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REGULAR PAPER

Additional evidence for recent divergence of Chinese *Epimedium* (Berberidaceae) derived from AFLP, chloroplast and nuclear data supplemented with characterisation of leaflet pubescence

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Background – The genus *Epimedium* is well known for its ornamental representatives. However, species boundaries and evolutionary relationships within the genus remain uncertain due to several difficulties. First, potentially diagnostic characters are generally not described with enough detail, hampering linkage of specimens to recognized taxa. Second, previous molecular studies failed to gain resolution, especially within the Chinese distribution area of the genus. Nevertheless, growing scientific interest in the medicinal properties of *Epimedium* has prompted the need for reliable identification of species.
Aims and methods – This study aims at: (1) assessing genetic diversity within *Epimedium*, using nuclear and chloroplast DNA sequences in combination with AFLP fingerprinting, (2) delivering a detailed description for one potentially diagnostic character, pubescence of leaflets.
Key results – The DNA sequences and AFLP fingerprints resulted in an unresolved polytomy for the Chinese representatives of *Epimedium*. Furthermore, this study provided detailed scanning electron microscope images of four clearly distinguishable types of leaflet pubescence.
Conclusions – As AFLP is considered capable of detecting rare genetic differences in groups with low sequence variation, we suggest the lack of resolution in the Chinese clade to represent a hard polytomy. This is interpreted as additional evidence for the hypothesis of a recent origin for these taxa. As this implies that several recognized species are still in the process of differentiation, these difficulties in resolving evolutionary relationships are linked to difficulties in species delimitation. Furthermore, the lack of details in some species descriptions has led to the description of new taxa based on small variations. To tackle these problems, we propose a change in view on *Epimedium* taxonomy. In this view, broader taxonomic entities are recognized, characterized by clearly defined characters. The four types of pubescence described in this study can serve as a first step towards this new interpretation of *Epimedium* taxonomy.

Key words – *Epimedium*, recent divergence, AFLP, *trnK-matK*, ITS, leaflet pubescence, SEM.

INTRODUCTION

The genus *Epimedium* L. is represented by perennial woodland herbs, restricted to two widely separated Old World regions: temperate Asia and the areas surrounding the Mediterranean and Black Sea. Within each of these regions, areas can be found in which the genus is entirely absent (map given in Zhang et al. 2007). None of the species inhabit a very wide range (Stearn 2002). Based on geographical distribution, C-banding of chromosomes, flower and leaf morphology, the most recent classification of the genus recognizes two subgenera, four sections and four series (Stearn 2002). Subgenus *Rhizophyllum* comprises an endemic species from Algeria (*E. perralderianum*) and *E. pinnatum* from Caucasia. Subgenus *Epimedium* consists of four sections: (1) section *Macroceras* limited to Japan and Korea, (2) section *Polyphyllon* from the western Himalaya, (3) section *Epimedium* with species from the Mediterranean and Balkan areas, while (4) section *Diphyllon* is uniting all Chinese species. This last section contains 43 of the 54 species recognised by Stearn (2002), as well as six more recently described taxa (He & Xu 2003, Guo et al. 2007, Xia & Li 2009, Zhang & Li 2009, He et al. 2010). *Epimedium* reaches its highest species diversity in central to southeastern China.

A recent scientific interest in the reputed pharmaceutical properties of *Epimedium* (e.g. Hu et al. 2010, Li et al. 2010) has prompted the need for rigorous methods of correct species identification. Indeed, as the concentration of active components seems species-dependent (Chen et al. 2007, Quan et al. 2010), the procurement of extracts with reproducible properties requires correct identification of collected individuals. Several morphological (e.g. pubescence of leaflets, Lü et al. 1981), chemical (e.g. flavonoid profiling, Shen et al. 2007), and genetic characters (5S rRNA gene spacer, Sun et al. 2004) have been proposed for characterisation of taxa traditionally used in herbal medicine. As some of these characters seemed successful at distinguishing between a limited subset of species, they should be tested on a larger sample of taxa, for their applicability in *Epimedium* systematics. Furthermore, diagnostic characters were often described with little detail, even in descriptions of new species. Additionally, unequivocal terminology is used to describe a certain character (e.g. pubescence of leaflets), a problem described as the 'linguistic problem of morphology' (Vogt et al. 2010). The abovementioned lack of accuracy in species descriptions hampers the assignment of specimens to described species and thus might result in publication of 'unnecessary' new species. A detailed survey of potentially diagnostic characters for a large sample of *Epimedium* taxa would contribute to the recognition of species and their boundaries, thus aiding pharmaceutically oriented studies by supplying reliably identified source material.

Several molecular markers (ITS and *atpB-rbcL* spacer, Zhang et al. 2007; 5S rRNA gene spacer, Sun et al. 2005) have been sequenced for representatives of *Epimedium*, in order to infer evolutionary relationships between taxa, and to establish a natural classification. All sections and subgenera as recognized by Stearn (2002) have been shown to be monophyletic, with the exception of subgenus *Epimedium* which was recovered as paraphyletic in relation to subgenus *Rhizophyllum* (Zhang et al. 2007). As subgenus *Rhizophyllum* was not included in the study by Sun et al. (2005) the latter was not informative concerning this paraphyly. Additionally, none of the studies was capable of providing resolution within the Chinese section *Diphyllon*, which resulted in a polytomy for these species. Both studies (Sun et al. 2005, Zhang et al. 2007) concluded that more variable markers were needed to gain insight into evolutionary relationships within *Epimedium*.

As the amplified fragment length polymorphism (AFLP) approach provides a broad genomic coverage, it is more likely to detect rare genetic differences in groups with low sequence variation (Mendelson & Shaw 2005), compared to DNA sequence information. Therefore, this approach is a potential candidate for elucidating species boundaries or evolutionary relationships between recognized taxa. On the level of species delimitation this would require dense population level sampling of wild populations, in order to potentially detect processes of hybridisation and differentiation. On the level of reconstructing evolutionary relationships between recognized entities, AFLP datasets have been successfully used in phylogenetic reconstructions for closely related species (e.g. Després et al. 2003, Jacobs et al. 2008). Therefore, this approach might yield more resolution for the evolutionary relationships between the Chinese representatives of *Epimedium* (table 1). However, several drawbacks are associated with the AFLP technique (reviewed by Koopman 2005). Specifically, the problems of non-independence of fragments and of identifying homologous fragments potentially limit phylogenetic interpretation of restriction fragment data. The percentage of homoplasy in AFLP data is suggested to be correlated with the evolutionary distance between the taxa under study. Koopman (2005) suggests that a robust phylogenetic hypothesis can be inferred from AFLP data for accessions differing at 0 to 7 nucleotide positions in their ITS-1 sequences. For accessions with 6–19 differing nucleotides in their ITS-1 sequences, AFLP data is suggested to contain phylogenetic signal, but not enough for the construction of a robust phylogenetic hypothesis due to saturation. The ITS sequences obtained by Sun et al. (2005) for 22 species of *Epimedium* were 0–14 nucleotides apart, suggesting that AFLP genotypes should contain phylogenetic information for this genus.

The aim of this study is to examine genetic diversity within *Epimedium*, assessing whether the low nucleotide diversity obtained in earlier studies (Sun et al. 2005, Zhang et al. 2007) is characteristic of the genus, especially the Chinese section *Diphyllon*. In order to achieve this, a highly representative sampling of *Epimedium* species was assembled. For these samples, nucleotide sequences of the nuclear internal transcribed spacer (ITS) and a chloroplast region (*matK* gene, together with the 5' *trnK* exon and intron, and 3' *trnK* intron and exon) were sequenced. In addition an AFLP fingerprint dataset was obtained. The resulting data were used to assess genetic diversity and evolutionary relations within the genus. Furthermore, this study aims at offering a detailed description of characters with supposed diagnostic value, the pubescence of leaflets. This will provide a new impulse regarding description of species.

MATERIAL AND METHODS

Sampling

Fresh leaf material was collected for 56 accessions growing in the Ghent University Botanical Garden. These accessions represented 53 of sixty currently recognized *Epimedium* species and all three recognized *Vancouveria* C.Morr. & Decne. species. Identification followed the revision of *Epimedium* by Stearn (2002), or species descriptions published after this revision. A list of accessions including voucher information, origin of material and GenBank accession numbers is provided in table 1.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted according to the Mini Protocol delivered with the Dneasy® Plant Mini Kit (Qiagen, Hilden, Germany). PCR amplification was carried out as 50 µl reactions for both the chloroplast and nuclear region, and consisted of 1 µl extracted DNA; 27.8 µl H_2O; 14 µl 10x *Taq* buffer (15 mM $MgCl_2$); 10 µl dNTP's (containing 1.25 mM of each dNTP); 0.2 µl *Taq* polymerase (5 units/µl) and 2 µl of each primer (forward and reverse). All PCR reactions were carried out in a Mastercycler® gradient (Eppendorf, Hamburg, Germany). For amplification of ITS, the program

Table 1 – List of taxa included in this study.
For each accession country of origin, systematic placement and accession numbers for Botanical Garden Ghent University and Genbank are given. No *matK* sequences where obtained for *Epimedium macrosepalum*, *E. alpinum* and *E. pubigerum* in this study.

Subgenus	Section	Series	Taxon	Accession number	Country/ region of origin	Genbank accession *matK*	Genbank accession ITS
Epimedium	*Diphyllon*	*Campanulatae*	*Epimedium campanulatum* Ogisu	20051739	China	JN010366	JN010313
			Epimedium platypetalum K.Meyer	20051151	China	JN010323	JN010297
			Epimedium ecalcaratum G.Y.Zhong	20041594	China	JN010342	JN010274
			Epimedium shuichengense S.Z.He	20081320	China	JN010365	JN010306
		Davidianae	*Epimedium davidii* Franch.	20051674	China	JN010339	JN010270
			Epimedium fangii Stearn	20071617	China	JN010354	JN010278
			Epimedium hunanense (Hand.-Mazz) Hand.-Mazz	20071618	China	JN010341	JN010283
			Epimedium flavum Stearn	20051204	China	JN010357	JN010280
			Epimedium ilicifolium Stearn	20081132	China	JN010326	JN010284
			Epimedium epsteinii Stearn	20081145	China	JN010322	JN010277
			Epimedium latisepalum Stearn	20051152	China	JN010346	JN010285
			Epimedium ogisui Stearn	20041580	China	JN010324	JN010292
			Epimedium pauciflorum K.C.Yen	20051179	China	JN010351	JN010294
			Epimedium mikinorii Stearn	20051150	China	JN010340	JN010290
		Dolichocerae	*Epimedium elongatum* Kom.	20081083	China	JN010327	JN010276
			Epimedium membranaceum K.Meyer	20051144	China	JN010347	JN010289
			Epimedium rhizomatosum Stearn	20021002	China	JN010348	JN010302
			Epimedium lishihchenii Stearn	20081147	China	JN010359	JN010287
			Epimedium acuminatum Franch.	20081123	China	JN010358	JN010262
			Epimedium franchetii Stearn	20051147	China	JN010352	JN010281
			Epimedium sutchuenense Franch.	20091239	China	JN010361	JN010309
			Epimedium chlorandrum Stearn	20051140	China	JN010344	JN010268
			Epimedium wushanense T.S.Ying	20051178	China	JN010360	JN010311
			Epimedium leptorrhizum Stearn	20051139	China	JN010350	JN010286
			Epimedium brachyrrhizum Stearn	20051746	China	JN010363	JN010266
			Epimedium simplicifolium T.S.Ying	20080597	China	JN010367	JN010307
			Epimedium zhushanense K.F.Wu & S.X.Qian	20081081	China	JN010370	JN010312
			Epimedium baojingense Q.L.Shen & B.M.Yang	20080591	China	JN010338	JN010264
		Brachycerae	*Epimedium pubescens* Maxim.	20021306	China	JN010321	JN010299

Table 1 (continued) – List of taxa included in this study.

Subgenus	Section	Series	Taxon	Accession number	Country/ region of origin	Genbank accession *matK*	Genbank accession ITS
Epimedium	*Diphyllon*	*Brachycerae*	*Epimedium brevicornu* Maxim.	20051146	China	JN010362	JN010267
			Epimedium reticulatum C.Y.Wu ex S.Y.Bao	20080595	China	JN010328	JN010317
			Epimedium sagittatum Maxim.	20081166	China	JN010319	JN010304
			Epimedium myrianthum Stearn	20071616	China	JN010331	JN010291
			Epimedium stellulatum Stearn	20081135	China	JN010325	JN010308
			Epimedium dolichostemon Stearn	20071615	China	JN010332	JN010273
			Epimedium fargesii Franch.	20051683	China	JN010343	JN010279
			Epimedium parvifolium S.Z.He & T.L.Zhang	20071598	China	JN010345	JN010293
			Epimedium truncatum H.R.Liang	20091238	China	JN010349	JN010310
			Epimedium coactum H.R.Liang & W.M.Yan	20080593	China	JN010318	JN010269
			Epimedium borealiguizhouense S.Z.He & Y.K.Yang	20081080	China	JN010320	JN010265
	Macroceras		*Epimedium grandiflorum* C.Morren	20081319	Japan	JN010335	JN010282
			Epimedium sempervirens Nakai ex F.Maek	20081121	Japan	JN010364	JN010305
			Epimedium macrosepalum Stearn	20051740	Korea	/	JN010288
			Epimedium diphyllum Lodd.	20091568	Japan	JN010369	JN010272
	Polyphyllon		*Epimedium elatum* C.Morren & Decne.	20102080	Kashmir	JN010368	JN010275
	Epimedium		*Epimedium alpinum* L.	20031319	southern Europe	/	JN010263
			Epimedium pubigerum C. Morren & Decne.	20071525	Caucasia	/	JN010300
Rhizophyllum			*Epimedium pinnatum* Fisch.	20051148	Iran	JN010336	JN010296
			Epimedium perralderianum Coss.	20061553	North Africa	JN010356	JN010295
not in Stearn (2002)			*Epimedium dewuense* S.Z.He, Probst & W.F.Xu	20081117	China	JN010330	JN010271
			Epimedium pseudowushanense B.L.Guo	20081321	China	JN010334	JN010298
			Epimedium qingchengshanense G.Y.Zhong & B.L.Guo	20081322	China	JN010333	JN010301
			Epimedium rupestre (nom.prov.)	20080594	China	JN010329	JN010303
Outgroup			*Vancouveria hexandra* (Hook.) C.Morren & Decne.	20031432	California	JN010337	JN010315
			Vancouveria planipetala Calloni	20061566	California	JN010355	JN010316
			Vancouveria chrysantha Greene	19950562	California	JN010353	JN010314

Table 2 – Comparison of observed vs. described type of leaflet pubescence.
Pubescence types observed in this study are indicated as roman numerals corresponding to those used in figs 5 & 6, with "0" indicating absence of mature hairs on the lower leaf surface.

Taxon	Observed Type	Pubescence in description of species
Epimedium acuminatum	III	short appressed fairly stout bristle-like hairs
Epimedium alpinum	0	subglabrous
Epimedium baojingense	II	dark yellow hairs
Epimedium borealiguizhouense	II	lanate
Epimedium brachyrrhizum	0	glabrous
Epimedium brevicornu	0	almost glabrous
Epimedium campanulatum	III	minute erect hairs
Epimedium chlorandrum	III	minute appressed hairs
Epimedium coactum	IV	brown lanate
Epimedium davidii	III	short erect hairs
Epimedium dewuense	II	pilose
Epimedium diphyllum	III	minute spreading hairs
Epimedium dolichostemon	0	glabrous
Epimedium ecalcaratum	0	pilose
Epimedium elatum	II	pilose
Epimedium elongatum	0	glabrous or few hairs
Epimedium epsteinii	III	minute appressed bristle-like hairs
Epimedium fangii	III	appressed minute hairs
Epimedium fargesii	0	glabrous or pilose
Epimedium flavum	0	glabrous
Epimedium franchetii	III	minute appressed hairs
Epimedium grandiflorum	IV	pubescent
Epimedium hunanense	III	pubescent or almost glabrous
Epimedium ilicifolium	II	minute erect hairs
Epimedium latisepalum	II	short erect hairs
Epimedium leptorrhizum	II	spreading or curled reddish hairs
Epimedium lishihchenii	II	long multicellular hairs
Epimedium macrosepalum	0	short reddish hairs
Epimedium membranaceum	I	spreading slender multicellular hairs
Epimedium mikinorii	0	glabrous
Epimedium myrianthum	III	minute appressed hairs
Epimedium ogisui	I	erect short hairs
Epimedium parvifolium	0	glabrous
Epimedium pauciflorum	0	glabrous
Epimedium perralderianum	0	subglabrous
Epimedium pinnatum	0	nearly glabrous
Epimedium platypetalum	IV	pilose
Epimedium pseudowushanense	IV	villous
Epimedium pubescens	IV	fine, multicellular, spreading or curled grey hairs
Epimedium pubigerum	IV	soft white hairs
Epimedium qingchengshanense	III	glabrous
Epimedium reticulatum	0	pilose
Epimedium rhizomatosum	0	minute erect hairs
Epimedium sagittatum	III	stout short unicellular appressed hairs
Epimedium sempervirens	0	glabrous or fine appressed hairs
Epimedium shuichengense	III	white appressed hairs

Table 2 (continued) – Comparison of observed vs. described type of leaflet pubescence.

Taxon	Observed Type	Pubescence in description of species
Epimedium simplicifolium	IV	white-sericeous
Epimedium stellulatum	I	hairs short, slightly ascending
Epimedium sutchuenense	0	grey hairs
Epimedium truncatum	0	glabrous
Epimedium wushanense	I	erect hairs
Epimedium zhushanense	IV	tomentose

and primers described in Zhang et al. (2007) were used. The program for the chloroplast region amplification described by Hilu et al. (2003) was adapted in the following manner: 3 min at 96°C; followed by 35 cycli consisting of 30 s at 94°C, 60 s at 54°C and 3min at 72°C; and a post treatment of 5 min at 72°C. The *matK* gene, together with the 5' *trnK* exon, 5' *trnK* intron, 3' *trnK* intron and 3' *trnK* exon was amplified using the primers 1394F (Johnson & Soltis 1995) and MG15R (Hufford et al. 2001). Owing to the length of this sequence, two internal primers, 1100R (Hilu et al. 2003) and 3385F (CGGGTTGCAAAAATAAAGGA), were also used. Next, the PCR products were cleaned by adding 0.3 µl enzyme mix to 2 µl product. This mix contained CIAP (calf intestine alkaline phosphatase) (Fermentas, Maryland, USA) and EXO (Exonuclease I, *E. coli*) (Fermentas) in a 2:1 ratio. Samples were consequently held at 37°C (15 min) for digestion, followed by 15 min at 80°C to deactivate the enzymes. The cleaned PCR products were cycle-sequenced with a BigDye® Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA). These reactions were carried out as 10 µl reactions, consisting of 0.4 µl BigDye; 1.8 µl 10x sequencing Buffer; 2.8 µl H_2O; 3 µl of the respective primer at a concentration of 4 µM; 2 µl cleaned PCR product. The program was: 1 min at 96°C followed by 30 cycles of: (1) 10 s at 96°C, (2) 10 s at 50°C and a final step of 75 s at 60°C. The resulting cycle-sequencing products were cleaned by ethanol precipitation, and loaded into a 3130xl Genetic Analyzer (Applied Biosystems) for sequencing.

AFLP analysis

Considering the broad genomic sampling of AFLPs, this technique was used to assess the presence of genetic differentiation between the sampled species, especially the Chinese representatives. For each sample, the extracted DNA was digested with *EcoRI* and *MseI* and ligated to an adapter. Preamplification used an *EcoRI*(+A)/*MseI* (+C) preselective primer pair. Each sample was submitted to four specific PCR amplifications, using different primer combinations. Combinations A (Eaca/Mcac), B (Eact/Mcac) and C (Eact/Mctg), were adapted from Shen et al. (2007), and the *MseI* primers labelled with FAM. The fourth combination, D (Eaac/Mcag), was taken from Bottini et al. (2002), and labelled with HEX. After mixing with DNA size standards labelled with ROX, amplified fragments were resolved in a LI-COR Global Edition IR^2 system (Li-COR, Lincoln, Nebraska, USA). Fragments were detected and analysed in ABI Prism®. GeneMapper (Applied Biosystems). Fragments were scored in a length range from 60–515 base pairs. The scores for the different primer combinations were combined into a single binary data matrix (presence = 1, absence = 0).

Sequence alignment and phylogenetic analysis

The ITS and *trnK-matK* sequences were edited and aligned manually in PhyDE 0.995 (Müller et al. 2006). Using Modeltest 3.8 (Posada & Crandall 1998), the best model of evolution for both markers was selected under AIC (Akaike Information Criterion). This resulted in the Sym+I model for the ITS dataset, whereas K81uf+I+G was selected for the chloroplast dataset. Both datasets were submitted to maximum likelihood (ML) and Bayesian analysis. The ML analysis was performed in PAUP* 4.0b10 (Swofford 2003), using the abovementioned models of evolution. Starting from a stepwise addition tree, 1000 replicate heuristic searches were performed with TBR and MULTREES options in effect. Statistical branch support was obtained using 200 bootstrap replicates.

All Bayesian analyses in this study were performed in Mr Bayes 3.1.2. (Huelsenbeck & Ronquist 2001) For *trnK-matK* and ITS only the general models proposed by Modeltest were used, thus estimating the parameters during the analysis. This approach was preferred because only a slight computational penalty is associated with this estimation. Furthermore, Modeltest uses several simplifications to recommend a specific model and parameters. Five runs of 25 million generations (ITS data) and five runs of 20 million generations (chloroplast dataset) have been calculated in the Bayesian analysis. Each run contained 10 independent chains with a temperature parameter of 0.01. Burnin values were selected with Tracer v1.5 (Drummond & Rambaut 2007) resulting in 10000 and 2 million generations for ITS and *trnK-matK* respectively.

For the AFLP dataset, the restriction site model implemented in MrBayes was used, with the option 'noabscencesites' selected. This analysis consisted of five runs, each comprising ten independent chains, running for 70 million generations, with a temperature parameter of 0.01. The burnin value for this dataset was 7 million.

Scanning electron microscopy of leaflet pubescence

Basal leaves were collected from living specimens and fixated in Kew mix (53% industrial methylated spirit, 37% water, 5% formaldehyde solution and 5% glycerol). The fixated material was dehydrated followed by critical point drying with carbon dioxide, using a BAL-TEC CPD 030 critical-point-dryer. Using carbon adhesive tape (LET-TABS, Plano GmbH), sam

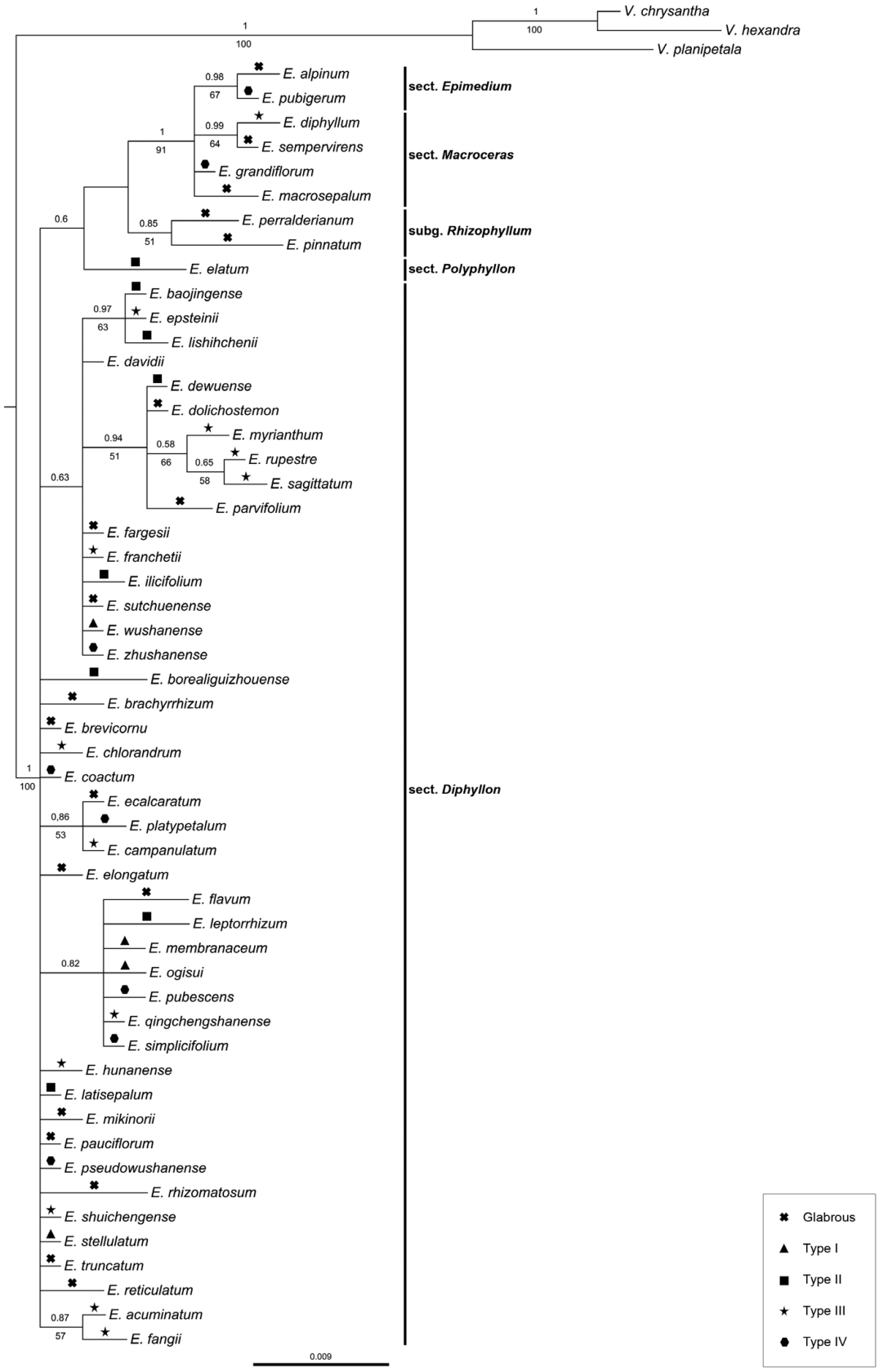

Figure 1 – The 50% majority rule consensus tree resulting from Bayesian inference for the ITS dataset. Posterior probabilities are indicated above the relevant branches, ML bootstrap support is indicated below branches. Vertical bars indicate sections as proposed by Stearn (2002).

Figure 2 – The 50% majority rule consensus tree resulting from Bayesian inference for the *matK* dataset. Posterior probabilities are indicated above the relevant branches, ML bootstrap support is indicated below branches. Vertical bars indicate sections as proposed by Stearn (2002).

ples were fixed to aluminium stubs (Plano GmbH) in order to be sputter-coated with a 20 nm thick layer of gold under an argon atmosphere. This was done using an EMITECH K550 sputter-coater. Once prepared in this manner, the abaxial side of the leaf was visualised using a Supra40VP scanning electron microscope (Carl Zeiss NTS, Oberkochen, Germany) at an acceleration voltage of 5kV. The resulting images were compared to the descriptions of leaflet pubescence in Stearn (2002). For species described later than this revision, the descriptions from protologues were used. Moreover, the observed type of pubescence was plotted on the phylogenetic tree obtained from the ITS data, in order to inspect for phylogenetic value for this character.

RESULTS

Sequence characteristics

The ITS sequences consisted of 706 bp, and could be obtained for all collected specimens. This resulted in a datamatrix of 56 taxa and 706 positions, of which 632 positions were constant. Therefore 89.5% of the ITS sequences were identical across the sampled specimens.

Despite several attempts, the *trnK-matK* region could not be amplified for three accessions representing the species *Epimedium alpinum*, *E. macrosepalum* and *E. pubescens*. Therefore the *matK* dataset comprised 53 taxa, and 2556 positions, of which 2366 positions (92%) were constant.

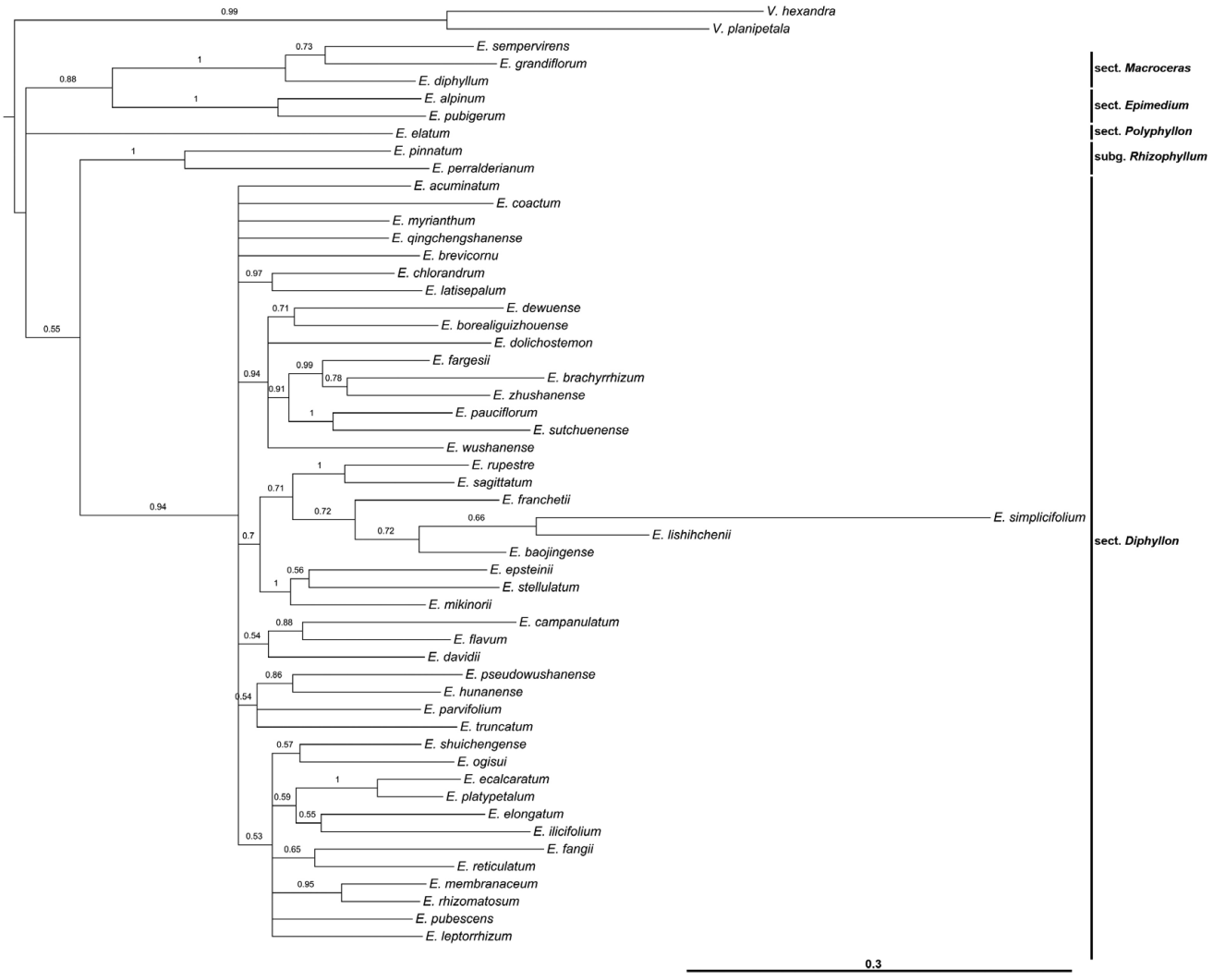

Figure 3 – The 50% majority rule consensus tree resulting from Bayesian inference for the AFLP dataset. Posterior probabilities are indicated above the relevant branches. Vertical bars indicate sections as proposed by Stearn (2002).

For the AFLP dataset, we were unable to amplify primer combination D for *Epimedium macrosepalum* and *Vancouveria chrysantha*. Consequently, these taxa were removed from the dataset, resulting in a binary matrix of 54 taxa and 1370 positions for the AFLP data. Of these 1370 positions, 25 were constant, and 1239 were parsimony informative.

Phylogenetic analyses

All resulting phylogenetic trees showed low resolution, especially for the Chinese representatives belonging to Stearn's (2002) section *Diphyllon* (table 1). Maximum likelihood analysis of the ITS dataset yielded a tree with log likelihood -1649.04, tree topology was similar to the tree resulting from Bayesian inference (BA). The only difference consisted of the position of *Epimedium elatum*. In the Bayesian analysis, this species is sister to a clade congruent with Stearn's (2002) section *Diphyllon*, whereas in the ML analysis, *E. elatum* was found to be sister to the rest of the genus. However, the relationships are unsupported (PP 0.6 and ML-BS 30 respectively), resulting in the same tree when unsupported branches were collapsed. Therefore, only the 50% majority rule consensus tree resulting from Bayesian inference is shown here (fig. 1), with addition of posterior probabilities (PP) and maximum likelihood bootstrap (ML-BS) values higher than 50. Both tree-inference methods (ML and BA) resulted in identical trees for the chloroplast region, with a log likelihood of -4844.80 for the tree resulting from the ML analysis. The 50% majority rule consensus tree resulting from the Bayesian analysis, with addition of PP and ML-BS supports is depicted in figure 2. The phylogenetic tree (50% majority rule consensus tree) resulting from Bayesian analysis of the AFLP data is depicted in figure 3.

Bayesian analysis of the AFLP fingerprints resulted in a limited number of supported clades. First, a highly supported clade (PP 1), containing all sampled species classified in Stearn's section *Macroceras* (*E. diphyllum*, *E. grandiflorum* and *E. sempervirens*). This group is sister (PP 0.88) to a clade, which receives high support (PP 1), containing all species from section *Epimedium* (*E. alpinum*, *E. pubigerum*). These

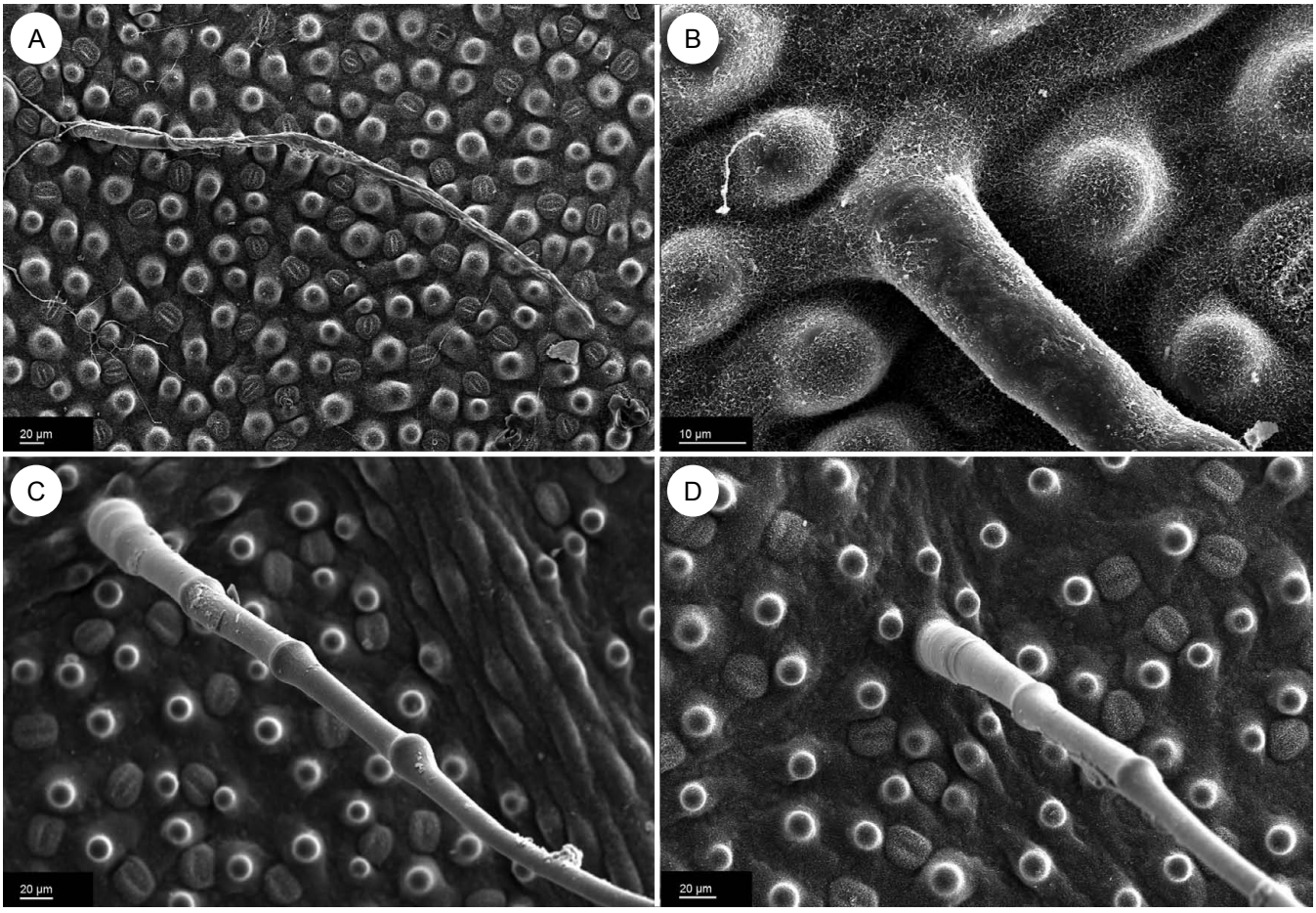

Figure 4 – SE micrographs of the abaxial leaflet surface, showing two types of pubescence described in this study. A & B, type I in *Epimedium fargesii*; C & D, type II in *E. ilicifolium*.

two sister-lineages are part of a polytomy, together with *E. elatum*, the only species in section *Polyphyllon*, and an unsupported clade (PP 0.55) containing two highly supported groups. The first of these clades consists of *E. pinnatum* and *E. perralderianum*, the only species in Stearn's (2002) subgenus *Rhizophyllum*, while all other sampled species belong to subgenus *Epimedium*. The second clade (PP 0.94) contains all Chinese species sampled in this study (belonging to section *Diphyllon*). Within this Chinese clade, resolution is low, and only a few, morphologically diverse clades are supported. A first highly supported clade (PP 1) includes *E. ecalcaratum* and *E. platypetalum*, two species with flat, spurless petals. Two comparable species, *E. campanulatum* and *E. flavum* are recovered in a rather poorly supported clade (PP 0.88). Another highly supported clade (PP 0.95) unites *E. rhizomatosum* and *E. membranaceum*, two taxa first considered variations of the same species, and mainly differing in shape of their rhizome (Stearn 2002). The analysis also revealed a highly supported (PP 0.97) clade containing *E. chlorandrum* and *E. latisepalum*. Finally, a large clade containing morphologically diverse taxa, belonging to different series (series *Davidianae*: *E. pauciflorum*; series *Brachycerae*: *E. borealiguizhouense*, *E. dolichostemon*, *E. fargesii*; series *Dolichocerae*: *E. brachyrrhizum*, E. *zhushanense*, *E. sutchuenense*, *E. wushanense*), received high support (PP 0.94), with several species pairs highly supported (e.g. *E. pauciflorum* and *E. sutchuenense*, PP 1).

Phylogenetic trees obtained from the nuclear and chloroplast regions differ from those obtained from the AFLP data in the position of *E. elatum* (section *Polyphyllon*). This species was found as sister to section *Macroceras* (chloroplast data) or a clade consisting of section *Epimedium*, section *Macroceras* and subgenus *Rhizophyllum* (nuclear data). However, these relationships are unsupported (PP 0.58 and PP 0.6 respectively) causing the same tree to result from collapsing branches with low support. Furthermore, the analysis of the ITS sequences was unable to resolve section *Macroceras* as a monophyletic clade. Instead the sampled Japanese species (*E. diphyllum*, *E. sempervirens*, *E. grandiflorum* and *E. macrosepalum*) are recovered as a polytomy, which also contains the clade corresponding to section *Epimedium*. The trees resulting from the analyses of the nucleotide sequences also differ from the AFLP results in being unable to recover the monophyly of section *Diphyllon*, containing all Chinese species sampled in this study. The highly supported pairs of species recovered in the AFLP analysis within section *Diphyllon* are not supported in the analysis of the nuclear or chloroplast markers, with exception of a clade formed by *E. chlorandrum* and *E. latisepalum*.

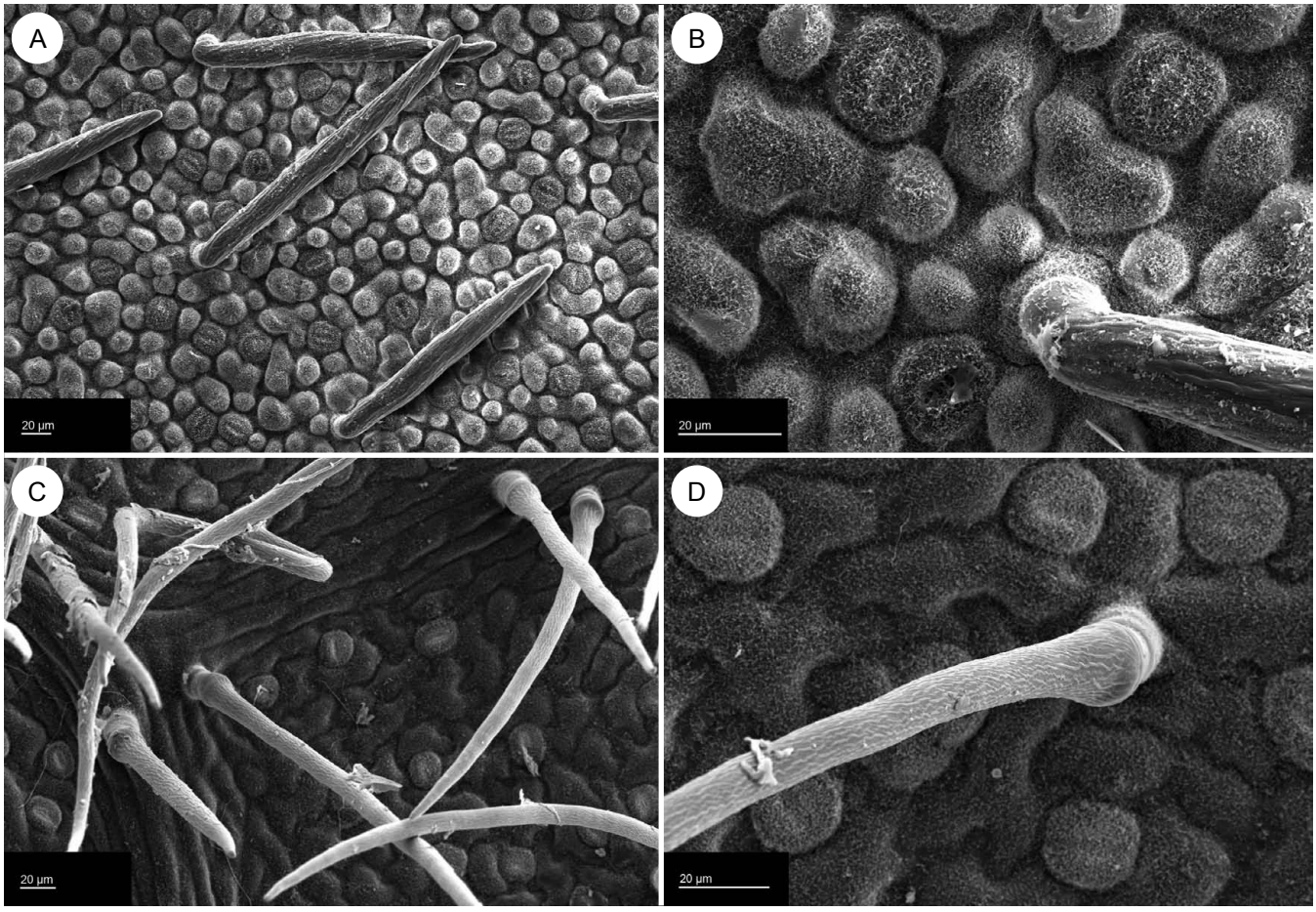

Figure 5 – SE micrographs for the abaxial leaflet surface, showing two types of pubescence described in this study. A & B, type III in *Epimedium acuminatum*; C & D, type IV in *E. pubescens*.

Leaflet pubescence

Based on our sample of 56 accessions, we distinguish four types of mature pubescence for the abaxial leaflet-surface (figs 4 & 5). Within these types, further subdivisions could be made according to dimensions of individual hairs, or density of the pubescence. Here only four types are described, generating clear-cut, discrete characters without the vagaries inherent to splitting up continuous characters into discrete categories (reviewed by Stevens 1991).

(1) Type I consists of long, slender hairs, with no apparent bulges along their length (here referred to as articulations) (fig. 4A & B). These hairs are flexible, as a result of which multiple hairs can appear entangled. (2) Type II shows rigid, tapering hairs, with clear articulations along their entire length (fig. 4C & D). In most cases these structures are upright, almost perpendicular to the leaf surface. (3) As type III we distinguish the hairs generally described as 'appressed hairs' (fig. 5A & B). Closer evaluation of these structures reveals a small stalk, perpendicular to the leaf surface, often showing several articulations. At the distal part, this stalk is connected to a spindle-shaped structure, in a 90° angle. This type appeared to be the most variable, with specimens showing long, slender spindle-shaped parts, as well as small, broad ones. (4) Finally, type IV consists of erect hairs, with articulations limited to the basal portion (fig. 5C & D). Furthermore, the distal part of these hairs is smooth and slightly curved. In some species pubescence was absent (table 2).

Some of the leaflets showed an additional type of pubescence, sometimes together with the abovementioned types. These hairs are long, slender and compressed in alternating planes resulting in a chain-like appearance (fig. 6A & B). This compression seems to result from dehydration of these structures, which becomes evident when considering they are often damaged or withered. Careful evaluation of developing leaflets revealed this type of hairs to correspond with the early pubescence present on developing plant organs in *Epimedium* (described by Stearn 2002). The majority of these hairs are shed during leaflet development, but some can remain in a deteriorated state, in addition to the four mature types described above.

Plotting the observed type of pubescence on the phylogenetic tree resulting from the ITS analysis showed no clear evolutionary patterns for this character. Species descriptions are inconsistent with respect to terms used to describe leaflet pubescence (table 2). Most descriptions seemed to coincide with the hairs observed in this study, taking into account the low detail of some descriptions. Exceptions were *E. campanulatum* and *E. davidii* for which erect hairs have been described, while we found type III hairs, which are more consistent with the description 'appressed hairs'. Additionally, we observed

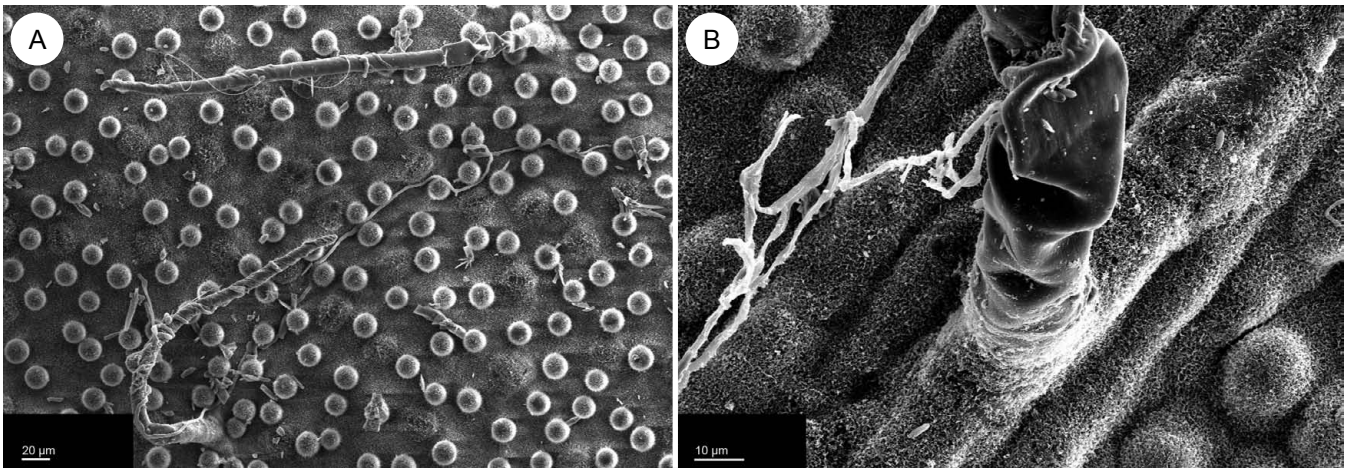

Figure 6 – SE micrographs for the abaxial leaflet surface, showing deteriorated juvenile hairs. A & B in *E. pauciflorum*.

no mature pubescence for *E. ecalcaratum*, *E. macrosepalum*, *E. reticulatum*, *E. rhizomatosum* and *E. sutchuenense* while species descriptions mention pubescence. On the other hand, we observed type III hairs in *E. qingchengshanense*, while the description mentions glabrous leaflets.

DISCUSSION

Implications for classification

The major clades recovered in our analyses are generally congruent with the sections proposed by Stearn (2002). However, relationships between clades are not in line with the recognition of the two subgenera. Subgenus *Rhizophyllum* is sister to sect. *Macroceras* and sect. *Epimedium* in the analysis of our ITS data. In the chloroplast dataset this subgenus is sister to sect. *Macroceras* and sect. *Polyphyllon*, whereas it is placed sister to sect. *Diphyllon* in the AFLP analysis. Therefore, our results suggest subg. *Epimedium* to be paraphyletic in relation to subg. *Rhizophyllum*, corroborating the findings of Zhang et al. (2007).

The single species classified in section *Polyphyllon*, *E. elatum*, is resolved in different positions depending on the marker used. As the respective nodes show low statistical support, no reliable inference can be made concerning the evolutionary relationship of this species to the rest of the genus. Therefore, the sequence of range formation in *Epimedium* proposed by Zhang et al. (2007) should be interpreted carefully, as our results do not support an early separation of *E. elatum* from the rest of the genus.

The finding of a closer relationship between the Japanese lineage (sect. *Macroceras*) and the western Eurasian lineage (sect. *Epimedium*) rather than between the Japanese lineage and the geographically closer Chinese lineage (sect. *Diphyllon*) is in line with the findings by Zhang et al. (2007). This provides support for the hypothesis of a continuous belt of deciduous broad-leaved forest, connecting the Japanese and western Eurasian lineages during the Tertiary (Stearn 2002, Zhang et al. 2007).

Phylogenetic trees resulting from AFLP data should be interpreted carefully. As stated by Koopman (2005), problems of non-independence of fragments and identification of homologous fragments potentially limit phylogenetic interpretation of restriction fragment data. Indeed, despite the high amount of parsimony informative characters in our AFLP dataset (1239 of 1370 positions) the obtained resolution in our phylogenetic reconstruction was rather low. Comparison of non-homologous fragments could have obscured the phylogenetic signal in our dataset, resulting in a low support for evolutionary relationships. Nevertheless, several preliminary remarks can be made. The AFLP dataset revealed a monophyletic, supported (PP 0.94) clade, containing all Chinese specimens sampled in this study (sect. *Diphyllon*), while the nucleotide sequence data was unable to confirm the monophyly of this section. Within this clade none of the markers provided enough resolution to be informative concerning the subdivision into series (Stearn 2002). However, species lacking petal spurs (*E. ecalcaratum*, *E. platypetalum*, *E. campanulatum*, *E. flavum*, and some forms of *E. davidii*) were recovered in moderately to highly supported clades in both the AFLP and ITS analyses. Additionally, this type of petal does not seem to be ancestral in the genus (Stearn 2002, Ying 2002). Instead, spurs have likely been lost at least twice independently (within sect. *Diphyllon* and sect. *Macroceras*).

Lack of resolution within section *Diphyllon*

Congruent with earlier studies (Sun et al. 2005, Zhang et al. 2007), our analyses of ITS, *trnK-matK* and AFLP datasets were unable to provide resolution within the Chinese section *Diphyllon*. The resulting polytomy, containing all Chinese representatives of genus *Epimedium*, can represent two different phenomena: hard or soft polytomies. In the case of a soft polytomy, the lack of phylogenetic resolution obtained in this study would be caused by the choice of molecular markers. Therefore, other markers might provide sufficient resolution to reconstruct the evolutionary relationships within *section Diphyllon*. However, the observed polytomy could be caused by a recent radiation in the Chinese distribution area of *Epimedium*, thus representing a hard polytomy. Here, the latter hypothesis is preferred, as it is supported by previous molecular studies (Sun et al. 2005, Zhang et al. 2007) and karyomorphology of Chinese species (Zhang et al. 2008).

Furthermore, despite the ability of AFLP fingerprinting to provide resolution in phylogenetic reconstructions of closely related species (e.g. Després et al. 2003, Jacobs et al. 2008), this technique did not resolve the abovementioned polytomy. Therefore this study provides an additional line of evidence in support of the hypothesis of a recent origin for the Chinese taxa in *Epimedium*.

The diversification of Chinese *Epimedium* representatives was dated to the Quaternary, between 0.6 and 0.46 Mya (Zhang et al. 2007). This dating coincides with 'Stage II' of China's 'Third Glacial Period' (c. 0.52–4.6 Mya) as defined by Yi et al. (2005). This suggests a connection between climatic oscillations in Asia and differentiation of Chinese taxa of *Epimedium*. Additionally, reconstruction of vegetation in East Asia during this period (Harrison et al. 2001) has led Zhang et al. (2007) to propose a shift in geographical distribution of *Epimedium* as causal factor for the Chinese diversity in the genus. Such shifts combined with the complex topology of East Asia could explain the high morphological diversity of the genus in China (Qian & Ricklefs 2000). However, debate exists concerning the response of temperate vegetation to glacial-interglacial climatic oscillations during the Quaternary in East Asia. Key to this debate is the question whether temperate forest communities merged or fragmented during glacial periods (Qian & Ricklefs 2001, Harrison et al. 2001). As *Epimedium* is intricately associated with such temperate forests (Stearn 2002), closer study of the genus could shed new light on this debate.

Leaflet pubescence

This study describes four types of pubescence for the abaxial surface of *Epimedium* leaflets. These types form clear-cut, discrete character states which can be readily observed in living material. As stated earlier, one of the difficulties regarding *Epimedium* species recognition is the low detail in which diagnostic characters are often described (table 2). Additionally, different terms were used to describe the same character state (table 2). Therefore we offer a first step towards clarifying species descriptions, by providing detailed descriptions and figures for one new character, pubescence of the abaxial leaflet surface. As there are only four character states, pubescence of leaflets can not be used as a single character to appoint any given specimen to a species. However, if used in combination with other clear-cut characters this could greatly aid species identification for different purposes, for example pharmacological studies. Therefore we suggest that future research should focus on producing detailed descriptions for other potentially diagnostic characters in the genus. Candidates for this are characteristics of the leaf margin (e.g. shape of spines) and petals (e.g. characterisation of spur opening). In addition, combination of such clear-cut, well-defined characters would yield more insight in species boundaries.

The differences in type of leaflet pubescence observed in this study, and mentioned in species descriptions can have different sources. First, the type of hair mentioned in a certain species description can be based on juvenile, withering hairs. Second, the low level of details in descriptions can render comparison impossible.

Species recognition in *Epimedium*

In this study, nuclear and chloroplast markers together with AFLP fingerprints were used in an attempt to provide insight in the evolutionary relationships between recognized taxa. The methodology utilised here does not warrant delineation of species, which would require extensive infrapecific sampling. However, assessing the evolutionary relationships between recognised taxa of *Epimedium* provided us with additional evidence for the hypothesis of a recent origin of the diversity in section *Diphyllon*. This hypothesis is intrinsically linked to problems regarding species recognition, as it suggests that some of the recognized taxa in section *Diphyllon* might still be in the process of differentiation (Stearn 2002, Zhang et al. 2008). Indeed, such incompletely differentiated entities can be recognized as one or several species, depending on the species definition utilised (de Queiroz 1998). Furthermore, species boundaries in the genus are blurred by several additional factors, among which the lack of detail in species description, hampering linkage of specimens to recognized species. This in turn results in recognition of slightly aberrant specimens as new species.

When combined, abovementioned difficulties in species recognition in *Epimedium* suggest an overestimation of the number of Chinese species. We therefore propose a different approach in *Epimedium* taxonomy, recognizing broader, more variable species. Each of these species can be characterized using characters with clear-cut, standardized character states. The description of four types of leaflet pubescence can be a first step towards this new view on *Epimedium* taxonomy. However, the recognition of morphologically more variable species will not render collection and documentation of infraspecific variation obsolete. Detailed samplings of new morphological variations are essential in order to document the process of differentiation as it occurs. Habitats for this group are likely to deteriorate and become even more fragmented in the future. This will reinforce speciation through isolation of small populations. As similar deteriorations of distribution areas have already been documented (e.g. in *Magnolia*, Cicuzza et al. 2007); this scenario is not unlikely to occur in *Epimedium*.

Future research

Future research along different lines is possible in *Epimedium*. First, a priority should be given to delineating clearly defined species, especially in section *Diphyllon*. As suggested by de Queiroz (1998) this is best achieved using multiple lines of evidence. For section *Diphyllon*, extensive sampling of natural populations for all recognized taxa could shed light on the status of these entities. Considering the drawbacks associated with AFLP fingerprinting, other markers should be explored for their capacity to delimit species within the Chinese section of *Epimedium* (e.g. microsatellites: Xu et al. 2008). These molecular insights can then be combined with clearly defined morphological characters in order to generate useful species descriptions. Once species delimitation is clear, further studies can address evolutionary relationships between these recognised entities, testing the hypothesis of a hard polytomy. Finally these results can contribute to the abovementioned discussion concerning the response of tem-

perate vegetation to glacial-interglacial climatic oscillations during the Quaternary in East Asia.

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