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ORIGINAL RESEARCH

Molecular Systematic Study of *Chrysosplenium* Series *Pilosa* (Saxifragaceae) in Korea

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Abstract To address systematic issues pertaining to series *Pilosa* of Korean *Chrysosplenium*, the phylogenetic relationships of the 35 accessions of Korean *Chrysosplenium* taxa were examined using the DNA sequence data obtained from the nuclear ribosomal internal transcribed spacer (ITS) regions and the *psb*K-I gene of the plastid genome. The results from both ITS and *psb*K-I sequence data indicated that *Chrysosplenium flaviflorum* formed a well-supported clade that merits species status of the taxon. The ITS phylogenetic trees detected two distinct lineages in *Chrysosplenium pilosum* var. *fulvum*. Geographically, one lineage was widely distributed throughout the country whereas the other was confined to Gyeonggi and Gangwon provinces. The plants sampled from Jeju Island, which have been recognized as *C. pilosum* var. *sphaerospermum* [*C. hallaisanense*] exhibited no DNA sequence feature segregating them from *C. pilosum* var. *fulvum* [*C. barbatum*] distributed in the mainland area of Korea. The plastid DNA sequence data was not fully in concordance with the nuclear ribosomal ITS data and the current taxa delimitation. These results suggest incomplete lineage sorting or that plastid genome capture may have occurred during the evolution of some taxa examined in the present study.

Keywords *Chrysosplenium* · Series *Pilosa* · ITS · *psb*K-I · Incomplete lineage sorting

Chrysosplenium L. (Saxifragaceae) consists of approximately 70 species of perennial herbs distributed in temperate regions of the Northern Hemisphere with two disjunctive species in Chile (Hara 1957). A total of seven species, classified into five series (*Oppositifolia* Maxim., *Sinica* Maxim., *Pilosa* Maxim., *Alternifolia* Maxim. and *Flagellifera* Maxim.), are known to occur in Korea (Hara 1957; Chung and Kim 1988). Among the five series, plants included in series *Pilosa* present a serious problem for identification and taxon delimitation due to severe variations in the morphology of leaves, seed coat, and hairiness (Hara 1957; Kim 2007). A seasonal change in the morphology of vegetative parts of the plants adds to the difficulty of correctly identifying many herbarium specimens.

In series *Pilosa*, *C. flaviflorum* Ohwi and *Chrysosplenium pilosum* Maxim. are morphologically very similar to each other, and taxonomy of the two taxa has been problematic. While the former has been treated as a variety of the latter (Ohwi 1937), Hara (1957) and Kim (2007) kept the species status of *C. flaviflorum* based on a distinctness in seed coat morphology. *C. flaviflorum* is confined to the northern and central parts of the Korean Peninsula (Chung and Kim 1988), but *C. pilosum* is distributed more widely in Ussuri, China, Korea, and Japan (Hara 1957). Hara (1957) recognized four varieties in *C. pilosum*: var. *pilosum*, var. *fulvum* [*C. barbatum* Nakai], var. *sphaerospermum* [*C. hallaisanense* Nakai], and var. *valdepilosum*, reflecting the wide distribution area and great morphological variations of the species. The number of varieties distributed in Korea, however, is controversial. Hara (1957) concluded that three varieties (var. *pilosum* excluded) occur in Korea, whereas Chung and Kim (1988) recognized only two varieties, var. *valdepilosum* and var. *fulvum*. They were not able to find any morphological characteristic that could be used to distinguish the plants distributed in Jeju Island (*C. pilosum* var. *sphaerospermum* sensu Hara) from the mainland area. Since then, the presence of *C. pilosum* var. *sphaerospermum* in Korea has been in dispute (Kim 2007). Therefore, the taxonomic status of *C. flaviflorum* and the

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identity of *C. pilosum* taxon on Jeju Island are considered as major taxonomic issues with regard to Korean *Chrysosplenium*.

Nakazawa et al. (1997) and Soltis et al. (2001) performed molecular phylogenetic studies on *Chrysosplenium* using *mat*K gene sequences. They found that the molecular data were mostly congruent with previous classification proposed by Hara (1957). Those studies, however, focused mainly on the relationships of some representative species within the genus. Thus far, no molecular approach was undertaken for *Chrysosplenium* series *Pilosa* in Korea. In this study, we analyzed DNA sequences of the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA and the *psb*K-I (intergenic spacer of *psb*K and *psb*I) of the plastid genome to address the taxonomic issues pertaining to Korean *Chrysosplenium* taxa included in series *Pilosa*.

Materials and Methods

Plant Samples, Polymerase Chain Reaction (PCR), and Sequencing

A total of 35 accessions representing series *Pilosa* of *Chrysosplenium* in Korea and two closely related outgroup taxa (*C. pseudofauriei* Lév. and *C. ramosum* Maxim.) were collected throughout the country (Table 1). The outgroup taxa were selected based on the phylogenetic studies of Nakazawa et al. (1997) and Soltis et al. (2001). All voucher specimens were deposited at the Herbarium of Hallym University (HHU). Total DNA was extracted from young fresh leaves using DNeasy Plant Mini Kits (Qiagen, Germany) following the manufacturer's instructions.

PCR amplifications of ITS and *psb*K-I regions were carried out in a total reaction volume of 50 μl containing 5× GoTaq Flexi buffer 10 μl, 25 mM $MgCl_2$ 4 μl, 2.5 mM dNTPs 4 μl, forward and reverse primers each 0.5 μl, 5 U/μl Taq polymerase 0.25 μl, 10 to 20 ng template DNA and distilled water up to the final volume. The thermocycling profile consisted of an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 1 min at 95°C, 1 min at 55.5°C, 1 min at 72°C, and a final extension step of 10 min at 72°C. The products were purified with a QIAqiuck PCR purification kit (Qiagen) according to the manufacturer's protocol. Purified double-stranded PCR products were used for determining the DNA sequences of ITS and *psb*K-I regions using the automatic DNA sequencer, ABI PRISM 377 (PE Applied Biosystems). The ITS regions were amplified and sequenced using primers designed by White et al. (1990) except ITS1, which differed by the two italicized bases (5′-GGAAG*GA*GAAGTCGTAACAAGG-3′). The *psb*K-I region of chloroplast DNA was amplified and sequenced using primers psbK (5′-TTAGCCTTTGTTTGG CAAG-3′) and psbI (5′-AGAGTTTGAGAGTAAGCAT-3′). All sequences were deposited in GenBank (Table 1).

Sequence and Phylogenetic Analyses

The DNA sequences were aligned with Clustal X (Thompson et al. 1997). The alignment was further examined and slightly edited manually as necessary. Gaps introduced from the alignment were treated as missing characters in subsequent analyses. Phylogenetic analyses were performed using the maximum parsimony and the Bayesian methods. The maximum parsimony phylogenetic analyses were conducted in PAUP* ver. 4.0b10 (Swofford 2002) using heuristic searches with the MULTREES option, ten random entries of taxa, and TBR (Tree Bisection–Reconnection) branch swapping. Bootstrap analysis (Felsenstein 1985) with 1,000 replicates was conducted to evaluate the degree of support for given clades of maximum parsimonious (MP) trees (PAUP* ver. 4.0b10, Swofford 2002). Bayesian phylogenetic analyses (Rannala and Yang 1996) were conducted with MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck 2003). The model selection implemented in the program Modeltest 2.2 (Posada and Crandall 1998) resulted in TPM + G for ITS and F81 for *psb*K-I. Each Markov chain was started from a random tree and run for 1,000,000 generations, sampling a tree every 100 generations. We ran four chains simultaneously, three heated and one cold, with the default settings for the priors. To check that stationarity had been reached, we monitored the fluctuating value of the log-likelihood and repeated each analysis twice. After discarding burn-in samples (initial 1,000 trees) the remaining samples were retained for the construction of a final consensus tree with posterior probabilities for given clades.

Results

The sequences of the ITS regions determined from 31 accessions of series *Pilosa* taxa had lengths of 547 to 550 base pair (bp) (including 5.8s): *C. pilosum* var. *fulvum* 550 bp, *C. pilosum* var. *valdepilosum* 548 bp, and *C. flaviflorum* 547 bp. Length variation in the DNA sequence was not observed among the accessions within each variety. After sequence alignment, a 550-bp sequence data file was obtained. A total of 49 nucleotide sites were variable and among them 40 sites were parsimony informative.

The MP analysis using the ITS sequences generated 162 equally parsimonious trees with a retention index (RI) of 0.9629. A strict consensus tree of the equally parsimonious trees is shown in Fig. 1. *C. flaviflorum* formed a distinct clade with a 100% bootstrap value. Within the *C. pilosum*

Table 1 Plant accessions included in the phylogenetic analyses. Voucher specimens are deposited at the herbarium of Hallym University (HHU)

Taxon	Locality	Voucher	GenBank no. ITS; *psbK*-I
C. pilosum var. *valdepilosum* 01	GW, Mt. Bong-bok	KYI-2009003	HQ896895; HQ896860
C. pilosum var. *valdepilosum* 02	GW, Mt. Bong-bok	KYI-2009004	HQ896896; HQ896861
C. pilosum var. *valdepilosum* 03	GW, Mt. Tae-gi	KYI-2009006	HQ896898; HQ896863
C. pilosum var. *valdepilosum* 04	GW, Mt. Oh-dae	KYI-2009007	HQ896899; HQ896864
C. pilosum var. *valdepilosum* 05	GG, Mt. Chun-ma	KYI-2009008	HQ896900; HQ896865
C. pilosum var. *valdepilosum* 06	GW, Mt. Hwa-ak	KYI-2009012	HQ896903; HQ896868
C. pilosum var. *valdepilosum* 07	GW, Mt. Oh-dae	KYI-2009021	HQ896909; HQ896874
C. pilosum var. *valdepilosum* 08	GW, Mt. Gwang-duk	KYI-2009030	HQ896915; HQ896880
C. pilosum var. *valdepilosum* 09	GW, Mt. Gwang-duk	KYI-2009031	HQ896916; HQ896881
C. pilosum var. *valdepilosum* 10	GW, Mt. Oh-dae	KYI-2009039	HQ896923; HQ896888
C. pilosum var. *fulvum* 01	JJ, Jul-mul	KYI-2009002	HQ896894; HQ896859
C. pilosum var. *fulvum* 02	JJ, Mul-chat-oh-reum	No Voucher	HQ896897; HQ896862
C. pilosum var. *fulvum* 03	JN, Mt. Jo-gae	KYI-2009009	HQ896901; HQ896866
C. pilosum var. *fulvum* 04	GB, Temple Jik-Ji	KYI-2009010	HQ896902; HQ896867
C. pilosum var. *fulvum* 05	GW, Mt. Gwang-duk	KYI-2009013	HQ896904; HQ896869
C. pilosum var. *fulvum* 06	GW, Mt. Gwang-duk	KYI-2009015	HQ896905; HQ896870
C. pilosum var. *fulvum* 07	GW, Mt. Gwang-duk	KYI-2009025	HQ896911; HQ896876
C. pilosum var. *fulvum* 08	GW, Mt. Gwang-duk	KYI-2009026	HQ896912; HQ896877
C. pilosum var. *fulvum* 09	GW, Mt. Gwang-duk	KYI-2009027	HQ896913; HQ896878
C. pilosum var. *fulvum* 10	GW, Mt. Gwang-duk	KYI-2009032	HQ896917; HQ896882
C. pilosum var. *fulvum* 11	JJ, Eo-ri-mok	KYI-2009035	HQ896919; HQ896884
C. pilosum var. *fulvum* 12	GN, Mt. Mi-reuk	KYI-2009036	HQ896920; HQ866885
C. pilosum var. *fulvum* 13	GG, Gwang-reung	KYI-2009037	HQ896921; HQ896886
C. pilosum var. *fulvum* 14	JJ, Min-oh-reum	KYI-2009041	HQ896924; HQ896889
C. pilosum var. *fulvum* 15	GG, Gwang-reung	KYI-2009043	HQ896925; HQ896890
C. pilosum var. *fulvum* 16	GW, Mt. Dae-sung	YoungdongKim2008-0062	HQ896926; HQ896891
C. pilosum var. *fulvum* 17	GG, Mt. Yeon-in	KYI-2010001	HQ896927; HQ896892
C. pilosum var. *fulvum* 18	GG, Mt. Yeon-in	KYI-2010002	HQ896928; HQ896893
C. flaviflorum 01	GW, Mt. Dae-sung	KYI-2009029	HQ896814; HQ896879
C. flaviflorum 02	GW, Mt. Dae-sung	KYI-2009034	HQ896918; HQ896883
C. flaviflorum 03	GW, Mt. Keum-dang	YoungdongKim2009-0021	HQ896922; HQ896887
C. pseudofauriei 01	GW, Mt. Gwang-duk	KYI-2009017	HQ896906; HQ896871
C. pseudofauriei 02	GW, Mt. Hwa-ak	KYI-2009018	HQ896907; HQ896872
C. pseudofauriei 03	JJ, Eo-ri-mok	No Voucher	HQ896910; HQ896875
C. ramosum	GW, Mt. Hwa-ak	No Voucher	HQ896908; HQ896873

GG Gyeoggi-do, *GW* Gangwon-do, *GB* Gyeongsangbuk-do, *GN* Gyeongsangnam-do, *JN* Jeollanam-do, *JJ* Jeju-do

clade, monophyly of two taxa, *C. pilosum* var. *valdepilosum* and *C. pilosum* var. *fulvum*, were supported by relatively high bootstrap values (100 and 75, respectively). Two well-supported lineages were observed in *C. pilosum* var. *fulvum* (referred to hereafter as Fulvum clades I and II, respectively). Fulvum clade I consisted of the plants collected throughout the country including Gyeonggi-do, Gyeongsangbuk-do, Gyeongsangnam-do, Jeollanam-do, and Jeju-do, whereas Fulvum clade II included accessions sampled from limited areas of Gyeonggi-do and Gangwon-do. Support for the monophyly of *C. pilosum* taxa was relatively poor (67% bootstrap value). The Bayesian tree (not shown) exhibited a topology and degree of support similar to the strict consensus tree.

The sequence length of the *psb*K-I region for 31 samples of *Chrysosplenium* series *Pilosa* ranged from 323 to 342 bp. Sequence length variation was observed from *C. pilosum* var. *fulvum* (332 to 342 bp) and *C. pilosum* var. *valdepilosum* (323 to 339 bp) while the sequence lengths of *C. flaviflorum* were invariable. All ingroup taxa had multiple haplotypes: two types from *C. flaviflorum*; three types from *C. pilosum* var. *fulvum*; and five types from *C.*

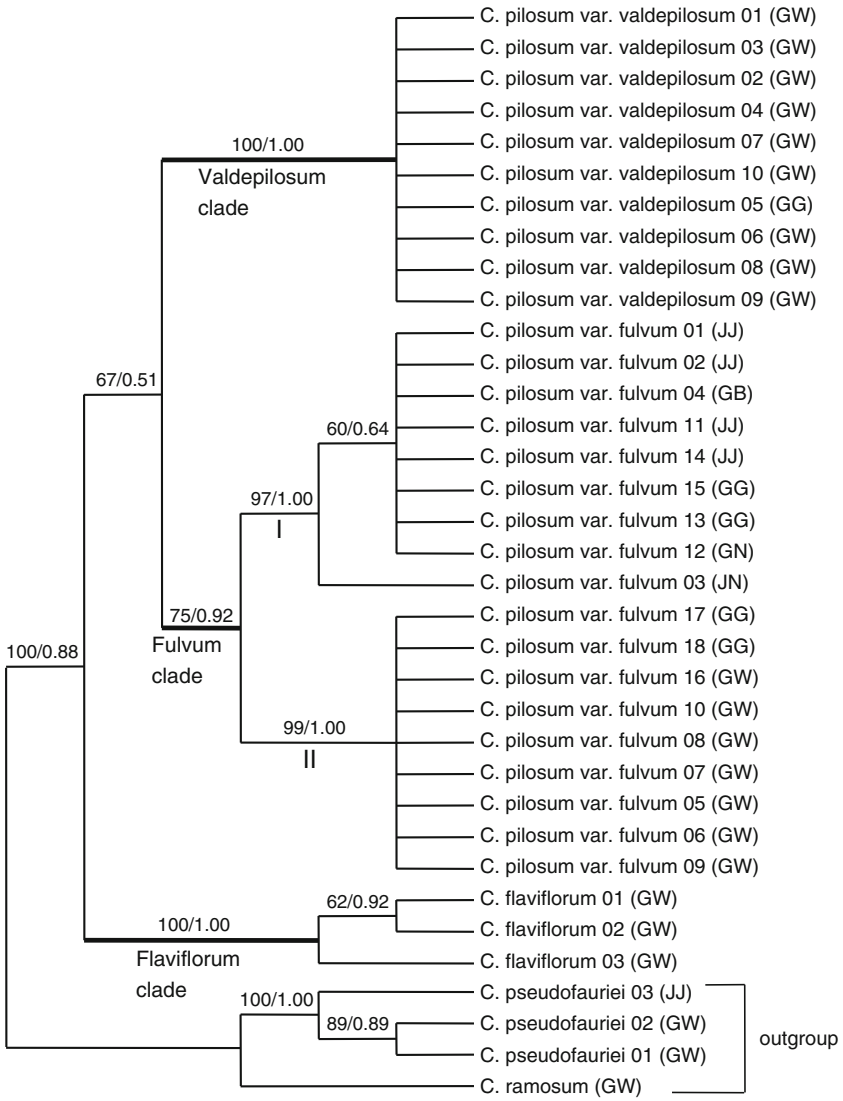

Fig. 1 A strict consensus tree of 162 equally parsimonious trees (RI=0.9629) of *Chrysosplenium* series *Pilosa* based on nuclear ribosomal ITS sequences. Numbers above branches denote support values from maximum parsimony and Bayesian analyses, respectively: bootstrap support percentages/posterior probabilities. (*GG* Gyeoggi-do, *GW* Gangwon-do, *GB* Gyeongsangbuk-do, *GN* Gyeongsangnam-do, *JN* Jeollanam-do, *JJ* Jeju-do)

pilosum var. *valdepilosum*. In a strict consensus tree of 34 equally parsimonious trees reconstructed by the *psb*K-I sequences (RI=0.9808, Fig. 2), the monophyly of *C. flaviflorum* was evident as in the ITS trees; however, neither *C. pilosum* nor two varieties within the species formed a clade. Two accessions of *C. pilosum* var. *valdepilosum* (accession numbers 03 and 05) sampled from Mt. Tae-gi (Gangwon-do) and Mr. Cheon-ma (Gyeonggi-do), respectively, were strongly clustered with the accessions of *C. pilosum* var. *fulvum*, making these varieties nonmonophyletic. A similar topology was observed from the Bayesian tree (not shown).

Discussion

This study provided important clues to the systematic issues pertaining to series *Pilosa* of the genus *Chrysosplenium* in Korea. The monophyly of *C. flaviflorum* was strongly supported by both ITS and *psb*K-I trees, suggesting it merits species status. The taxon was first described as a species by Ohwi (1934), but later it was changed in rank and placed as a variety of *C. pilosum* (Ohwi 1937) considering their close morphological similarity. The molecular sequence data of ITS and *psb*K-I, however, support the taxonomic view of Hara (1957) who restored the rank of taxon by emphasizing the importance of its seed morphology characterized by the lack of longitudinal ridges on the surface. Other than the seed morphology, *C. flaviflorum* is discernible in the field by the smallest flower (ca. 2 to 3 mm in diam.) among the taxa in series *Pilosa* (personal observation). Meanwhile, the affinity of *C. flaviflorum* to the *C. pilosum* var. *valdepilosum* in the *psb*K-I tree (Fig. 2) may support the idea that the species is closely related to *C. pilosum* through var. *valdepilosum* (Hara 1957).

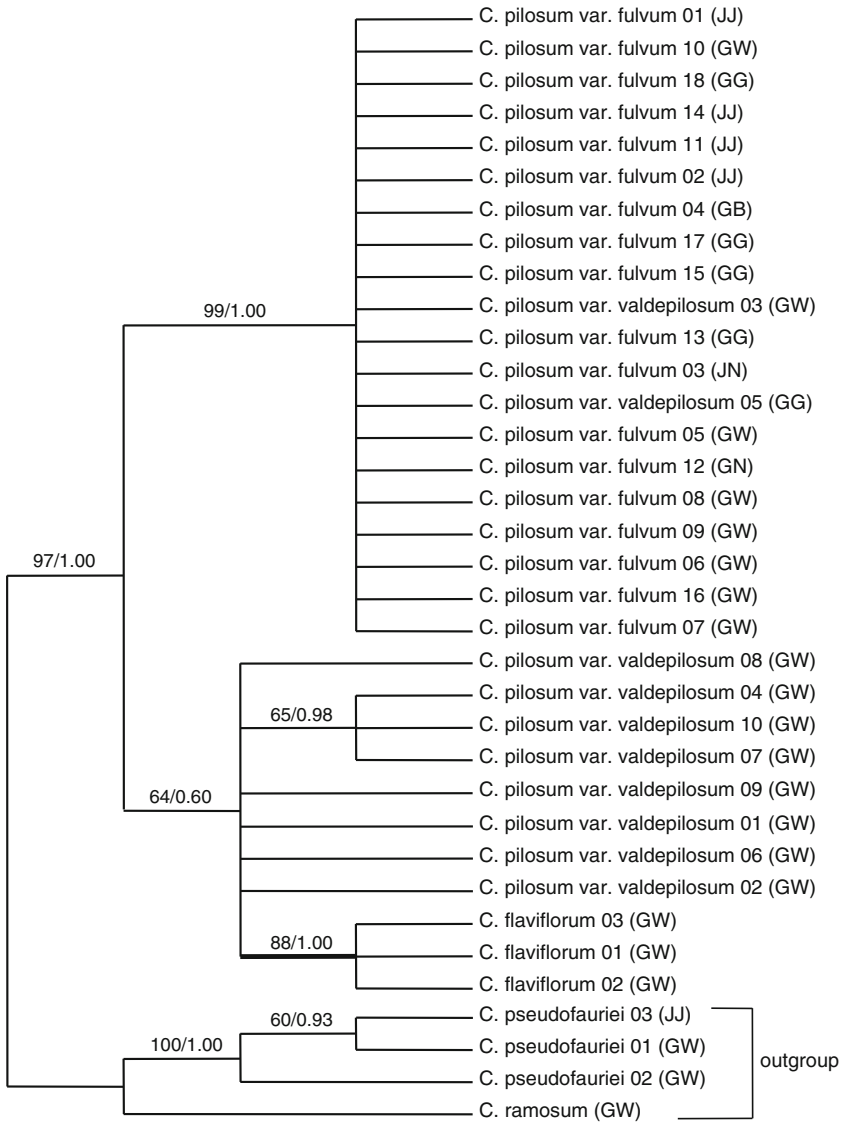

Fig. 2 A strict consensus tree of 34 equally parsimonious trees (RI=0.9808) of *Chrysosplenium* series *Pilosa* based on *psb*K-I sequences. Numbers above branches denote support values from maximum parsimony and Bayesian analyses, respectively: bootstrap support percentages/ posterior probabilities. (*GG* Gyeoggi-do, *GW* Gangwon-do, *GB* Gyeongsangbuk-do, *GN* Gyeongsangnam-do, *JN* Jeollanam-do, *JJ* Jeju-do)

The DNA sequence data did not provide any evidence for recognizing the plants of series *Pilosa* in Jeju Island (*C. pilosum* var. *sphaerospermum* sensu Hara) as a distinct taxon different from the plants distributed in the mainland area of Korea (*C. pilosum* var. *fulvum* sensu Hara). Hara (1957) mentioned the two taxa could be distinguished by the hairiness and the shape of the leaf margin. On observing the continuity of those characteristics between the populations in Jeju Island and the inland area, however, Chung and Kim (1988) questioned the distinctness of the island taxon, making the entity of the Jeju Island taxon controversial (Kim 2007). Because *C. pilosum* var. *sphaerospermum* Hara is very widely distributed in Japan, more extensive morphological and molecular examinations encompassing enough accessions from Japan are necessary to determine whether the two varieties represent the same taxonomic entity or not.

The *psb*K-I trees exhibited limited utility in taxon delimitation, especially in circumscribing *C. pilosum* var. *valdepilosum*. The placement of two accessions of *C. pilosum* var. *valdepilosum* in the *C. pilosum* var. *fulvum* clade (Fig. 2) implies that there may have been incomplete lineage sorting of haplotypes in the ancestral population (Nei 1987; Wendel and Doyle 1998) or plastid capture by hybridization and subsequence introgression (Rieseberg and Soltis 1991; Soltis et al. 1991; Rieseberg and Brunsfeld 1992; Soltis et al. 1996). Lacking evidence for hybridization, incomplete lineage sorting could be the better explanation for the polymorphisms shared among the examined taxa. Unlike *psb*K-I, the ITS trees were concordant with current taxa recognition based on morphology, suggesting that the ITS sequence is useful for the phylogenetic study of *Chrysosplenium*. This result suggests that the nuclear ribosomal ITS sequences, despite several drawbacks for phylogeny recon-

struction (Álvarez and Wendel 2003), still produce insightful results in phylogenetic studies, provided that they are used carefully (Feliner and Rosselló 2007).

Another remarkable result obtained from the ITS sequence analysis is the discovery of two distinct lineages in *C. pilosum* var. *fulvum*: Fulvum clades I and II (Fig. 1). Morphologically, plants included in those clades differ slightly in the color of bracteal leaves in flowering season and microstructure of the seeds (personal observation). The bracteal leaves tend to be more yellowish in the plants of Fulvum clade II whereas ridges on the seed surface are more clearly developed in the plants of Fulvum clade I. Retention of the same plastid DNA haplotype within the two clades (Fig. 2), however, suggests that they may be in the initial stage of evolutionary divergence. At this point, more extensive examination employing additional samples, especially individuals from the populations where the plants of the two clades cooccur is necessary to decide whether they can be recognized as separate taxa or not.

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