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Genetic differentiation and relationship of populations in the *Aconitum delavayi* complex (Ranunculaceae) and their taxonomic implications

F.-M. Zhang, W.-L. Chen, Q.-E. Yang, and **S. Ge**

Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Beijing, China

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Abstract. The *Aconitum delavayi* complex consists of six climbing diploid species distributed mainly in the Hengduan Mountains of China. In order to clarify the circumscription and relationships among species within the complex, RAPD markers were employed to examine the differentiation of 18 populations representing all the six species. The PCO and cluster analyses of RAPD data indicated that the *Aconitum delavayi* complex comprises three different clusters. The first cluster consists exclusively of *A. episcopale* populations, which indicates that *A. episcopale* is a very distinct species, in good agreement with our previous allozyme and ITS data. The second cluster includes one *A. campylorrhynchum* population and all the *A. henryi* populations from the northern Hengduan Mountains region and its neighbor areas. The third cluster consists of the populations of *A. delavayi*, *A. stapfianum*, *A. tuguancunense* and *A. campylorrhynchum* from the southern Hengduan Mountains region. The little genetic differentiation among populations of *A. stapfianum*, *A. delavayi* and *A. tuguancunense* suggests that they would better be treated as a single species. The fact that *A. campylorrhynchum* populations appear in two different clusters demonstrates that *A. campylorrhynchum* comprises of two different species.

Key words: *Aconitum delavayi* complex, RAPD, Genetic differentiation, Hengduan Mountains, Ranunculaceae.

Aconitum L. (Ranunculaceae) is a diverse genus with nearly 300 species worldwide, mainly in the temperate regions of the northern hemisphere (Wang 1979, Kadota 1987). Climbing species with twining stems are unusual in the genus. They are distributed only in East Asia and mostly in the Hengduan Mountains of China, which is the diversity and endemism center of the genus *Aconitum* (Wang 1979; Li 1988, 1993). The total number of the climbing species is nearly 20 and all of them were described in subgenus *Aconitum* distinguished from the other two subgenera by its biennial tubers (Wang 1979, Tamura 1990).

The *Aconitum delavayi* complex is a group of diploid climbing species and can be separated easily from other climbing species by its trisect leaves (Yang et al. 1994, Yang 1999). Currently, six species are recognized within the complex—*A. delavayi* Franch., *A. episcopale* Lévl., *A. stapfianum* Hand.-Mazz., *A. tuguancunense* Q. E. Yang, *A. campylorrhynchum*

Hand.-Mazz. and *A. henryi* Pritz., and they are mainly distributed in the Hengduan Mountains, with the exception of *A. henryi* that occurs widely across the Hengduan Mountains and its neighbor areas (Fig. 1). Although all populations in this complex are highly localized and isolated in narrow areas quite small sizes, they frequently show overlapping patterns of variation in many morphological characters. Therefore, the circumscription and relationships among species within the complex have long been problematic, as is the case among other members of the genus *Aconitum* (Wang 1979, Brink 1982, Kadota 1987, Yang 1999, Utelli and Roy 2000, Cole and Kuchenreuther 2001, Luo 2003). Based

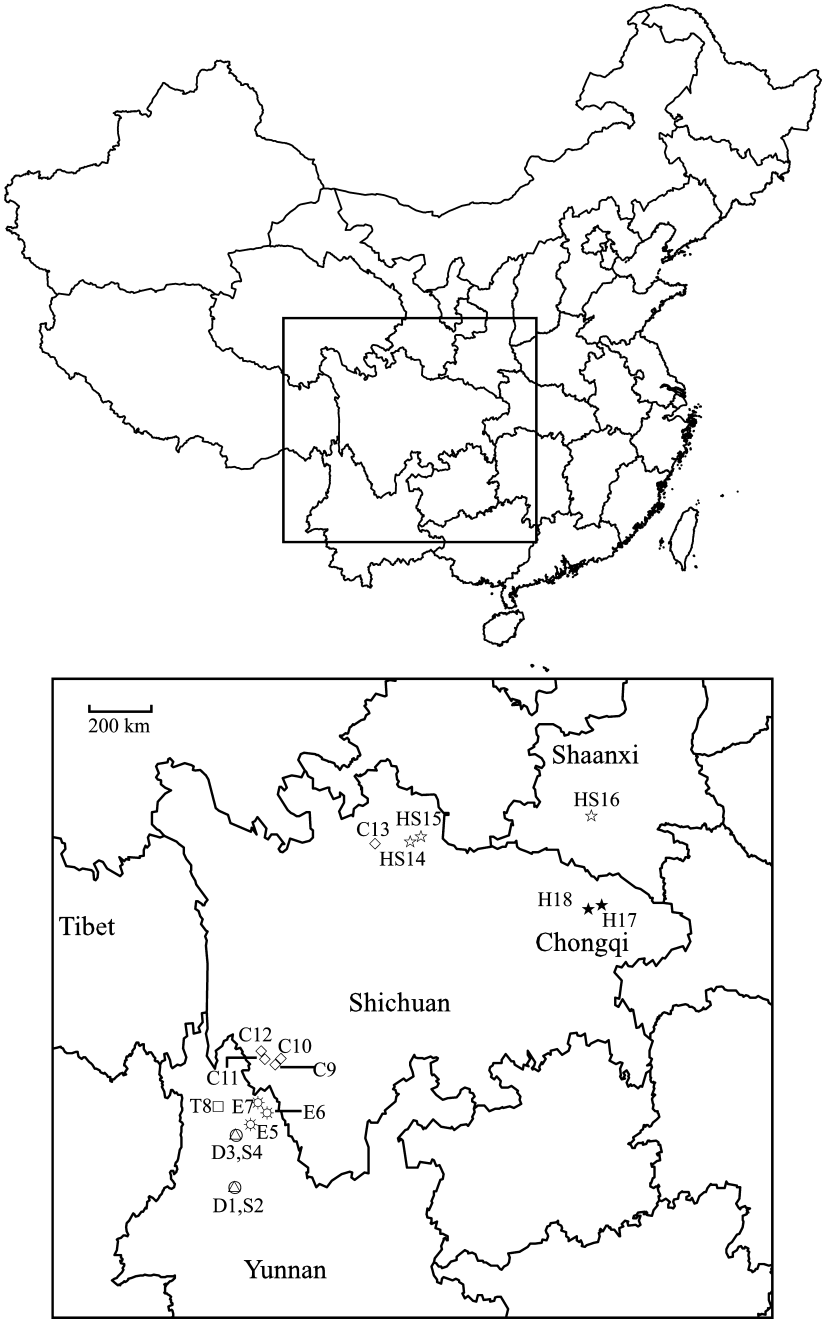

Fig. 1. Locations of 18 natural populations of *Aconitum delavayi* complex sampled in this study (see Table 1 for population names) ○: *A.delavayi* △: *A.stapfianum* ☼: *A.episcopale* □: *A.tuguancunense* ◊: *A.campylorrhynchum* ☆: *A. henryi,* corresponding to the former *A. sungpanense* ★: *A. henryi*

upon observations of four species of the complex in Yunnan, Yang (1999) found that there exist spreading hairs on pedicels of *A. delavayi*, but sparse curled hairs on pedicels of *A. episcopale*. He also indicated that *A. stapfianum* can be distinguished from *A. tuguancunense* by shorter galeate upper sepals although both of them usually have no hairs on pedicels. Similarly, *A. campylorrhynchum* could be identified by its long beak of upper sepal (hood) and *A. henryi* by sparse reflexed and curled hairs on pedicels (Wang 1979, Luo 2003). It appears that only a few slight morphological differences, such as status of hairs on pedicels, height and shape of upper sepal, were employed for distinguishing the species of the complex from each other. However, field investigations on the complex showed that there are many intermediate types between its populations or species, which lead to the difficulties in the identification of species. Therefore, Yang (1999) pointed out that if the specimens of these "species" were not accompanied with precise locality records, it would be rather difficult to identify them correctly.

Random amplified polymorphic DNA (RAPD) is a sensitive method of detecting genetic variation and has the advantage of being quick and easy, requiring little plant material, and having a high resolution (Williams et al. 1990, Fischer et al. 2000). In plant taxonomy and systematic studies RAPD has been used as a DNA marker for population genetic studies (e.g. Demeke et al. 1992, Hilu and Stalker 1995), and has contributed significantly to the understanding of the population genetics of *Aconitum* species in Europe and North America (Cole and Kuchenreuther 2001, Fico et al. 2003). Here, we used RAPD markers (1) to examine the levels of genetic differentiation among populations within the *Aconitum delavayi* complex; (2) to clarify the circumscription and relationships among species within the complex. Such information will contribute to a better understanding of

Table 1. The sampled populations of the *Aconitum delavayi* complex

Location	Species	Population	Altitude	Population size	Sample size
Hengduan Mountains					
Changshan, Dali	*A.delavayi*	D1	2900	30–40	13
Changshan, Dali	*A.stapfianum*	S2	3400	25–35	18
Yulongshan, Lijiang	*A.delavayi*	D3	3200	40–50	22
Yulongshan, Lijiang	*A.stapfianum*	S4	3600	50–60	24
Yunshanpin, Lijiang	*A.episcopale*	E5	2900	40–50	20
Heishui, Lijiang	*A.episcopale*	E6	2600	30–40	12
Baishui, Lijiang	*A.episcopale*	E7	2600	40–50	22
Tuguancun, Zhongdian	*A.tuguancunense*	T8	3200	20	18
912, Muli	*A.campylorrhynchum*	C9	3450	18	16
912, Muli	*A.campylorrhynchum*	C10	3400	30	20
912-1, Muli	*A.campylorrhynchum*	C11	3350	20–30	16
912-2, Muli	*A.campylorrhynchum*	C12	3450	60–80	25
Shuajingsi, Hongyuan	*A.campylorrhynchum*	C13	3300	11	11
Huanglong, Songpan	*A. henryi*	HS14	2750	50–60	24
Huanglong, Songpan	*A. henryi*	HS15	2750	11	11
The neighbor areas of Hengduan Mountains					
Baohuqu, Fuopian	*A. henryi*	HS16	2200	30–40	17
Qiganshan, Chengkou	*A. henryi*	H17	1700	24	20
Baicishan, Chengkou	*A. henryi*	H18	1550	22	19

speciation and evolution in this complex and the genus as a whole as well as it will facilitate a better classification of the genus *Aconitum*.

Materials and methods

Plant materials. A total of eighteen populations covering the entire distribution of the *Aconitum delavayi* complex were sampled in August and September of 2000 and 2001. Fifteen of them were located in Hengduan Mountains region while the remaining three (*A. henryi*) were from outside the region. It is worthy to mention that two populations from each of *A. delavayi* and *A. stapfianum* were collected from the Cangshan and Yulong Mountains, where these two species are altitudinal vicariants. *A. tuguancunense* is an extremely narrow endemic species and so far only one population has been found in Tuguancun. The location, original identification of species, estimated and sampled sizes of each population are listed in Table 1 and shown in Fig. 1. Because *A. henryi* used to be recognized as two different species (Handel-Mazzetti 1939, Wang 1979), two codes (H, HS) were used to represent the two "species" with HS standing for the previous *A. sungpanense*. For each individual sampled, one or two fresh leaves were collected and immediately stored in a small sealed plastic bag with about 50g silica gel for drying the leaves quickly. After the leaves were dried completely, the samples were transported to the laboratory and stored at room temperature.

DNA isolation. Genomic DNA was extracted using a modification of the protocol of Doyle and Doyle (1987). Dried leaf materials were ground to fine powder in a 2 ml Eppendorf tube, and then mixed with 900 μl of preheated 2×CTAB extraction buffer containing 0.3% mercaptoethanol. The homogenate was incubated at 65°C for 60 min prior to adding 900 μl of chloroform:isoamyl alcohol (24:1,v/v). After mixing by inversion for 5 min the mixture was centrifuged at 9168 g for 10 min at 15°C, and the supernatant reserved in another 1.5 ml Eppendorf tube and mixed with 550 ml (nearly 2/3, v/v) ice-cold isopropanol. The DNA was recovered as a pellet by centrifugation at 13201g for 10 min at 4°C, washed twice with 200 μl of 70% ethanol, dried, and dissolved in 200 μl of 0.1× TE buffer. DNA quality and quantity were determined in 0.8% agarose gels.

Polymerase chain reaction. DNA amplification was performed in a Rapidcycler 1818 (Idaho Tech.), and commenced with 2 cycles of 1 min at 94°C, 10s at 37°C and 20s at 72°C, followed by 42 cycles of 2s at 94°C, 10s at 37°C and 70s at 72°C, and ended with 5 min at 72°C. Each reaction was conducted in a glass capillary with 10 μl of mixture solution containing 50 mmol/l Tris-HCL (PH 8.3), 500 μg/ BSA, 10% Ficoll, 1mmol/l Tartrazine, 2 mmol/l $MgCl_2$, 200 μmol/l dNTP, 1 μmol/l primer, 10 ng target of DNA, and 0.5 units of Taq polymerase. RAPD fragments were separated electrophoretically in 1.5% agarose gels, stained with ethidium bromide, and photographed under ultraviolet light using Gel Doc 200 (Bio-Rad Inc.). Molecular weights were estimated using 100 bp DNA ladder.

Data analysis. Since RAPD markers are dominant, we assumed that each band represented the phenotype at a single biallelic locus (Williams et al. 1990). Thus, RAPD bands scored as present (1) or absent (0) at every locus for each target DNA. The RAPD phenotype data of all individuals were organized in a binary matrix. Using the matrix, we conducted a principal coordinates analysis (PCO) in Euclidean distance with software Mvsp3.13b (download from the website: http://www.kovcomp.com) and a cluster analysis using the unweighted pair-group method algorithm (UPGMA) based on mean character difference with software PAUP* 4.04ß4a (Swofford 1999). 1000 bootstrap replicates were calculated to indicate percentage support of the cluster dendrogram branches.

Results

RAPD banding. After screening 140 decamer primers against six plants selected from two *A. episcopale*, two *A. delavayi* and two *A. henryi* populations, 20 were selected for further analyses based on the following criteria: (1) consistent production of strong amplification; (2) production of uniform, reproducible fragments between replicate PCRs (see Table 2). Among 136 bands yielded by the 20 primers, 12 were monomorphic, and thus 91.2% of the RAPD loci detected were polymorphic. The number of loci varied from 5 to 12 among 20

primers with the mean number per primer being 6.2.

The principal coordinates analysis. Based on the principal coordinates analysis, relationships among populations of *Aconitum delavayi* complex were visualized best as two two-dimensional graphs (Fig. 2). A decision to use only the first three factors in the PCO, which contained the clear majority of variance (54.35%), would satisfy the Kaiser criterion (Kaiser 1960). One graph (Fig. 2a) based on axis 1 and 2 contained 46.0 % of variance and shows that the 328 individuals form three distinct clusters. The first cluster comprises all populations of *A. episcopale* (E5, E6, E7), which were located in southern Hengduan Mountains region and this cluster is far away from the other two on the graph. The second cluster includes all six populations from the northern Hengduan Mountains region and its neighbor areas, representing two species: *A. campylorrhynchum* (C13) and *A. henryi* (HS14, HS15, HS16, H17, H18). The third cluster or group consists of the populations of *A. delavayi* (D1, D3), *A. stapfianum* (S2, S4), *A. tuguancunense* (T8) and *A. campylorrhynchum* (C9, C10, C11, C12) in the southern Hengduan Mountains region. The other graph (Fig. 2b) based on axis 1 and 3 contained 35.90 % of variance and fails to show a clear delimitation within the *A. episcopale* (first) cluster and the northern (second) cluster. However, the southern (third) cluster was divided into two distinct subgroups: one consists of the populations of *A.campylorrhynchum* (C9, C10, C11, C12) in Sichuan (see Fig. 1) and the other consists of the rest (D1, S2, D3, S4, T8, C9) in Yunnan (see Fig. 1). Both graphs show that *A. campylorrhynchum* populations were unexpectedly divided into two parts based on their geographic localities, appearing in the southern or northern cluster respectively.

The UPGMA cluster analysis. The UPGMA cluster analysis indicated that 328 individuals were grouped into three distinct clusters (Fig. 3). The populations of *A. episcopale* form the *A. episcopale* cluster in the southern Hengduan Mountain region, show-

Table 2. The primers used in this study

Primer	Sequence	No. of loci	No. of polymorphic loci
S46	ACCTGAACGG	6	5
S50	GGTCTACACC	7	7
S55	CATCCGTGCT	5	5
S60	ACCCGGTCAC	5	2
S85	CTGAGACGGA	7	7
S87	GAACCTGCGG	5	5
S88	TCACGTCCAC	8	7
S90	AGGGCCGTCT	9	9
S92	CAGCTCACGA	6	6
S406	CTGGGCAACT	6	5
S419	CCTTCAGGCA	6	4
S425	ACTGAACGCC	7	7
S427	CAGCCCAGAG	9	7
S428	ACCTCAGCTC	6	6
S439	GTCCGTACTG	11	10
S462	TCGGCACGCA	8	7
S464	GTGTCTCAGG	4	4
S465	CCCCGGTAAC	12	12
S504	CCCGTAGCAC	4	4
S506	GTCTACGGCA	5	5

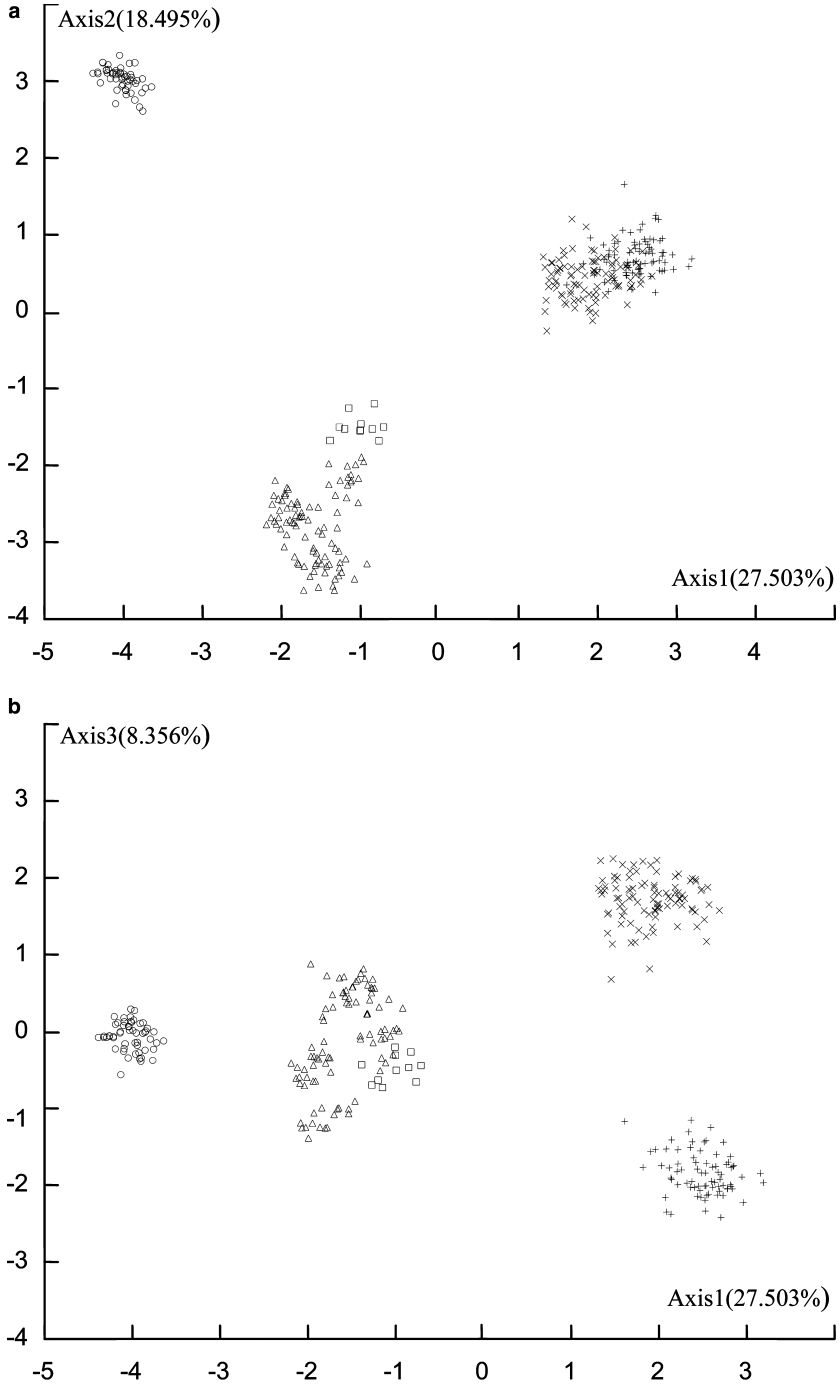

Fig. 2. Principal coordinates analysis of 18 populations within the *Aconitum delavayi* complex. **a** Based on axis 1, 2; **b** Based on axis 1, 3; ×: D1, S2, D3, S4, T8; +: C9, C10, C11, C12; □:C13; △:HS14, HS15, HS16,H17,H18. ○:E5, E6, E7

ing clear separation from the other two. The populations of *A. campylorrhynchum* and *A. henryi* from the northern Hengduan Mountains region and its neighbor areas form the northern cluster; and the populations of *A. delavayi*, *A. stapfianum*, *A. tuguancunense* and *A. campylorrhynchum* from the southern Hengduan Mountains region form the southern cluster. In addition, *A. campylorrhynchum* populations appear in both southern and northern clusters. These results parallel exactly those obtained by PCO analysis. More impor-

Fig. 3. UPGMA dendrogram based on mean character differences estimated from RAPD data from 327 individuals in 18 populations of the *Aconitum delavayi* complex. Numerals above branches indicate percentage of support in 1000 bootstrap replicates

tantly, UPGMA cluster analysis gave us more detailed information about the relationships among the populations. In the *A. episcopale* cluster, all the individuals from the same population clustered together tightly before clustering with individuals of any other population. It is more interesting that two geographically close populations (E6, E7) cluster together before forming the *A. episcopale* cluster with the more geographically distant population (E5). In the northern cluster, although all the *A. henryi* populations cluster

together initially, they showed different clustering patterns. The individuals from each of the three populations of *A. henryi* in the neighbor areas of the Hengduan Mountains (HS16, H17, H18) formed their own cluster before clustering with those from other populations. In contrast, the individuals from the two populations of *A. henryi* within the Hengduan Mountains (HS14, HS15) entirely mixed together. In this species, geographically close populations also form clusters before clustering with those far away, such as HS14 and HS15, H17 and H18. In the southern cluster, the populations from four species are divided into two subgroups: one consists of four populations of *A. campylorrhynchum* in Sichuan and the other comprises the rest of the species that occur in Yunnan. In the former subgroup, individuals from a particular population of *A. campylorrhynchum* did not form a cluster before clustering together with individuals from other populations. In contrast, individuals from a particular population clustered initially in the latter subgroup comprising five populations of three species. Finally, the two *A. delavayi* populations (D1 and D3) did not form their own cluster because one of them clustered with an *A. tuguancunense* population (T8) initially.

Discussion

The circumscription and relationships among the species of the *Aconitum delavayi* complex have long been in a great confusion (Finet and Gagnepain 1904, Handel-Mazzetti 1939, Fletcher and Lauener 1950, Wang 1979). In his recent revision, Yang (1999) believed that there were very close relationships among all species within the *Aconitum delavayi* complex and that these species formed so-called geographical vicarious relationships. The results of our previous ITS research on the phylogeny of the complex indicated that its species were separated into three different clades (Zhang et al. 2003a). Nevertheless, the ITS information was not sufficient to resolve the relationships among species, as evidenced in other ITS studies on the genus *Aconitum* (Utelli and Roy 2000, Kita and Ito 2000). In the present study, both the PCO and cluster analysis indicated that the *A. delavayi* complex comprises three distinct clusters: *A. episcopale*, and the northern and the southern clusters, among which there is significant genetic differentiation. This result is consistent with the ITS research on this complex.

Aconitum episcopale shows clear RAPD differentiation from the other species. This evidence is not only consistent with our allozyme study (Zhang 2003b), supporting Yang's opinion that *A. episcopale* is a distinct species, but also confirms that *A. episcopale* is the most divergent cluster of the complex as inferred from our ITS data (Zhang et al. 2003a). All individuals of each population of *A. episcopale* clustered tightly, suggesting that there is obvious genetic differentiation among populations in this species. The fact, that the geographically closer populations have tighter genetic relationship indicates that the differentiation among *A. episcopale* populations is possibly caused by geographical isolation.

Aconitum henryi had long been treated as two different species: *A. sungpanense* Hand.-Mazz. and *A. henryi*, because it was thought that different leaf shapes existed between them (Handel-Mazzetti 1939, Wang 1979). Nevertheless, after investigating the available specimens, Luo (2003) believed that their leaves share the same pattern of morphological variability, and so they should be treated as a single species. In the present study, though all populations of *A. henryi*, including those previously identified as *A. sungpanense* (HS14, HS15, HS16), clustered initially before clustering with those of other species, there is still obvious differentiation between former *A. henryi* and former *A. sungpanense*. Therefore, the present study does not offer enough evidence to support the recent treatment of combining *A. sungpanense* and *A. henryi* as a single species. Besides populations of *A. henryi*, the northern cluster includes one population of *A. campylorrhynchum* and there is no

significant differentiation between this population and those of *A. henryi* based on bootstrap support.

Both the PCO and cluster analyses show that the southern cluster is divided into two distinct subgroups. One comprises four populations of *A. campylorrhynchum* in Sichuan while the other comprises five populations of *A. stapfianum*, *A. delavayi* and *A. tuguancunense* in Yunnan. In the Sichuan subgroup, individuals of four *A. campylorrhynchum* populations mixed together, indicating that the four populations are closely related genetically. Althoug the delimitations of each population of the Yunnan subgroup were clear, there were still high genetic similarities among the five populations, which is consistent with the allozyme analysis (Zhang et al. 2003b). Similar to the allozyme results in which neither *A. delavayi* populations nor *A. stapfianum* populations clustered initially, two populations (D1, D3) from *A. delavayi* did not form their own cluster because one of them (D1) clustered with the *A. tuguancunense* population and the other (D3) with two *A. stapfianum* populations (S2, S4). The present result, in conjunction with the allozyme evidence, indicates that there is no significant genetic differentiation among the populations of *A. stapfianum*, *A. delavayi* and *A. tuguancunense*. Recent morphological study also suggested that high morphological similarity occurs among the three species (Yang 1999). Therefore, the three species would better treated as a single one.

One unexpected outcome of this study is that *A. campylorrhynchum* populations appear both in the Southern (C9, C10, C11, C12) and in the Northern (C13) clusters. Therefore, *A. campylorrhynchum*, as a species, is doubtlessly problematic and would better be treated as two different species. This conclusion is consistent with our field observations on *A. campylorrhynchum*; the long beaks (hood) of the individuals in the southern Hengduan Mountains were pendent while those individuals in the northern part of the range have the long beaks (hood) reflexed upwards.

In conclusion, the *Aconitum delavayi* complex comprises three different clusters. *A. episcopale* is the most divergent cluster and thus a distinct species. There is not enough evidence to support the recent treatment of combining *A. sungpanense* and *A. henryi* as a single species. The present study also suggests that *A. stapfianum*, *A. delavayi* and *A. tuguancunense* would better be treated as single species, while *A.campylorrhynchum* should be treated as two different species.

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Addresses of the authors: Fu-Min Zhang, Wen-Li Chen, Qin-Er Yang, Song Ge (e-mail:gesong@ibcas.ac.cn), Laboratory of Systematic and Evolutionary Botany, Institute of Botany, the Chinese Academy of Sciences, 20 Nanxincum Xiangshan, Beijing 100093, China.