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A cladistic analysis of medical *Astragalus penduliflorus* Lam. complex (Leguminosae: Papilionoideae) in China and its taxonomic implications

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ABSTRACT

To test the taxonomy of medical *Astragalus penduliflorus* complex and specific status of *A. petrovii*, eleven accessions (covering four species) in China were sampled herein for an AFLP study. A total of 346 informative polymorphic bands from eight primers pairs were obtained, dendrogram was generated using UPGMA analysis. The results indicated *A. membranaceus* is more morphologically and generically diverse than *A. mongholicus* on the populational level. Both of them and the former species *A. minhensis* were degraded as three varieties of *A. penduliflorus*. In the light of morphological and cladistic evidence, a new combination, *A. penduliflorus* ssp. *mongholicus* var. *petrovii* was proposed based on former species *A. petrovii*.

Key wods: AFLP, *Astragalus penduliflorus* complex, *Astragalus petrovii*, Taxonomy.

INTRODUUCTION

Astragalus L. (Leguminosae; Papilionoideae; Galegeae) is the largest genus of Angiosperm, containing ca. 2300-2500 species, widely spreading in Eurasia, N Africa, N & S America (Lock and Schrire, 2005; LPWG, 2013). Some taxa in this genus are used as Chinese traditional medicine, e.g. roots of *A. membranaceus* (Fisch.) Bunge. and *A. mongholicus* Bunge (The State Pharmacopoeia Commission, 2010). These medical *Astragalus* species belong to *A. penduliflorus* Lam. complex, which includes a group of morphologically similar species, varieties and formae, mainly distributed in China (Chen and Zhu, 1990; Shi, 2003). This complex is characterized by erect stems with basifixed hairs, imparipinnate leaves with 12-25 leaflets, reflexed standard (longer than wings) and semi-elliptical, inflated, membranous legumes (Zhu, 2003; Xu and podlech, 2010).

Flora of U.S.S.R. recorded four species of *Astragalus penduliflorus* complex (Komarov, 1946), agreed by Yakovlev *et al*. (1996). Hsiao (1964) merged *A. mongholicus* as a subspecies of *A. membranaceus*, followed by Ho and Fu (1993), Liu (2008) and Qian *et al*. (2009). Based on karyotype studies, Chen and Zhu (1990) treated this complex as one single species: *A. penduliflorus*, with three subspecies. Zhu (2003) in turn revised this species into two subspecies and five varieties, with *A. minhensis* X.Y.Zhu & C.J.Chen and *A. sericeocanus* Gontsch. being included as varieties, which are endemic to Qinghai (China) and South of Lake Baikal (Russia), respectively. Shi (2003) and Zhao (2006) recovered the specific status of *A. membranaceus* and *A. mongholicus*, whereas *Flora of China* merged them as one species again, which also accepted the delimitation of *A. petrovii* N.Ulziykhalso and treated *A. minhensis* and *A. purpurinus* (Y.C.Ho) Podlech & L.R.Xu as independent species (Xu and Podlech, 2010). According to our field and herbarium observation, *A. petrovii* morphologically fit into *A. penduliflorus* complex, although no previous study noted it.

A few molecular phylogenetic study lent some supports to above-mentioned revisions (Dong *et al*., 2003; Zhang *et al*., 2009), which indicated three species of *A. penduliflorus* complex (*A. membranaceus*, *A. mongholicus* and *A. propinquus* B.Schischk.) formed a clade. Some molecular marking investigations focused on medical species in this complex, showing the infra-specific diversity of *A. membranaceus* is higher than that of *A. mongholicus*, both of which fail to cluster (Wang, 2013; Zhang, 2014). Based on morphological and AFLP analyses, four species of the *A. penduliflorus* complex in China were sampled in this study to test (a) taxonomic revisions of this complex, and (b) specific status of *A. petrovii*.

MATERIALS AND METHODS

Taxon sampling: Our sampling was designed largely following Ho and Fu (1993) and Xu and Podlech (2010),

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with all taxa temporarily treated as separated species before conclusion was made. Within the *Astragalus penduliflorus* complex, five species distributed in China, four were sampled herein (except for *A. purpurinus*), represented by eleven accessions: six of *A. mongholicus*, three of *A. membranaceus*, one for each of *A. minhensis* and *A. petrovii* (Table 1). Samples were kept in silicon gel before DNA extraction, vouchers were stored in herbarium of Northwest A&F University (WUK).

DNA extraction, restriction enzyme digestion and AFLP reaction: Gel-dried leaves were ground in liquid nitrogen, genomic DNAs were isolated using CTAB method (Doyle and Doyle, 1990). The purity of extractions was tested by 0.8% agarose gel electrophoresis and nucleic acid detector, and the latter yielded acceptable OD260/OD280 values (1.93-2.00) for AFLP analysis.

Method of enzyme digestion followed Huang *et al*. (2011), using Mse I/EcoRI adapters: MseI adapter: 5'-GACGATGAGTCCTGAG - 3'TACTCAGGACTCAT - 5'; EcoRI adapter: 5'- AATTGGTACGCAGTCTAC - 3'CCATGCGTCAGATGCTC - 5'. AFLP reactions were carried out as suggested by Zhou (2009), including pre-amplification, selective amplification, polyacrylamide gel electrophoresis, silver stain and photoing.

Data analysis: AFLP data was scored for the presence (1) or absence (0) of amplification products (bands) at corresponding positions. Applying the NTsys2.0 program, genetic distances of the scores were cladistically analyzed using UPGMA (Sneath and Sokal, 1973) with Nei - Li coefficient, a clustering dendrogram was consequently generated.

RESULTS AND DISCUSSION

Eight primer pairs were selected for our AFLP amplification from list of Zou *et al*. (2001): E-ACG/M-CAT; E-ACG/M-CTG; E-ACA/M-CTT; E-ACT/M-CAA; E-AAG/M-CAT; E-AAG/M-CAA; E-AAG/M-CTG; E-ACT/M-CAT. A total of 414 bands were obtained, within which 346 bands were informative polymorphic. The corresponding proportion of polymorphic loci was 83.58%, with an average of 43.25 bands per primer pair. The primer pair E-ACG/M-CAT produced the most polymorphic loci in the present study (56 bands), contrarily, E-ACT/M-CAT yielded the least (34 bands). The highest proportion of polymorphic loci, 92.21%, attributed to primer pair E-ACT/M-CAA. The significant differences between fingerprints of samples rendered AFLP an acceptable method to test the relationships within the *Astragalus penduliflorus* complex (see Fig. 1, showing fringerprints generated from seven primer pairs, except for E-ACG/M-CAT).

In our dendrogram (Fig. 2), both of *Astragalus mongholicus* and *A. membranaceus* were unresolved. Five accessions of *A. mongholicus* clustered into two branches (B and G); on the other hand, accessions of *A. membranaceus* failed to group. *A. minhensis* and *A. petrovii* formed a branch at generic distance of 0.80 (F), both of which nested within *A. penduliflorus* complex.

Due to its medical use, the *Astragalus penduliflorus* complex attracted much attentions from taxonomists and pharmacologist (Zhu, 2003; Wang, 2013; Zhang, 2014). On the inter- and infra-specific level, it is a morphologically diverse complex with green/reddish stems, white/yellow/crimson/purple corollas, inflated/compressed young pods, *etc* (Liu, 2008; Xu and Podlech, 2010). Such diversity makes it difficult to unambiguously delimit species in this complex. For example, some workers kept specific status for *A. membranaceus* and *A. mongholicus* (Komarov, 1946; Shi, 2003; Zhao, 2006; Zhang *et al*., 2009), whereas more studies merge them as one species (Hsiao, 1964; Chen and Zhu, 1990; Ho and Fu, 1993; Dong *et al*., 2003; Zhu, 2003; Liu, 2008; Qian *et al*., 2009; Xu and Podlech, 2010).

Hsiao (1964) attribute such morphological diversity to adaptation to different habitats, and "*A. membranaceus* and *A. mongholicus* tend to be undistinguishable when habitats are convergent". In addition, Lavin *et al*. (2005) illuminated *Astragalus* as a newly emergent genus around middle Miocene, experiencing a rapid diversification. Thus, it is possible that subspecies or varieties diverged within one ancient widely spreading *Astragalus* species to adapt to

Table 1: Species and voucher information of samples in this study

Taxon name	Abbreviation	Locality (China)	Voucher (WUK)
Astragalus membranaceus (Fisch.) Bunge	MEM1	Hunyuan, Shanxi Province	Xu & Sheng 2009011
Astragalus membranaceus (Fisch.) Bunge	MEM2	Ledu, Qinghai Province	Xu & Sheng 2009021
Astragalus membranaceus (Fisch.) Bunge	MEM3	Fufeng, Shaanxi Province	Xu & Sheng 2009127
Astragalus minhensis X.Y.Zhu & C.J.Chen	MIN	Minhe, Qinghai Province	Xu & Sheng 2009001
Astragalus mongholicus Bunge	MON1	Jiexiu, Shanxi Province	Xu & Sheng 2009009
Astragalus mongholicus Bunge	MON2	Qingciyao, Shanxi Province	Xu & Sheng 2009012
Astragalus mongholicus Bunge	MON3	Guaner, Shanxi Province	Xu & Sheng 2009013
Astragalus mongholicus Bunge	MON4	Liangcheng, Inner Mongolia Province	Xu & Sheng 2009014
Astragalus mongholicus Bunge	MON5	Laoguanfang, Shanxi Province	Xu & Sheng 2009015
Astragalus mongholicus Bunge	MON6	Jining, Inner Mongolia Province	Xu & Sheng 2009126
Astragalus petrovii N.Ulziykh.	PET	Sunan, Gansu Province	Xu & Sheng 2009007

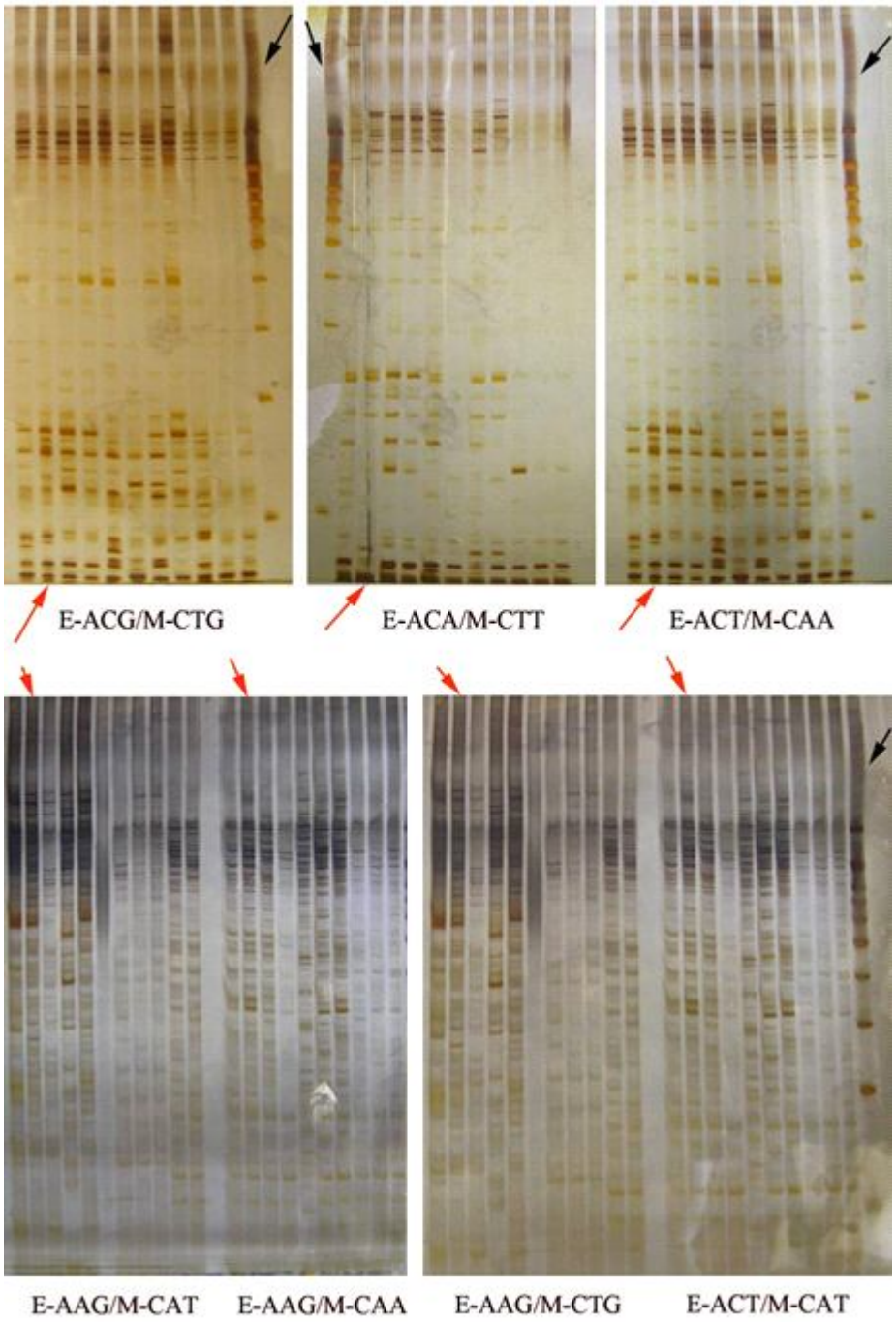

Fig 1: Seven groups of AFLP fringerprints, which were generated from seven primer pairs. Samples were arranged in the same order in each group: MIN, PET, MON1, MEM1, MON2, MON3, MON4, MON5, MEM2, MON6, MEM3 (from left to right). Black arrows: ladder markers; red arrows: *Astragalus penduliflorus* ssp. *mongholicus* var. *petrovii* (N.Ulziykhutag) X.L.Liu & L.Duan.

rapid shifting habitat environment. While such speciation is still underway, the morphological variation is not significant enough to ensure *A. membranaceus* and *A. mongholicus* as independent species. Such hypothesis consists with our field and herbarium studies. We thus agree with Zhu (2003), treating them as two varieties of *A. penduliflorus* (this species constitute the monotypic *Astragalus pendulijlorus* complex): *A. pendulijlorus* ssp. *mongholicus* var. *dahuricus* X.Y.Zhu and *A. penduliflorus* ssp. *mongholicus* var. *mongholicus* (Bunge) X.Y. Zhu, respectively. Similarly, *A. purpurinus* is mainly recognized by purple corollas in this complex (Ho and Fu, 1993), it is also valid to be degraded as a variety: *A. pendulijlorus* ssp. *mongholicus* var. *pallidipurpureus* (Hsiao) X.Y. Zhu (Zhu, 2003).

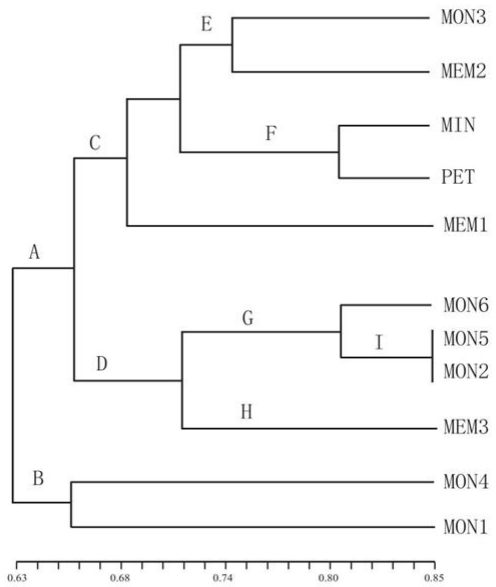

Fig 2: UPGMA clustering dendrogram of *Astragalus penduliflorus* complex based on AFLP markers.

Some molecular marking analyses also concentrated on *A. membranaceus* and *A. mongholicus*, these results showed that the former had lower level of inter-populational gene exchange than the latter, leading to higher infra-specific diversity (Wang, 2013; Zhang, 2014). This is concurred by our analysis: all four accessions of *A. membranaceus* failed to cluster, while five *A. mongholicus* accessions formed two branches. Our dendrogram supported neither of their specific status (Fig. 2), such cladistic results are consistent with the treatment merging *A. membranaceus* and *A. mongholicus* as varieties of *A. penduliflorus* (Zhu, 2003).

A. minhensis is morphologically similar to *A. membranaceus* and *A. mongholicus*, differed by the former's brown root (vs. grayish white), non-reflexed standard (vs. reflexed) and shorter ovary stipe (Fig. 3A-C). Zhu (1996) included *A. minhensis* into the *A. penduliflorus* complex, Zhu (2003) in turn treated it into the species *A. penduliflorus*, however, Xu and Podlech (2010) recovered its specific status. In our dendrogram (Fig. 1), *A. minhensis*, although forming a branch with *A. petrovii*, failed to diverge from *A. membranaceus* and *A. mongholicus*. Plus, based on our field observation, its morphological differentiation may not be obvious enough to support its specific rank. Thus, it seems appropriate to accept Zhu (2003)'s treatment, reducing it as *A. penduliflorus* ssp. *mongholicus* var. *minhensis* (X.Y.Zhu & C.J.Chen) X.Y.Zhu.

A. petrovii embedded in the *A. penduliflorus* complex based on our dendrogram (Fig. 2). It is different from *A. membranaceus* and *A. mongholicus* mainly by corolla colors (blackish purple vs. white/yellow/crimson). Accordingly, this species may also belong to the *A. penduliflorus* complex. *A. petrovii* clustered with *A. minhensis* at a genetic distance of 0.80, displaying their close relationship. While unlike *A. minhensis* (with dense white or black hair), the stems, rachises and both surfaces of leaflets of *A. petrovii* are glabrous or sparsely hairy (Fig. 3 D-H; Zhu and Chen, 1995; Ulziykhutag, 1996; Xu and Podlech, 2010). Additionally, *A. minhensis* is endemic to Qinghai province of China, which is adjacent to the distribution region of *A. petrovii* (Gansu province), but more arid (Zhu and Chen, 1995; Xu and Podlech, 2010). Possibly it is the reason that *A. minhensis* possesses denser hair than *A. petrovii*: more efficiently decreasing water transpiration. Combining cladistic, morphological and geographical evidence, we propose to treat *A. petrovii* as a variety of *A. penduliflorus* (see below).

Taxonomic treatment

***Astragalus penduliflorus* ssp. *mongholicus* var. *petrovii* (N.Ulziykhutag) X.L.Liu & L.Duan, comb. & stat. nov.** (Fig. 3 D-H): – *Astragalus petrovii* N.Ulziykhutag in Novosti Sist. Vyssh. Rast. 30: 109. 1996; Xu et Podlech in Wu *et al*. Fl. China 10: 344. 2010.

Type. China. Gansu Province: Mt. Qilian, alt. 2600m, 12 July, 1958, M. Petrov s.n. (holotype, LE).

Additional specimens Examined. China. Gansu Province, Sunan County: Mati temple, alt. 2006m, 23 July, 2009, Xu & Sheng 2009007 (WUK).

Description. Herb. Plant 70-90 cm tall. Stems 7 mm thick, glabrous or with sparse hair. Stipules ciliate, reflexed. Leaves 6–8 cm, subsessile; rachis glabrous or sparsely hairy; leaflets in 9-11 pairs, 10-15 × 4-6 mm, loosely hairy. Racemes densely flowered; peduncle longer than leaves. Calyx campanulate, teeth ca. 1mm. Corolla blackish purple; standard oblong; wings with claws longer than limbs; keels shorter than or as long as to standard; stamens diadelphous (9 + 1); style curved. Pods 3–3.5 cm long, stipitate; valves membranous with short hairs (see Fig. 2 D-H). This variety is restricted to Gansu Province, China.

Note. This variety resembles *A. pendulijlorus* ssp. *mongholicus* var. *minhensis*, but differs by less hairy plant, more leaflets per leaf (9-11 pairs vs. 5-9 pairs), oblong standards (vs. ovate standards) and longer wing claws (Fig. 3). It also possesses more leaflets per leaf (9-11 pairs vs. 3-9 pairs) than *A. pendulijlorus* ssp. *mongholicus* var. *pallidipurpureus*. *A. penduliflorus* ssp. *mongholicus* var. *petrovii* is recognizable from other taxa (except for two above-mentioned ally varieties) of this complex by its blackish purple flowers.

Fig 3: *Astragalus penduliflorus* ssp. *mongholicus* var. *minhensis* (X.Y.Zhu & C.J.Chen) X.Y.Zhu (A, B & C) and *A. penduliflorus* ssp. *mongholicus* var. *petrovii* (N.Ulziykhutag) X.L.Liu & L.Duan (D, E, F, G & H). A-F: photoed by Peiliang Liu 2015; G & H: photoed by Xiaolin Liu 2013.

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