Pak. J. Bot., 44(2): 477-484, 2015.

SYSTEMATIC VALIDATION OF MEDICINALLY IMPORTANT GENUS *EPIMEDIUM* SPECIES BASED ON MICROSATELLITE MARKERS

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Abstract

Epimedium is taxonomically complicated in terms of identification and availability of limited phenotypical markers. Therefore in the present study, 36 SSR primers markers were used for 44 individuals belonging to 13 medicinal species of the *Epimedium* genus and one out group species *Vancouveria hexandra* W. J. Hooker to resolve their existing taxonomic problems. A total of 164 alleles by genomic SSR were detected. The markers were presented between 2-10 alleles per locus. Jacard index cluster analysis revealed two main and four subclusters. Principle component analysis indicated that genetic variability is analogous to geographical variability. It has been concluded that medicinally important species of the genus *Epimedium* possesses sufficient genetic variation for effective resolution of the existing taxonomic problems in combination of morphological markers. .

Key words: *Epimedium*, Genomic SSR/EST SSR markers, Genetic diversity, Systematics.

Introduction

Medicinally important temperate region genus of family Berberidaceae comprised of 60 species all over the world (Stearn, 2002). Out of these 60 species 52 are native to China (Ying *et al.*, 2011) with 52 native species (Geo & Xiao, 1999). The taxonomic information of this genus is chiefly based on morphometry. Leaves borne on peduncle, form and size of corolla (type, form and size) and flower dimension were frequently used for systematical studies of this genus (Fisher & Meyer, 1846; Morren & Decaisne 1834; Sun *et al.*, 2005). However less diversity of these markers leads to numerous taxonomic confusions. *Epimedium accuminata* and *E. pubescens*, *E. wushanense* and *E. nanchongense* were difficult to identify because of least variable leaf morphology. The inflorescence of *E. sagittatum, E. brevicornu* and *E. pubescens* is compound, loose and many flowered raceme (Stearn, 2002). There are very little characters for the distinction of these species. Number of leaves on the inflorescence is another important feature of this genus to differentiate the species. However, this character is not consistent in some species, such as *E. sagittatum*, *E. leptorrhizum* Stearn, and *E. elongatum* Komarov (Stearn, 1938). The morphological and chemical markers are very few that generate space for other markers to bring them in utilization for taxonomic purpose (Shinwari *et al.*, 1994; Shinwari, 1995). Epimedium has been explored for various DNA markers to resolve the taxonomic problems. Nakai *et al.*, 1996 characterized Japanese *Epimedium* species by RFLP. Intra specific relationship of the genus based on Internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA and 5S rRNA was explored by Sun *et al.*, 2005 and Kim *et al.*, 2004a /b. AFLP markers for *Epimedium* were investigated by Efron *et al.,* (1996). However species like *E. myrianthum*, *E. leishanense*, *E. brevicornu*, *E. membranaceum* and *E. koreanum* have high intra-specific variation and difficult to identify their specific status. In the same way samples for *E. acuminatum*, *E. sagittatum* and *E. pubescens*, could not form a group per species. For the *Epimedium* species, intra species similarity is close or even lower than interspecies similarity and hence AFLP pattern may not be suitable for species identification. Microsatellites or SSRs (Simple Sequence repeats) are tandem repeat sequences having less than six base pairs. They are also very abundant and randomly distributed in genome (Turi *et al.,* 2012). Therefore an effort was made to characterize the *Epimedium* medicinally important species applying SSR markers to assess intra and interspecific relationship, leading to resolve the existing taxonomic confusion among medicinally important species of the genus *Epimedium*.

Materials and Methods

Plant material: A total of 13 medicinally important species of the genus *Epimedium* including 45 individuals were selected for the taxonomical evaluation based on genomic and EST-SSR markers. According to the classification of Stearn, 2002, sampled species covered two sections (*Diphyllon* and *Macroeras*) and two series (*Brachycerae* and *Dolichocerae*) of section *Diphyllon* (Stearn, 2002). *Vancouveria hexandra* W. J. Hooker was used as out group species due to its close relationship to the genus *Epimedium* (Wang *et al.*, 2007).

Microsatellite marker analysis: Leaf sample of 13 medicinally important species of *Epimedium* were collected from the various regions of China (Fig. 1). DNA was extracted from leaves by using standard protocol (Doyle & Doyle, 1990). After the extraction of DNA, the purity and concentration of the obtained DNA was calculated using Nanodrop ND-2000 Spectophotometer (Wilmington, USA). Bulk genomic DNA samples were made from 45 individuals of selected species. A total of 26 genomic SSR and 10 EST SSR primers were randomly selected from already generated libraries in Key Laboratory of Plant Resources Conservation and Sustainable Utilization, South China Botanical Garden, Chinese Academy of Sciences, Gaungzhou, China. PCR amplification was performed in a total volume of 10µl reaction mixture containing 50ng template DNA, 20 pmol of each primer, 10×buffer [10 mM Tris-HCl (pH 8.4), (1-2mM) $MgCl_2$, 200µM NTPs and 0.5 unit of Taq DNA polymerase. The concentration of $MgCl_2$ is locus specific. The PCR reactions were performed under standard conditions for all primers in lab cycler (PCR machine). The annealing temperature was fixed for all primer pairs at 55˚C. After 5 minutes at 95˚C, 34 cycles were carried out with 30s at 95˚C, 30s at 55˚C, 90s at 72˚C for extension, and final extension step of 10 minutes at 72˚C.

Electrophoresis of amplified products: The separation of alleles was performed on 6% polyacrylamide gel. PCR products were mixed with 7.5µl of loading buffer. The mixture was denatured at 94˚C for 10 min before loading onto the gels. Gels (20 bp marker, by Beijing Yuanpinghao Biotech Co., Ltd) were stained with silver nitrate following the protocol as described in Bassam *et al.* (1991) that was used for calculating the length of genomic and EST-SSR amplicons.

Data analysis: Preliminary population genetics analyses were performed using GENEPOP version 3.4 (Raymond & Rousset, 1995). Principle component analysis (PCA) was done by GenAlex 6. Cluster analysis based on Nei genetic distance was performed using GenAlex 6 combination with Mega 4.

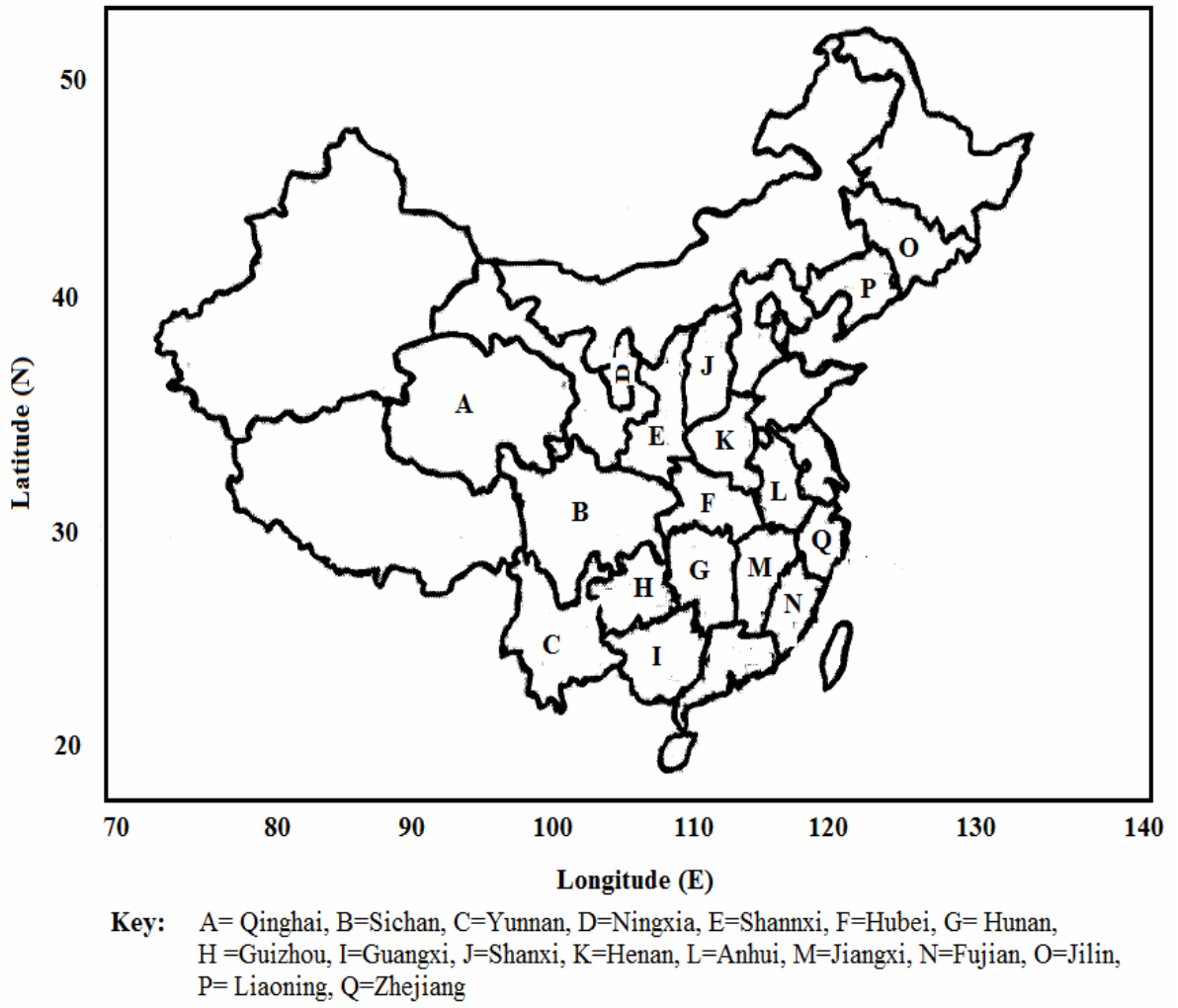

Fig. 1. Phytogeographical presentation of species of the genus *Epimedium* in China.

Results and Discussion

Epimedium is among the most important pharmaceutically important genus in China. Therefore this genus is continuously explored form cytological, molecular and phytochemical prospective. At the same time it was also investigated for the taxonomic purpose (Morren & Decaisne, 1834; Fischer & Meyer, 1846; Franchet, 1886; Komarov, 1908 and Stearn, 1938/2002) to enhance the effective utilization of this genus. However all of these taxonomic treatments are based on morphological characters. Hence insufficient morphological markers and natural existence of polymorphism of phenotypic markers leads toward the taxonomic confusion. Moreover this confusion contributes toward indefensible utilization of the medicinally important species. Along with over harvesting and reduction of habitat since 1990s incorrect identification resulted in dramatic reduction of wild resources of medicinal *Epimedium* species (Ward 2004; Xu *et al.,* 2007). Therefore in the present study efficacy of thirty six SSR markers were tested to resolve the identification problems related to thirteen medicinally important species of the genus *Epimedium.*

After studying the presence of diverse bands, a total 164 alleles were generated by SSR markers (Table 1). The number of alleles ranges from 2-10 with an average of 4.5 per locus. Among twenty six primers YYH-59, YYH-350, YYH-41, YYH-206, YYH-405, YYH-34, ET16OFH03GAAXJ, ETK79B003F5Z3V,Contig5231, ET16OFH03GK77E, and ET16OFH03FRSGL found to be most variable markers (Fig. 2). The least variation was observed at the locus of YYH-57 (*E. brevicornu*, *E. sagittatum*, *E. koreanum* and *E. wushanense*), YYH-63, YYH-234 (*E. stellulattum* Stearn), YYH-175 (*E. sagittatum*, *E. brevicornu*, *E. pubescens*, *E. stellulatum*), YYH-65 and GC-120 (*E. diphyllum* and *E. stellulatum*). Locus YYH-138 is monomorphic, however polymorphism for this locus is only found in *E. sagittatum.* For *Vancouveria hexandra* monomorphism was found at locus YYH-49, YYH-38, YYH-59 and YYH-405. However locus YYH-63, YYH-34, YYH-145, YYH-48, YYH-65, YYH-206 and GC-15 showed polymorphism in this species. Locus EWA9L9B0401 was not found in *E. Leptorrhizum*, *E. Koreanum*, *E. pubescens* and *E. brachyrrhizum*. Similarly, EWA9L9B04I75UH cannot be cross amplified in *E. brevicornu*, *E. chlorandrum*, *E. diphyllum* and *E. rhizomatosum.* Out of thirty six fifteen primers of genomic SSR primers and two EST-SSR ET16OFH03GK77E, ETK79B003F5Z3V could not cross amplified in out group species *V. hexandra.* The level of polymorphism among 45 individuals of 13 medicinally important species and one out group species *V. hexandra* was evaluated by calculating PIC (Polymorphism information contents) values for each of 36 SSR primers. The PIC values varied wiedly among loci and ranged from 0.17-0.79 with an average of 0.509 per locus (Table 1).

For evolutionary relationship among selected *Epimedium* medicinal species based on alleles information was generated by genomic SSR markers and used for the construction of dendrogram (Fig. 4). Nei genetic distance of 0.33 divided 14 species into two main clusters. The first cluster comprises of only *out* group species *vancouveria herandra*, whereas cluster second comprises of medicinally important species of the genus *Epimedium*. This cluster at the genetic distance of 0.25 is subdivided into four sub-clusters. *Epimedium membranceum* and *E. pubescence* comprises first sub-cluster (Fig 3). Second and third sub-clusters consist of one species each *E. chlorandrum* and *E. leptorrhizum* respectively. *Epimedium brachyrrhizum*, *E. wushanense*, *E. koreanum*, *E. brevicornu*, *E. accuminatum, E. diphyllum*, *E. rhizomatosum* Stearn, *E. stellulatum* and *E. Sagittatum* are the part of sub-cluster four. It is predicted from the dendrogram that species of section *Diphyllon* are more intact to their respective sections as compared to section *Macroceras* (Fig. 3).

Principle component analysis (PCA) was performed to produce scatter plots summarizing genetic variation among medicinally important species of the genus *Epimedium* across various geographical areas. Geographically, most of the *Epimedium* species are scattered between 100-110°E and 25-37°N (Fig. 1). It was predicted that gradient and wave patterns in these plots as signatures of inter specific genetic variability corresponding the geographical distribution (Fig. 4 and 5). Species from same or closely related geographical areas are genetically closer to each other than species of the distinct areas. *E. koreanum*, *E. brevicornu*, *E. wushanense* and *E. accuminatum* specimens were collected from Liaoning, Shanxi, Hubei and Sichuan province, respectively. When PCA was conducted, these species occupied very distinct place between two coordinates of the scatter plots. Similarly *E. chlorandrum*, *E. rhizomatosum* and *E. stellulatum* collected from Sichuan, Yaan and Shaanxi. Hence their geographical distribution is very close to each other and their location between two coordinate of scatter plots is clustering close to each other.

Relationship between geography and genetic variability was also determined by performing PCA the dataset produced by ten EST-SSR markers. The distribution of the species between two coordinates of scatter plots is parallel to their geographical distribution (Fig. 5). Species collected from the same or adjacent provinces marked the close location on PCA scatter plots. *Epimedium membranaceum* and *E. leptorrhizum* collected from Sichuan and Hubei province respectively. Both of these two species have close position on scatter plots too.

First taxonomic record of this genus was made by Linnaeus. The type species for this genus is *E. alpinum* L. (Linneaus, 1753). Subsequently, many taxonomists have performed systematic studies of this genus. However Stearn, 2002 gave the most comprehensive classification system for this genus. But the classification system was established wholly based on corolla characteristics, such as petal type, the form and relative size of the inner sepals and petals, flowers dimensions and geographical distribution of species. Resultantly, much taxonomic confusions were arisen. To answer these taxonomic question, many studies have been attempted using pollen morphology (Zhang & Wang, 1983; Liang & Yan, 1991), cytology (Sheng & Chen, 2007), isozymology (Koga *et al.,* 1991), molecular biology (Nakai *et al.,* 1996; Wang *et al.*, 2001; Sun, 2004; Sun *et al.*, 2005), biogeography (Ying, 2002; Zhang *et al.,* 2007), and chemical classification (Guo & Xiao, 1999; Koga *et al.,* 1991; Guo *et al.,* 2008). Unfortunately taxonomic questions still has to be answered.

Table 1. Characterization of twenty six polymorphic genomic SSR and ten EST-SSR microsatellite loci in taxonomically complicated medicinal species of the genus *Epimedium*.

Locus	Repeat motif	Total alleles	Polymorphic allele	Allele size range (bp)	Difference	PIC values
YYH-49	(CT)16	2	1	160-165	5	0.33
YYH-63	(CT)11CC(CT)10CCCT(CA)15	3	2	131-190	59	0.17
YYH-67	(TA)6(TG)9	4	2	140-160	20	0.41
YYH-212	(GT)10	3	2	180-195	15	0.42
YYH-138	(AC)9	2	1	155-185	30	0.33
YYH-34	(CT)26(AC)24	6	4	140-170	30	0.67
YYH-59	(CA)33(TA)(CA)26	7	6	130-200	70	0.75
YYH-57	(AG 14G2 AG 12	3	2	222-240	18	0.38
YYH-145	GA 43	4	2	195-222	27	0.55
YYH-175	(TC)19(AC)19	2	1	165-180	25	0.41
YYH-48	(CT)33TA(CA)15(TA)(CA)6	3	2	124-185	61	0.61
YYH-350	(GA)25	8	7	295-355	60	0.77
YYH-30	(CT)18(CA)11	2	1	185-193	8	0.52
YYH-41	(CA)5TA(CA)13	5	4	205-218	13	0.67
YYH-65	(AC)35	2	1	169-223	54	0.23
YYH-16	(CT)26	4	2	160-190	30	0.57
YYH-206	(GT)15	3	2	170-200	30	0.79
YYH-234	(TG)14	4	2	200-280	80	0.51
YYH-405	(AC)33	3	2	210-220	10	0.60
GC-15	(AC)20	4	3	233-299	66	0.44
GC-28	(TG)12	3	1	180-200	20	0.21
GC-102	(AC)10N20 (CA) 5 (AC) 2	4	2	275-292	17	0.19
YYH-336	(TG)12	4	2	119-181	62	0.65
YYH-152	(TC)14CC (TC) 8	5	3	200-235	35	0.44
YYH-254	(AC)22 (AT) 16	3	2	195-230	35	0.50
YYH-46	(AG) 39	6	4	158-192	34	0.35
EWA9L9B04H3M01	(AT) 6	5	3	256-265	9	0.25
EVNAJ6X02C8M94	(T) 12	3	2	280-290	10	0.33
ETK79B003HGO7V	(A) 23	4	3	90-105	15	0.67
EWA9L9B04I76NS	(A) 10	6	4	114-120	6	0.48
ET16OFH03FRSGL	(TC) 7	8	6	100-127	27	0.71
ET16OFH03GK77E	(CA) 9	7	6	95-105	10	0.79
Contig5231	(CTG) 6	7	5	180-205	25	0.76
ETK79B003F5Z3V	(A) 18	9	7	135-170	35	0.67
EWA9L9B04I75UH	(A) 11	6	3	185-205	20	0.55
ET16OFH03GAAXJ	(A) 10	10	7	120-155	35	0.67
Total		**164**	**109**			**18.35**
Average		**4.5**	**3.02**			**0.509**

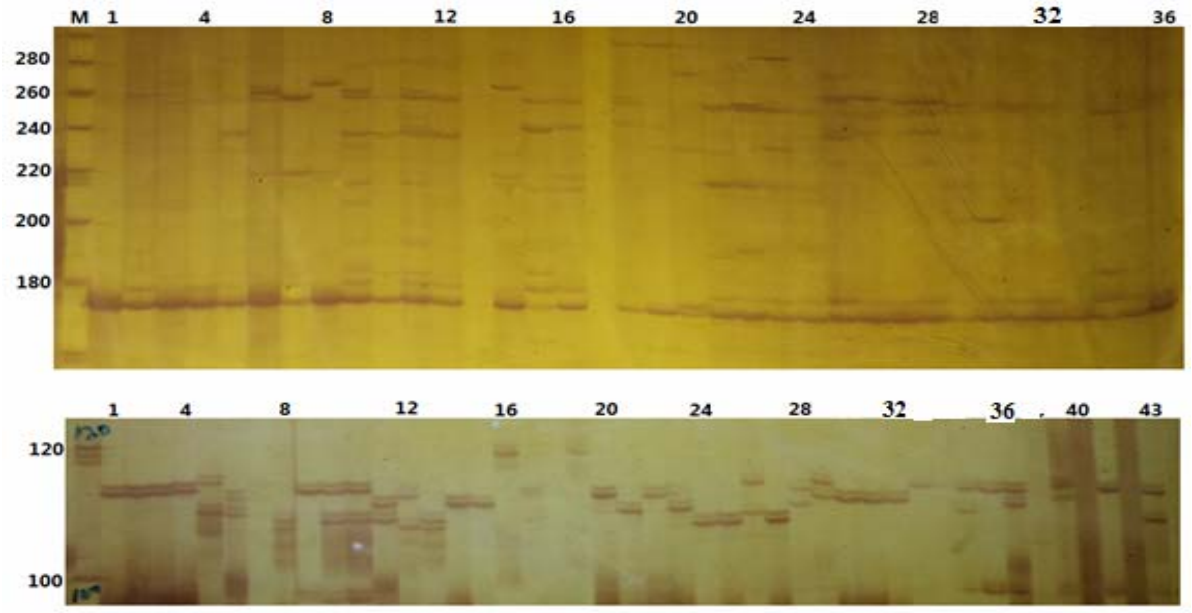

Fig. 2. Band patterns amplified by genomic SSR marker YYH-405 and EST-SSR marker EpSSR-08 key: *E. pubescens* (1-4); *E. sagittatum* (5-8); *E. accuminatum* (9-12); *E. brevicornu* (13-16); *E. koreanum* (17-20); *E. wushanense* (21-24); *E. leptorrhizum* (25-28); *E. brachyrrizhum* (29); *E. membranceum* (30-33); *E. rhizomatosum* (34); *E. chlorandrum* (35-38); *E. diphyllum* (39-40); *E. stellulatum* (41-43).

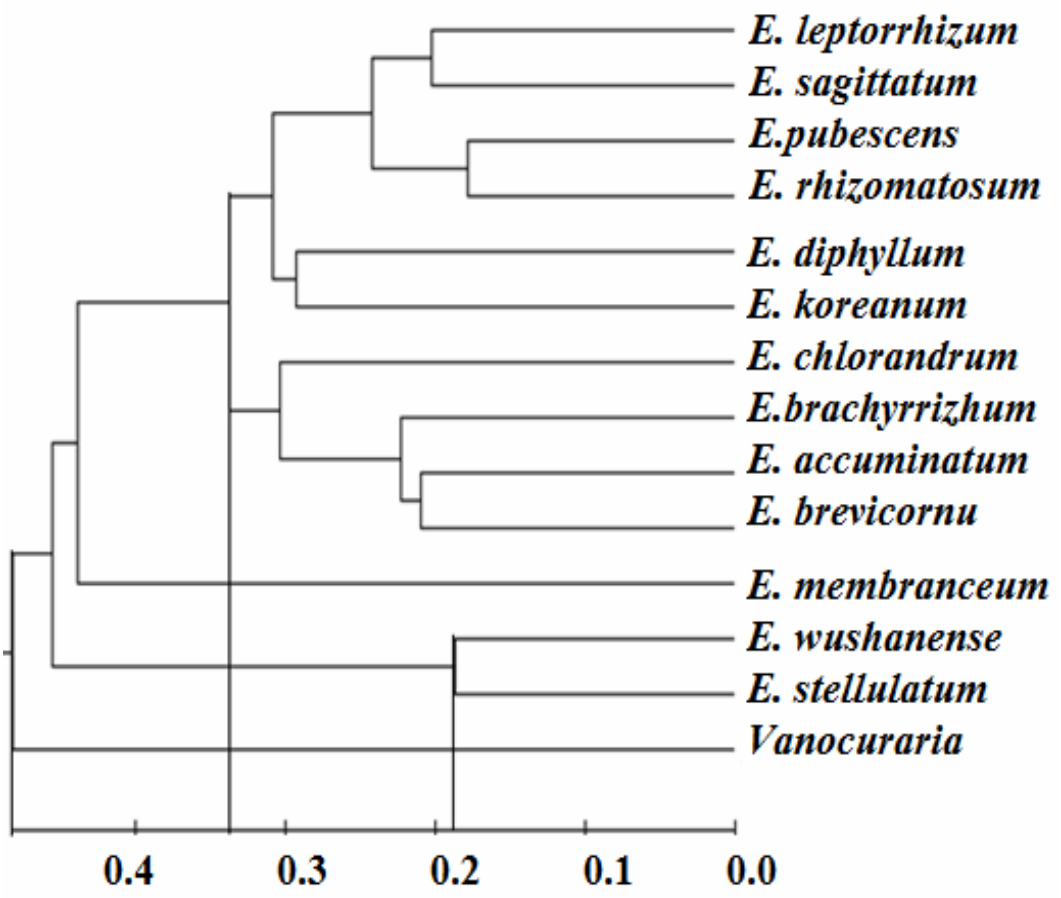

Fig. 3. Cluster analysis of medicinally important but taxonomically complicated species of the genus *Epimedium* based on thirty six SSR markers.

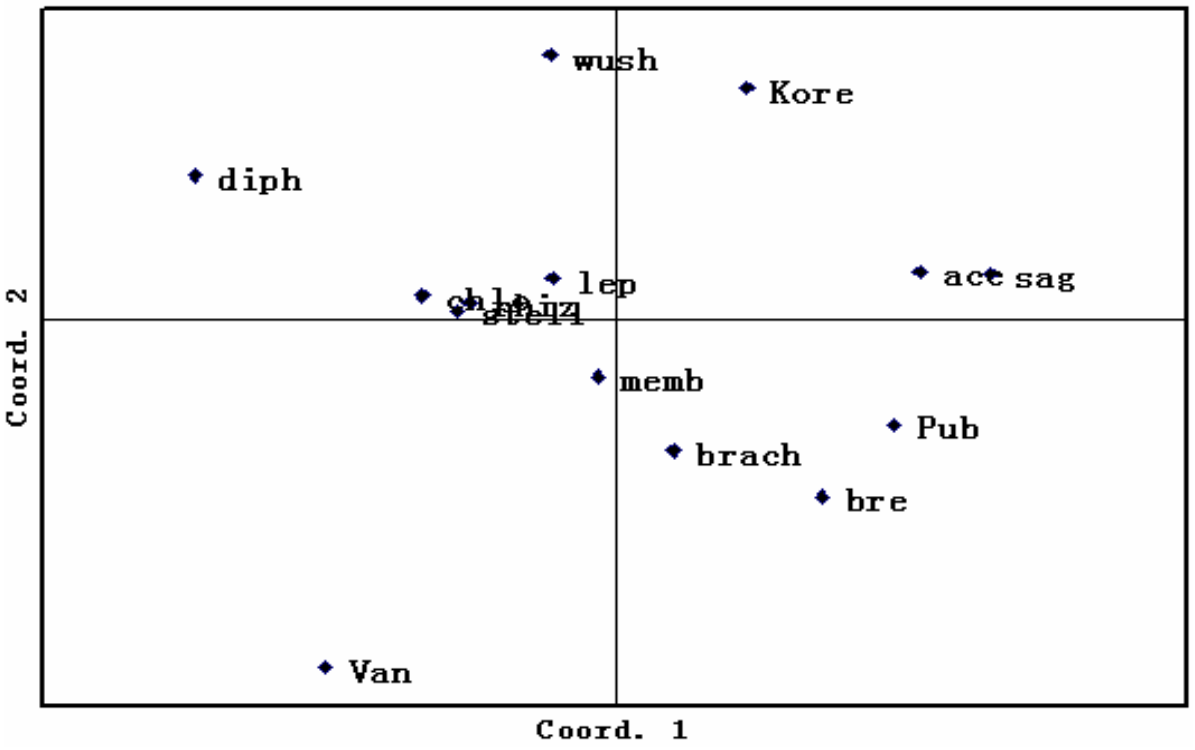

Fig. 4. Principle component analysis of allele frequencies from 26 genomic SSR markers typed in 13 medicinally important species of the genus *Epimedium.*

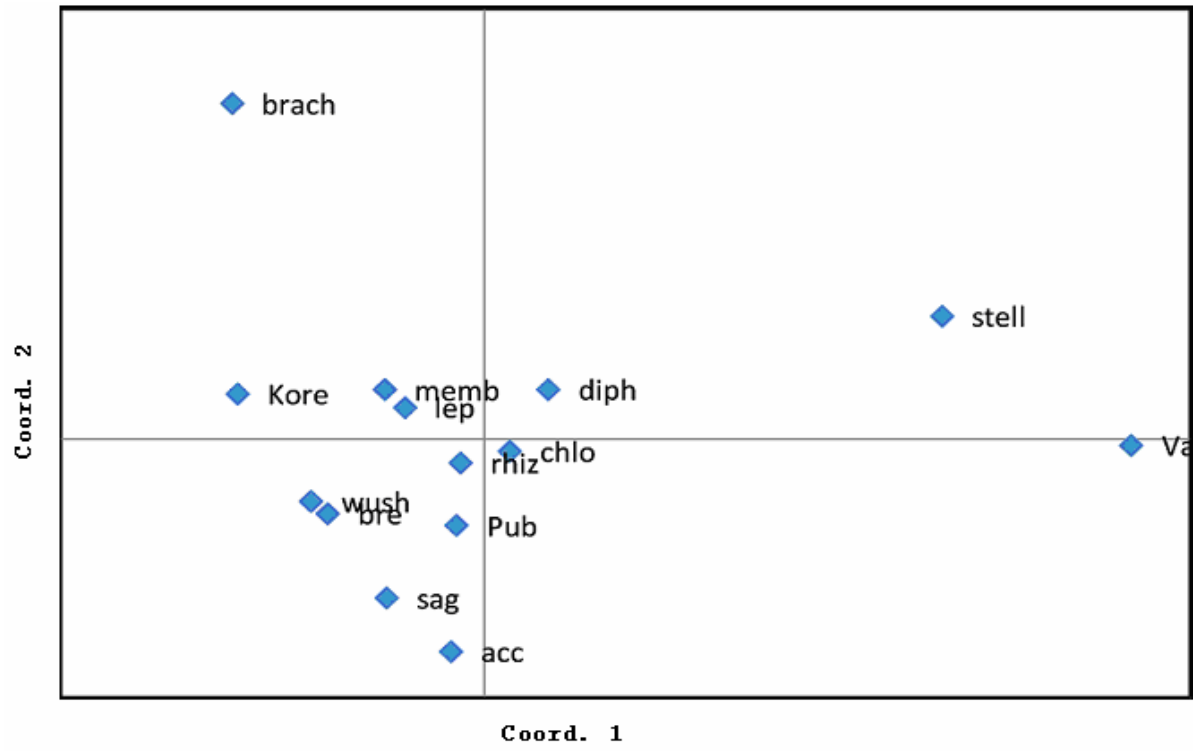

Fig. 5: Principle component analysis of allele frequencies from 10 EST-SSR markers typed in 13 medicinally important species of the genus *Epimedium*

Key: pub (*E. pubescens*), sag (*E. sagittatum*), acc (*E. accuminatum*), bre (*E. brevicornum*), kore (*E. koreanum*), wush (*E. wushanense*), lep (*E. leptorrhizum*), brach(*E. brachyrhizum*), memb (*E. membrance*), rhiz (*E. rhizomatosum*), chlo (*E. chlorandrum*), diph (*E. diphyllum*), stell (E. *stelluatum*)Van (*Vancouveria hexandria*)

Epimedium wushanense, *E. leptorrhizum*, *E. rhzomatosum*, *E. accuminatum*, *E. brachyrrhizum*, *E. chlorandrum*, *E. pubescens*, *E. sagittatum*, *E. brevicornu*, *E. koreanum*, *E. membranaceum*, *E. diphyllum* and *E. stellulatum* are pharmaceutical important species. Morphologically, *E. wushanense, E. leptorrhizum*, *E. rhizomatosum, E. accuminatum, E. brachyrrhizum* and *E. chlorandrum* are very close to each other (Ying *et al.,* 2011). Hence they are part of series *Dolichocerae* of section *Diphyllum*. The morphological relatedness of these species is because of long-spurred flowers without or almost no lamina to petal, which is longer or much longer than the inner sepal (Stearn, 2002). However genetic variation mapped by genomic and EST-SSR marker is providing sufficient basis for the differentiation of these species.

Epimedium pubescens, *E. brevicornu, E. sagittatum* and *E. koreanum* are considered as an integral part of Herb epimedii. *E. pubescens*, *E. brevicornu, E. sagittatum* are the part of series *Brachycerae* of section *Diphyllon*, while, *E. koreanum* is from section *Macroceras.* PCA based on genomic SSR and EST-SSR showed clear differentiation between these species (Figs. 4 and 5). The location of the species on scatter plots based on SSR and EST-SSR markers represent higher genetic variation analogue to their geographical distribution. *E. sagittatum* is found distinct from all other three species. It can be separated from other species at genetic distance of 0.35 and 0.24 based on SSR markers (Figs. 4 and 5). The difference in genetic diversity among species was likely due to geographical isolation. Nine polymorphic microsatellite markers for *E. sagittatum* were available based on the cross-species amplification from *E. koreanum* (Zhou *et al.,* 2007). However, some of those markers could be problematic for the genetic variation and population structure due to multiple/ambiguous bands or the presence of null alleles. Therefore, Xu *et al.* (2007) added 14 more genomic SSR markers for *E. sagittatum*. Through study, 26 genomic SSR and 10 EST-SSR were developed for the use in taxonomy, phylogenetics and conservation genetics.

Lower order taxonomy of the genus *Epimedium* always remained controversial. Taxonomist of the different era suggested different lower order taxon at different times. Morran & Decaine (1834) divided the whole genus only into two sections *Microceras* and *Macroceras*. Fischer & Meyer (1846) made the addition of another section *Rhizophyllum*. Franchet (1886) although divided genus into two subsections i.e. *Gymnocaulon* (Species with leafless flowering branch) and *Phyllocaulon* (species with leafy flowering branch). Komarov (1908) divided *phyllocaulon* into four series such as *Monophyll*, *Aceranth*, *Diphylla* and *Polyphylla* for one, two and many leaves on the flowering branch. Stearn (1938 and 2002) revised lower rank of this genus two times. In 1938, he divided the species into two section *Rhizophyllum* and *Phyllocaulons* with up gradation of series as suggested by Komarov (1908) into subsections. Each subsection except *Aceranthus* further divided into two series. In 2002, Stearn considered geographical distribution in combination with morphological markers for lower order taxonomic division of this genus and suggested classification as showed in the Fig. 1. Most recent phyllogenetic treatment of this genus are based upon AFLP markers (Shen *et al*., 2007). They suggested that at bootstrap values >50 clearly divided *Epimedium* into two main genetic trunks; a well-defined trunk consisting of *E. koreanum* and second trunk comprising of rest of species. However, as intra species similarity are lower than any of interspecies, suggesting higher level of variation between species hence AFLP pattern may not be suitable for species identification and phylogenetical studies (Effron *et al.,* 1996). Lower order taxonomic distribution of the medicinally important species was tried to explore by cluster analysis. Three different clusters were generated by using different computer software. It has been observed that lower order distribution of the species based on 10 EST-SSR markers was different from the traditional classification. However intactness of the sections were observed in the cluster based on 26 genomic SSR markers. However the classification based on 36 genomic and EST-SSR markers is closer to Stearn (2002) classification. Therefore it is suggested that genomic and EST-SSR markers based on interspecific variation is more suitable than intra specific variation are for taxonomic studies of the genus *Epimedium*. It is also predicted from the present study, as the number of primers increases in lower order taxonomy, it become closer to lower order distribution based on morphological markers.

Acknowledgements

Authors are thankful for the financial support from Knowledge Innovation Project of The Chinese Academy of Sciences [KSCX2-EW-J-20], TWAS-CAS Post doc fellowship 2010, National Natural Science Foundation of China [30900076], CAS/SAFEA International Partnership Program for Creative Research Teams Project.

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(Received for publication 27 November 2013)