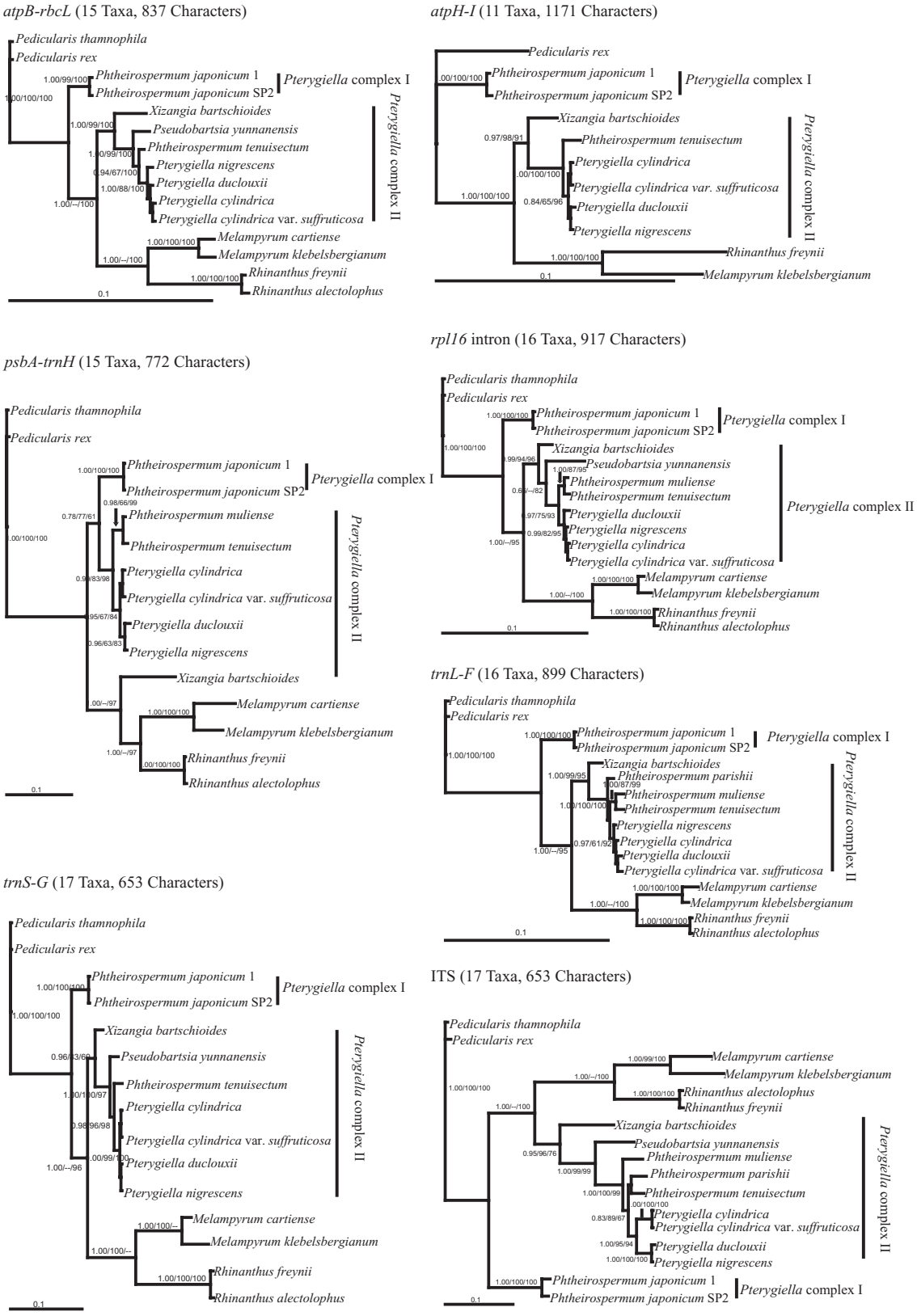

Figure 1. Fifty per cent majority rule consensus trees from Bayesian inference based on *atpB-rbcL*, *atpH-I*, *psbA-trnH*, *rpl16*, *trnL-F*, *trnS-G* and internal transcribed spacer (ITS). Numbers above branches indicate Bayesian posterior probability (PP)/maximum parsimony bootstrap (MPBS)/maximum likelihood bootstrap (MLBS) values.

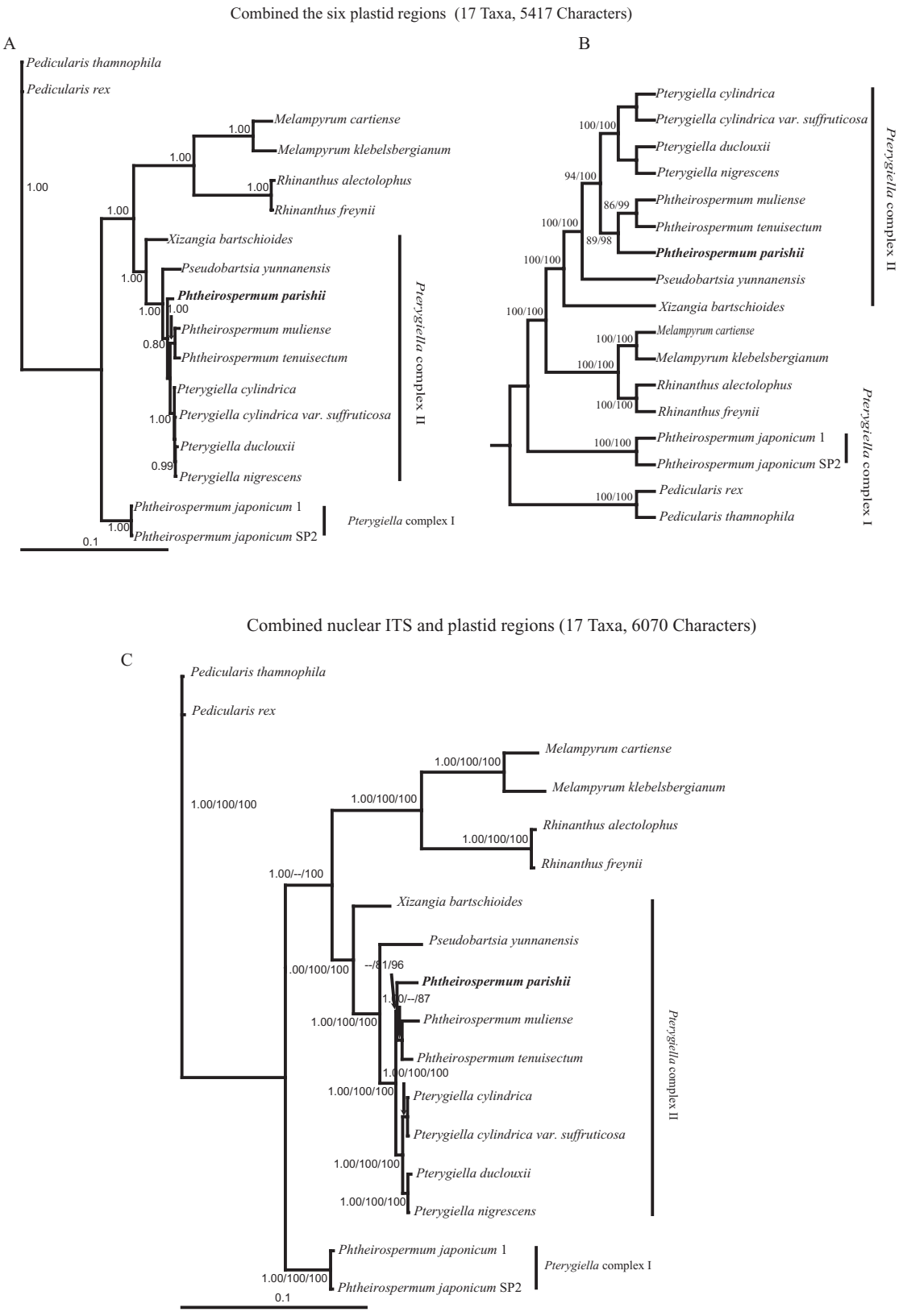

Figure 2. See caption on next page.

Figure 2. Phylogenetic inference based on the combined plastid and all regions data sets. A, 50% majority rule consensus tree from Bayesian inference based on the combined six plastid regions. Numbers above branches indicate Bayesian posterior probability (PP) values. B, the single most-parsimonious tree from parsimony analysis based on the combined six plastid regions. Numbers above branches indicate maximum parsimony bootstrap (MPBS)/maximum likelihood bootstrap (MLBS) values. C, 50% majority rule consensus tree from Bayesian inference analysis based on the combined all regions. Numbers above branches indicate PP/MPBS/MLBS values.

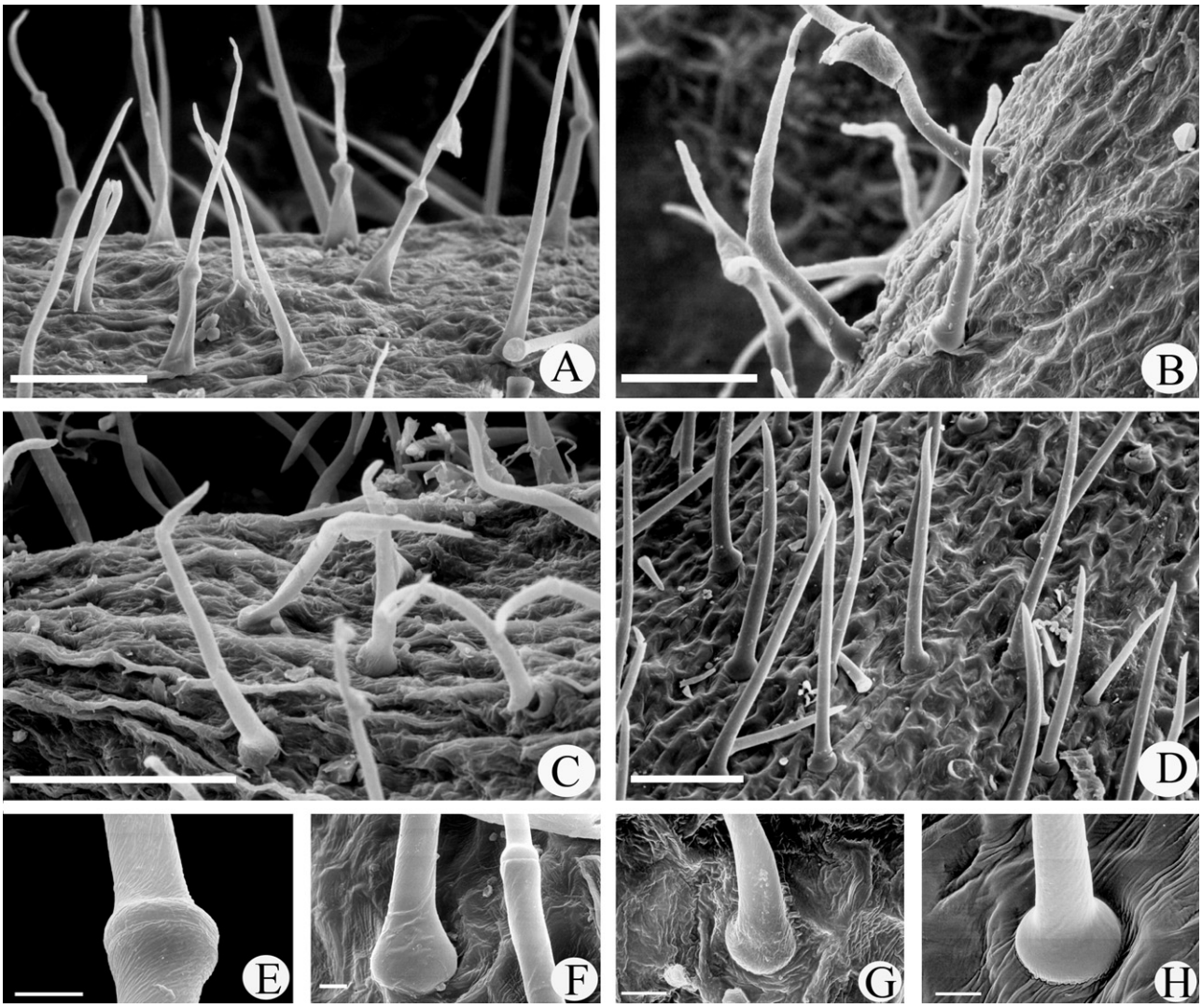

Figure 3. Characteristic features of capsule. Scale bars, 100 μm (whole capsule), 10 μm (higher magnification of hairs). A–B, E–F, muticellular hairs. C–D, G–H, unicellular hairs. A, E, *Phtheirospermum tenuisectum*. B, F, *Xizangia bartschioides*. C, G, *Pseudobartsia yunnanensis*. D, H, *Pterygiella cylindrica*.

brown. Capsule surface covered with eglandular unicellular pilose hairs with enlarged bases.

Seeds numerous, *c*. 0.5 × 0.2 mm, length to width ratio from 1.72 to 2.81, elliptic, brown or black. Hilum terminal, relatively large, protruding or strongly protruding. Seed coat striate, tight-fitting, possessing hook-like protuberances on the horizontal ridge, covered densely with granules, sometimes linked into a reticulum. Except in *P. cylindrica* var. *suffruticosa*, the seed coat is reticulate (Fig. 4M, N).

Xizangia D.Y. Hong (Figs 3B, F, 4O, P)

Capsule *c*. 8 × 6 mm, ovoid, laterally symmetrical, loculicidal, brown, coriaceous. Capsule surface

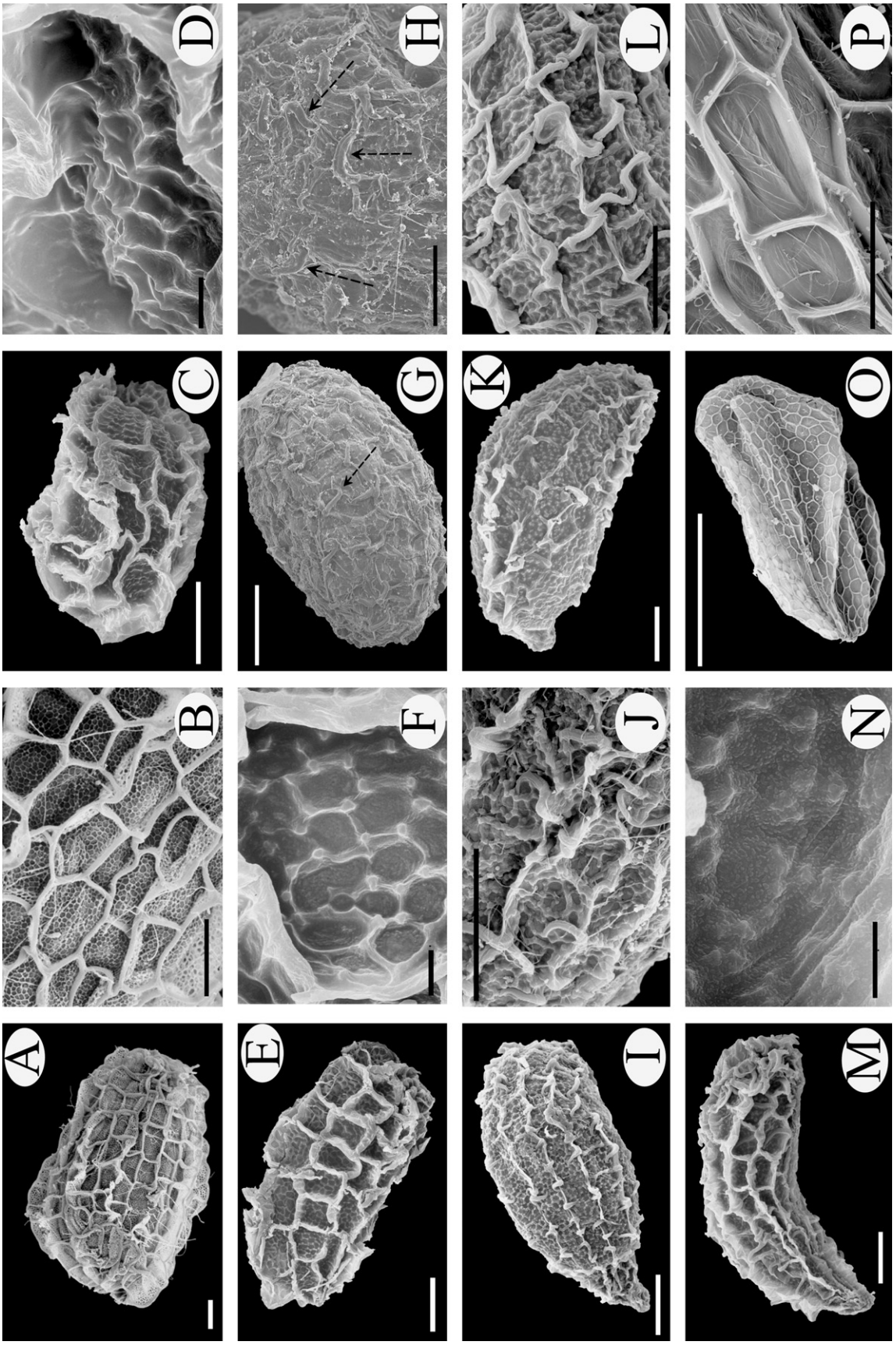

Figure 4. Characteristic features of seed. Scale bars, 1 mm (O), 200 μm (G), 100 μm (all other whole seeds), 50 μm (H) and 10 μm (all other higher magnification of seed coat). A, B, *Phtheirospermum japonicum*, showing reticulate feature (A) and reticulate secondary thickening (B). C, D, *Phtheirospermum tenuisectum*, showing reticulate feature (C) and papillate ornamentation (D). E, F, *Phtheirospermum parishii*, showing reticulate feature (E) and papillate ornamentation (F). G, H, *Pseudobartsia yunnanensis*, showing irregular feature (G) and hook-like protuberance (arrowed; H). I, J, *Pterygiella cylindrica*, showing striate feature (I) and hook-like protuberance and papillate ornamentation (J). K, L, *Pterygiella duclouxii*, showing striate feature (K) and hook-like protuberance and papillate ornamentation (L). M, N, *Pterygiella cylindrica* var. *suffruticosa*, showing reticulate feature (M) and papillate ornamentation (N). O, P, *Xizangia bartschioides*, showing reticulate feature (O) and smooth to sparsely granular protuberances (P).

KEY TO CAPSULE AND SEED MORPHOLOGY OF THE GENERA IN THE PTERYGIELLA COMPLEX

1. Capsule coriaceous, brown 2
   Capsule papery, yellow ..... *Phtheirospermum japonicum* (Thunb.) Kanitz
2. Capsule indumentum multicellular 3
   Capsule indumentum unicellular 4
3. Seed globose, seed coat loose-fitting ..... *Xizangia* D.Y. Hong
   Seed elliptic, seed coat tight-fitting ..... *Phtheirospermum* Bunge ex Fisch. & C.A. Mey
4. Hilum terminal, small, obscurely protruding ..... *Pseudobartsia* D.Y. Hong
   Hilum terminal, relatively large, protruding or strongly protruding ..... *Pterygiella* Oliv.

covered with eglandular multicellular pilose hairs with enlarged nodes and bases.

Seeds numerous, *c*. 2 × 1 mm, length to width ratio *c*. 2, globose, brown. Hilum terminal, small, obscurely protruding. Seed coat reticulate, loose-fitting, membranous. Cell wall nearly smooth with sparsely granular protuberances.

DISCUSSION

In this study, we analysed phylogenetic relationships in the *Pterygiella* complex. We used nucleotide substitution saturation and tree-length distributions to assess phylogenetic signal (Hillis & Huelsenbeck, 1992; Xia & Xie, 2001; Xia & Lemey, 2009). In addition, we used Bayesian inference, maximum parsimony and maximum likelihood analyses. The trees inferred from the different methods and assumptions were almost identical in terms of topology and branch supports. Although missing data can produce misleading estimates of phylogeny in maximum likelihood and Bayesian inference (Lemmon *et al*., 2009), this study implies that the phylogenetic signals involved in our data set are strong enough not to be overwhelmed by missing data.

Phylogenetic relationships based on the *psbA-trnH* data set were noticeably different from the other loci analysed. Although a random tree-length distribution and g_1 showed obvious skewness (see also Supporting Information, Fig. S2, Table 3), I_{ss} values indicated poor phylogenetic signals in the *psbA-trnH* data subsets (Table 3). This region contains high divergence and PIC values, mainly concentrated between the outgroup and the complex (see also Supporting Information, Fig. S1). When the outgroup was excluded, the results obtained were congruent with the analyses of all other data sets (data not shown).

Using the ITS data set, minor differences were seen in the sister relationships of *Phtheirospermum tenuisectum*. In most analyses, *P. muliense* was sister to *P. tenuisectum*, whereas based on the ITS data set *P. parishii* was sister to the latter. In comparing ITS-2 secondary structures, we found that *P. tenuisectum* and *P. parishii* diverge from *P. muliense* by the loss of a loop in the conservative helix II (data not shown). These changes imply that these sequences may be pseudogenes or paralogues (Schultz *et al*., 2005; Feliner & Rosselló, 2007). In our study, data set combination positively increased the accuracy of phylogenetic inference.

PHYLOGENETIC RELATIONSHIPS IN THE *PTERYGIELLA* COMPLEX

Based on our molecular phylogeny, the *Pterygiella* complex is polyphyletic and should be divided into two clades. One clade, '*Pterygiella* complex I', includes just one species, *P. japonicum*. Morphologically, *P. japonicum* is a divergent member of the complex, with significantly larger pollen (Lu *et al*., 2007), wide distribution, a yellow, papyraceus capsule, a seed coat with irregularly elongated epidermal cells and reticulate thickening (Table 4, Fig. 4A, B). The second clade, '*Pterygiella* complex II', includes the remaining taxa of the *Pterygiella* complex; namely, *Xizangia*, *Pseudobartsia*, *Phtheirospermum tenuisectum*, *P. muliense*, *P. parishii* and *Pterygiella*. This clade can be recognized by coriaceus capsules and lack of secondary thickening on the seed coat (Table 4, Fig. 4C–P).

The relationship between *Xizangia* and *Pterygiella* has been disputed (Hong, 1986, 2001; Tao, 1999; Wu, 1999). As noticed by Hong (2001), seed characters display apparent differences between *Xizangia* and *Pterygiella*, even in the indumentum of the capsule (Table 4, Fig. 3B, D, F, H). Based on proposed barcode DNA regions (*rbcL*, *matK* and ITS), *Xizangia* can clearly be discriminated from *Pterygiella* as a genus (Dong *et al*., 2011b). The monotypic nature of *Xizangia* is further supported by the present study. *Pterygiella* is a monophyletic group and can be recognized by the pilose hairs on the capsule and hooked appendages on the seed (Fig. 4I–N). In terms of the seed coat, two types are recognized in *Pterygiella*: Type I possesses striations on the seed coat and includes *P. cylindrica* (Fig. 4I), *P. duclouxii* (Fig. 4G) and *P. nigrescens*. Type II possesses a reticulate seed coat and includes only *P. cylindrica* var. *suffruticosa* (Fig. 4M). *Pterygiella cylindrica* var. *suffruticosa* was

Table 4. Fruit and seed characters in studied taxa, with voucher details

Taxon	Voucher specimen (herbarium code)	Fruit		Seed				
		Shape, colour, texture and mean size (length × width, in mm)	Gross appearance	Shape, colour, and mean size (length × width, in mm)	Hilum	Gross appearance	Tangential wall	Wall ornamentation
Phtheiroserpmum japonicum (Thunb.) Kanitz. (Fig. 4A, B)	Su guixing; 145 (KUN)	Ovoid, yellow, papery, 10.39 × 5.30	Multicellular pilose	Elliptic, yellow, 0.83 × 0.49	Inconspicuous	Reticulate	Apparently asymmetric	Reticulate, finely papillate and fibrous
Phtheirospermum parishii Hook.f. (Fig. 4E, F)	Ying junsheng; 4144 (KUN)	Ovoid, brown, coriaceous, 4.64 × 2.78	Multicellular pilose	Elliptic, black, 0.42 × 0.21	Inconspicuous	Reticulate	Apparently protuberant	Sparsely papillate
Phtheirospermum tenuisectum Bureau & Franch. (Figs 3A, E, 4C, D)	Qiu bingyun; 50196 (KUN)	Ovoid, brown, coriaceous, 5.00 × 2.71	Multicellular pilose	Elliptic, black, 0.41 × 0.19	Inconspicuous	Reticulate	Apparently protuberant	Sparsely papillate
Pseudobartsia yunnanensis D.Y. Hong (Figs 3C, G, 4G, H)	Zhang yingbai; 4 (KUN)	Oblong, brown, coriaceous, 3.25 × 1.85	Unicellular strigose	Elliptic, black, 0.37 × 0.28	Inconspicuous	Irregular	Irregular	Hook-like tubercules
Pterygiella cylindrica Tsoong (Figs 3D, H, 4I, J)	–;14889 (KUN)	Narrowly ovoid, brown, coriaceous, 9.61 × 4.23	Unicellular pilose	Elliptic, brown, 0.52 × 0.24	Terminal, strongly protruding	Striate	Horizontal ridge formed hook-like tuber	Papillate, sometime linked into a reticulum
Pterygiella cylindrica var. *suffruticosa* (D.Y. Hong) L.N. Dong & H. Wang (Fig. 4M, N)	Qingzang expedition; 14400 (KUN)	Narrowly ovoid, brown, coriaceous, 8.90 × 3.97	Unicellular pilose	Elliptic, black, 2.54 × 0.19	Terminal, protruding	Reticulate	Apparently protuberant	Papillate, procumbent glochidium present
Pterygiella duclouxii Franch. (Fig. 4K, L)	Yu Wenbing; 154 (KUN)	Narrowly ovoid, brown, coriaceous, 10.54 × 4.62	Unicellular pilose	Elliptic, black, 0.57 × 0.29	Terminal, slightly convex	Striate	Horizontal ridge formed hook-like tuber	Papillate, sometime linked into a reticulum
Pterygiella nigrescens Oliv.	Guangxi Expedition; 1281 (KUN)	Ovoid, brown, coriaceous, 9.74 × 5.44	Unicellular pilose	Elliptic, black, 0.55 × 0.32	Terminal, slightly convex	Striate	Horizontal ridge formed hook-like tuber	Papillate, sometime linked into a reticulum
Xizangia bartschioides (Hand.-Mazz.) C.Y. Wu & D.D. Tao (Figs 3B, F, 4O, P)	Qinghai-Tibet Expedition; 74-3801 (KUN)	Ovoid, brown, coriaceous, 7.84 × 5.79	Mullticellular pilose	Globose, brown, 1.82 × 0.92	Inconspicuous	Reticulate	Regular	Smooth to sparsely granular protuberances

first proposed as a new species because of its shrubby habit (Hong, 1996). Although the species possesses great similarity in morphology and molecular features to *P. cylindrica*, attributable to the reticulate seed coat, the taxon has been treated as a variety of *P. cylindrica* (Dong *et al*., 2011a).

Tao (1996) separated *Phtheirospermum* into two sections, *Phtheirospermum* (*P. japonicum* and *P. tenuisectum*) and *Minutisepala* H.W. Li & Tao (*P. glandulosum*, *P. muliense* and *P. parishii*) based primarily on the nature of the leaf, stem, corolla, calyx teeth, capsule and seed. However, phylogenetic analysis of sequence data from plastid and nuclear regions does not support this division, and the genus is found to be polyphyletic. Molecular data also show *P. japonicum* to be particularly divergent from the other members of the genus. This species is one of the most distinct and recognizable members of the genus, based primarily on the characteristic fruit (Table 4, Fig. 4A, B). In addition, *P. japonicum* has a wide distribution from China to Russia, whereas the other species are primarily distributed in the Himalayas and Hengduan Mountains.

The remaining species, *P. tenuisectum*, *P. muliense* and *P. parishii*, form a monophyletic group and can be identified by their coriaceous capsule and reticulate seed (Table 4, Fig. 4C, E). These species can also be recognized by lanceolate calyx teeth and a usually yellow corolla, but the shape of the leaf is highly variable. *Pseudobartsia* was proposed as a monotypic genus by Hong (1979), whereas Tao (1993) considered it to be a synonym of *Phtheirospermum glandulosum*. *Pseudobartsia* differs significantly from *Phtheirospermum* by having unicellular strigose hairs on the capsule and an irregular seed coat with hook-like protuberances (Figs 3C, G, 4G, H). The generic rank of *Pseudobartsia* has also been confirmed by this phylogenetic study. However, the sequences of *Phtheirospermum* used were mainly attained from herbarium specimens (e.g. *P. parishii*, *P. muliense* and *Pseudobartsia*). There is a need to gather more material from the field to conduct further study for the revision of the genus. In particular, *Pseudobartsia*, distributed in the western Himalayas, should be included in further studies, as it has not been collected from documented locations in China since 1940.

The significance of capsule and seed morphology in classification

Fruit characters were found to be diverse in the studied taxa, especially in terms of the indumentum of the capsule and ornamentation of the seed coat. These characters exhibited significant information for classification of the *Pterygiella* complex. We observed high variability in unicellular and multicellular hairs, a feature which is usually conservative at the generic level and useful in identifying different genera. Unicellular hairs were discovered in *Pseudobartsia* (Fig. 3C) and *Pterygiella* (Fig. 3D), but the former can be distinguished by the strigose nature of the hairs. The texture and colour of the capsule is stable and can be used to distinguish the two clades in the complex. Capsule size also provides a visual diagnostic character at generic level. *Pseudobartsia yunnanensis* has the smallest capsule in the studied taxa. The capsule of *Phtheirospermum japonicum* is much larger than that of *P. parishii* and *P. tenuisectum* (Table 4). However, the shape of the capsule provided little information for classification, because of variable and ambiguous characters states, depending on the condition of deposition and subjective judgments regarding the specimens.

Seed coat characters were also highly variable, from the generic to the varietal level. This character is the most useful character for identification of different species. In *Phtheirospermum*, *P. japonicum* can easily be identified by its reticulate secondary thickening. In addition to their shrubby habit, molecular and morphological characters showed high similarity between *Pterygiella cylindrica* and *P. cylindrica* var. *suffruticosa* (Dong *et al*., 2011a), but the seed coat is reticulate in the latter. Meanwhile, the generic ranks of *Pseudobartsia* (Fig. 4G, H) and *Xizangia* (Fig. 4O, P) are both supported by seed coat features. Although seed size has been considered a variable character in classification (Juan *et al*., 2000), it is found to be useful for identification of different species and even varieties in the studied taxa (Table 4). Chuang & Heckard (1983) noticed the question of the heterogeneity of seed shape; which they thought was directly related to the insertion of the seed in the fruit. In our study, the shape of the seed is defined using the ratio of length to width (Liu, Lin & He, 2004), because shape was found difficult objectively to judge by observation. In any case, shape provided little information for classification in the complex.

Fruit characters seem to suggest probable dispersal agents and adaptive significance. It has been tested in *Digitalis* L. that a seed coat with a reticulate surface is related to water dispersion because of these seeds having the capacity to trap air, thus increasing buoyancy (Juan, Fernández & Pastor, 1998). *Xizangia bartschioides* may be adapted to wetland habitats by means of water dispersion with its reticulate seed coat (Fig. 4O, P). The expanded epidermal cell has been observed in many genera of Antirrhineae and is also a possible adaptation to dispersion by water or wind (Elisens & Tomb, 1983). This kind of seed has been observed in *Phtheirospermum japonicum* (Fig. 4A, B), *P. tenuisectum* (Fig. 4C, D), *P. parishii*

(Fig. 4E, F) and *Pterygiella cylindrica* var. *suffruticosa* (Fig. 4M, N). In addition, we observed the hook-like protuberant decorations seen on *Pseudobartsia yunnanensis* (Fig. 4G, H), *Pterygiella cylindrica* (Fig. 4I, J) and *P. duclouxii* (Fig. 4K, L). Usually, the surfaces with more or less developed protuberances seem to be less susceptible to contamination by small particles or pathogens than smooth ones (Barthlott, 1981). With relation to the hooks, they may be adaptive for adhesive dispersal by animals (Sorensen, 1986). Thus, the hook-like protuberance points to the need for further investigation of dispersal agents and the adaptive significance of these characters.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. The comparison of sequence variation among studied regions.
Figure S2. Histogram of randomized tree-length distribution in studied data sets.