Pak. J. Bot., 44(4): 1261-1266, 2012.

DNA SEQUENCE AND RAPD INFORMATION RE-AFFIRMS THE TAXONOMIC RELATIONSHIPS BETWEEN *APOCYNUM VENETUM* L. AND *POACYNUM PICTUM* (SCHRENK) BAILL.

PENG XUEMEI[1,3], CAO YAN[1], YANG ZIYI[1], ZHANG WEIMING[1,2], LIU QITANG[4] AND LU CHANGMEI[1*]

[1]*Jiangsu Key Laboratory for Biodiversity and Biotechnology, College of Life Science, Nanjing Normal University, Nanjing 210046, China;*
[2]*Nanjing Institute for the Comprehensive Utilization of Wild Plants, Nanjing 210042, China;*
[3]*Analysis and Testing Center, Shanxi Datong University, Datong, 037009, China;*
[4]*Xinjiang Gebao Luobuhongma and Luobubaima Co1L td, Altai 836500, China*
[*]*Corresponding author's e-mail: luchangmei@njnu.edu.cn; Tel: (086)-025-85891971; Fax: (086)-025-85891526*

Abstract

Medicinal plants *Apocynum venetum* L. and *Poacynum pictum* (Schrenk) Baill. having similar efficacy belong to two genera. However, many similarities of morphological characteristics in pollen, seeds, leaves and stems *etc.* between the two species recommend it under one genus *Apocynum* L. To address this question, we selected DNA sequences [nuclear rDNA internal transcribed spacer (ITS), *trn*L intron and *trn*L-*trn*F intergenic spacer (IGS) sequence] and RAPD markers to assess the relationship between them, with samples from 10 populations of *A. venetum* and 5 populations of *P. pictum* from China. ITS sequences from all samples of *A. venetum* and *P. pictum* were same, and only two different sites were found in the *trn*L intron region and one different site in the *trn*L-*trn*F IGS region. The genetic distance between *A. venetum* and *P. pictum* calculated upon these regions was much smaller than both the intergeneric and interspecies genetic distances calculated upon the same regions in the vast majority of plants. This reaffirmed the notion that *A. venetum* and *P. pictum* had a close genetic relationship. Results from RAPD markers showed that there was high similarity and still stable difference between *A. venetum* and *P. pictum.* The genetic difference between *A. venetum* and *P. pictum* was greater than the differences among populations within species. Our studies recommend that *A. venetum* and *P. pictum* were genetic close-related species and they may be treated within one genus.

Introduction

Apocynum venetum L. locally known as "Luobuma" is a perennial wild herbaceous plant belonging to Apocynaceae (Jiang & Li, 1977). It has been treated as medicine for more than a thousand years in China and has been recorded by "Chinese Pharmacopoeia" (Anon., 2005). The components such as flavanoid contained in its leaves have the effects of preventing and relieving high blood pressure (Kamata *et al*., 2008). In China, there is another plant called "Luobuma" with similar efficacy. This plant was originally named *Apocynum pictum* Schrenk in 1844. Genus *Apocynum* has alternate leaves, cylindrical lower part and bell-shaped upper part of corolla, while this plant has opposite leaves and pelvis-shaped corolla, Hence, Baillon (1888) separated it from the genus *Apocynum* and placed in a new genus - *Poacynum*. He was followed by Woodson (1938), Pobedmova (1952) and Jiang & Li (1977).

This placement as a new genus became controversial when Chen & Li (1991) found that the leaf stomata characters in 2 specimens of *A. venetum and P. pictum* from China were similar; Su *et al*., (1997) reported that the anatomical structures of leaves and stems in 8 populations of *A. venetum and P. pictum* from Xinjiang China were similar, Ma *et al*., (2000) found that *A. venetum and P. pictum* from Ningxia China were very similar in seeds, such as in its size, shape, color and surface ornamentation *etc*. They proposed that *P. pictum* should be treated under genus *Apocynum*.

A. venetum is mainly distributed in large areas from southern Europe through northern Africa and Turkey all the way to China, where it is distributed in diversified natural conditions (sub-humid, quasi-arid and arid areas) (Table 1) (Jiang & Li, 1977; Liu *et al*., 2009). While *P. pictum* is just distributed in arid and alpine arid areas in China (provinces of Xinjiang, Gansu, Qinghai and Ningxia) and Russia (Table 1) (Jiang & Li, 1977; Liu *et al*., 2009). This controversy was not resolved because the data was limited and evidence from DNA was needed to rectify the error. Biological macromolecules, such as DNA *etc.* have successfully been used for analyzing the genetic relationships and classifications of plants, which are difficult to be determined by the classic taxonomy method due to their change to the environmental variations (Pérez-Barros *et al*., 2008). There are many reports where such controversies have been resolved through DNA data (Shinwari *et al*., 1994a, 1994b & 1995).

Table 1. Eco-geographical distribution of *A.venetum* and *P. pictum* in China.

Code		Eco-geographical type	Elevation (m)	Annual precipitation(mm)	Distribution in China	Species
1		Sub-humid	10~400	500~700, Jiangsu> 900	Beijing, Tianjing, Shandong, Jiangsu, Anhui, Henan, Shanxi, Shaanxi, Hebei, Liaoning	*Apocynum venetum* L.
2		Semi-arid	100~1000	250~500	Eastern Inner Mongolia, Ningxia	*A.venetum*
3	3-1	Arid	600~1300	<50	Tarim Basin of Xinjiang, Gansu, Western Inner Mongolia	*A.venetum*, *Poacynum pictum* (Schrenk) Baill.
	3-2	Quasi-arid	<1000	≈200	Area around the Junggar Basin of Xinjiang	*A.venetum*
	3-3	Alpine arid	≈2700	<30	Qinghai	*P. pictum*

Table 2. Plant materials. All the materials were collected by the Chinese Luobuma resource scientific investigation team in 2005 and identified by Xiao Zhengchun.

Species	Sample Codes	Origin/collector	Number of samples	Voucher number	Collection Date
A. venetum	$L1^{3-2}$	Altay, Xinjiang /Gu G.	5	200543-200546	2005-7-18
	$L2^{3-1}$	Korla, Xinjiang /Zhang W.	5	200535-200538	2005-7-14
	$L3^{2}$	Tongliaozha Qi, Inner Mongolia /Gong Z.	5	200565-200568	2005-8-15
	$L4^{3-1}$	Yumen, Gansu /Gong Z.	5	200569-200572	2005-8-30
	$L5^{2}$	Yinchuan, Ningxia /Xian X.	5	200557-200560	2005-8-5
	$L6^{1}$	Xingping, Shanxi /Qian X.	5	200553-200556	2005-7-27
	$L7^{1}$	Sanmenxia, Henan /Qian X.	5	200579-200582	2005-9-8
	$L8^{1}$	Dongtai, Jiangsu /Zhang W.	5	200587-200590	2005-9-15
	$L9^{1}$	Changling, Jilin /Gong Z.	5	200561-200564	2005-8-10
	$L10^{1}$	Xiaoxian, Anhui /Lu Z.	5	200583-200586	2005-9-12
P. pictum	$B1^{3-1}$	Bachu, Xinjiang /Gu G.	5	200547-200549	2005-7-20
	$B2^{3-1}$	Korla, Xinjiang /Zhang W.	5	200539-200542	2005-7-14
	$B3^{3-1}$	Yumen, Gansu/Gong Z.	5	200573-200575	2005-8-30
	$B4^{3-1}$	Dunhuang, Gansu /Gu G.	5	200550-200552	2005-7-26
	$B5^{3-3}$	Gomud, Qinghai /Gu G.	5	200576-200578	2005-9-2

Keeping in mind these reasons, we collected 10 representative populations of *A. venetum* and 5 representative populations of *P. pictum* from different eco-geographical areas in China (Tables 1 and 2), and DNA sequences of ITS, *trn*L intron and *trn*L-*trn*F IGS from various samples together with their RAPD information were used to re-affirm the taxonomic relationship between them.

Materials and Methods

Materials: All the plant materials were collected by the Chinese Luobuma resource scientific investigation team from various regions of China in 2005. Their concrete information was shown in Tables 1 and 2. Under natural conditions, *A. venetum* and *P. pictum* reproduce themselves by asexual reproduction. In order to avoid collecting samples with the same genetic composition, the distances between different samples were at least 1km or more. Leaves were dried by silica gel and identified by Xiao Zhengchun from Nanjing institute of comprehensive utilization of wild plants. Voucher specimens (collection No. from 200535 to 200590) were deposited at the herbarium of Nanjing institute of comprehensive utilization of wild plants for further reference.

DNA isolation: DNA was extracted with a Qiagen DNeasy plant mini kit (Qiagen, Valencia, CA). DNA extractions followed manufacturer's protocols with the modification of buffer AP1 lysis conditions by the addition of papain (Sigma) to the final concentration of 50mg/mL, and incubation at 37°C for 12h on a rocking platform.

DNA amplification and sequencing: The primer pairs of ITS4/ ITS5 (White *et al*., 1990) and c/e (Taberlet *et al*., 1991) were used for the amplifications of ITS and cpDNA of *trn*L intron and *trn*L-*trn*F IGS, respectively. Polymerase chain reactions (PCR) were performed using a DNA thermal cycler (Eppendorf, German) in 25μL reaction volumes containing about 1ng of DNA, 0.2 mM of each dNTP, 2 mM of $MgCl_2$, 10 pmol of each primer, 0.25U of *Taq* DNA polymerase (Promega, USA) and the corresponding buffer. Thermal cycling conditions consisted of an initial denaturation step of 95°C for 5 min followed by 30 cycles at 94°C for 30 s, 54°C (ITS), or 55°C (*trn*L intron and *trn*L-*trn*F IGS) for 1 min, 72°C for 1 min, and a final extension at 72°C for 7 min. All products were visualized under ultraviolet light in 1% agarose gels stained with ethidium bromide, with DNA ladders (Takara, Japan) as size markers to confirm the correct amplification. The correct amplification products were purified with the Qiagen PCR purification kit (Qiagen, German) and sequenced on ABI 377 automated sequencer (PE Applied Biosystem, USA). The boundaries of each DNA sequence was determined according to related sequences in GenBank. Sequences determined in this study were registered in GenBank and their accession numbers were shown in Table 3.

Table 3. GenBank accession numbers, lengths and variable sites in DNA sequences of ITS, *trn*L intron and *trn*L-*trn*F IGS from *A.venetum* and *P. pictum*. Numbers indicate nucleotide positions. Dashes indicate identity with no variable site.

Species	ITS			*trn*L intron				*trn*L-*trn*F IGS		
	GenBank accession number	Length (bp)	Variable site (No.)	GenBank accession number	Length/bp	Variable site (No.)		GenBank accession number	Length/bp	Variable site (No.)
						215	256			77
A. venetum	DQ449485	605	-	DQ463213	348	C	T	DQ463217	334	T

P. pictum	DQ451830	605	-	DQ463216	348	T	C	DQ463217	334	C

Data analysis: Nucleotide sequences were aligned using ClustalX (Jeanmougin *et al*., 1998), and then manually adjusted by eye. Pairwise genetic distances were calculated in MEGA 3.1 (Kumar *et al*., 2004). RAPD bands were scored as binary presence (1) or absence (0) characters, to assemble the matrix of the RAPD phenotypes. Genetic diversity was measured by the percentage of polymorphic bands (*PPB*), Shannon information index and Nei's gene diversity by using Popgene 32 (Yeh & Yang, 1999). Nei's genetic distances, genetic similarity, and a dendrogram demonstrating the genetic relationships among populations of *A. venetum* and *P. pictum* were performed with the Unweighted Pair Group Method of Arithmetic Average (UPGMA) method in NTSYS computer programs (Rohlf, 1993).

Primer selection and RAPD-PCR analysis: Eighty random primers (Shanghai Sangon. Inc., China) were initially tested using 2 samples of *A. venetum* and *P. pictum*. Seventeen primers (Table 4) that produced stable, clear and rich amplified polymorphic bands with these test templates were selected for RAPD-PCR analysis. The PCR amplification was performed in a 25μL reaction volume containing 10ng of DNA, 1U of *Taq* DNA polymerase (Promega, USA), 0.2mM of each dNTP, 1X *Taq* polymerase buffer with 2mM of $MgCl_2$, and 10pmol of each primer. The amplification was carried out in a DNA thermal cycler (Eppendorf, German) using following PCR temperature profile: 94°C for 5min; 42 cycles at 94°C for 45s, 36°C for 30s, and 72°C for 2min; and a final extension at 72°C for 7min. PCR products were subjected to 1.5% agarose gel, along with DNA ladders (Takara, Japan) as size markers. DNA was stained with ethidium bromide and photographed under UV light (GDS-8000, UVP, and U.S.A).

Table 4. Names and sequences of random primers which produced stable clear and rich amplified polymorphic bands. They were purchased from Shanghai Sangon. Inc. of China.

Primer	Sequence (5' →3')	Primer	Sequence (5' →3')
A02	TGCCGAGCTG	B11	GTAGACCCGT
A09	GGGTAACGCC	C06	GAACGGACTC
A14	TCTGTGCTGG	C09	CTCACCGTCC
A17	GACCGCTTGT	C10	TGTCTGGGTG
B01	GTTTCGCTCC	C15	GACGGATCAG
B02	TGATCCCTGG	D16	AGGGCGTAAG
B03	CATCCCCCTG	D18	GAGAGCCAAC
B05	TGCGCCCTTC	D20	ACCCGGTCAC
B08	GTCCACACGG		

Results

DNA sequence information: We got 225 sequences from 50 *A. venetum* individuals and 25 *P. pictum* individuals as following: 75 ITS sequences (605bp), 75*trn*L intron sequences (348bp) and 75 *trn*L-*trn*F IGS sequences (334bp) (Table 3). All the ITS sequences from samples of *A. venetum* and *P. pictum* were fully congruent (Table 3). The genetic distance between *A. venetum* and *P. pictum*, which was calculated upon ITS sequences, was zero. Both the *trn*L intron and *trn*L-*trn*F IGS sequences from all samples of *A. venetum* or from all samples of *P. pictum* were completely the same, but 2 sites (positions 215 and 256) in *trn*L intron region and one site (position 334) in *trn*L-*trn*F IGS region were different (Table 3). The genetic distance obtained from the integrated sequence of *trn*L intron and *trn*L-*trn*F IGS between *A. venetum* and *P. pictum* was 0.04.

RAPD information: Seventeen random primers (Table 4) were used for the RAPD amplification for 10 populations of *A. venetum* and 5 populations of *P. pictum*. The molecular weights of RAPD bands were mainly within the range of 200-2000bp (Fig. 1). In all, 165 bands were amplified from all samples of *A. venetum* and *P. pictum*, among which 141 were polymorphic (Table 5). The percentage of polymorphic bands (PPB) between *A. venetum* and *P. pictum* was 85.45%, which was higher than both the PPBs among populations of *A. venetum* (68.79 %) and among populations of *P. pictum* (66.9%) (Table 5), PPBs, together with indexes of Nei′s gene diversity and Shannon′s diversity (Table 5), indicated that the genetic diversity among all the populations of *A. venetum* and *P. pictum* were higher than both the genetic diversities among populations of *A. venetum* and among populations of *P. pictum.* Furthermore, 4 clear bands produced by primer A-17, B-03, B04 and B08 were observed in all populations of *A. venetum*, but none of them existed in populations of *P. pictum* (Fig. 1 showed the band produced by primer B-03); and one clear band produced by primer D-20 existed in all populations of *P. pictum*, but it did not exist in populations of *A. venetum*.

Table 5. Genetic variation analysis of *A.venetum* and *P. pictum*. TNB, total number of bands; NPB, number of polymorphic bands; PPB, percentage of polymorphic bands.

Populations	TNB	NPB	PPB(%)	Nei's gene diversity	Shannon's information
A.venetum	157	108	68.79	0.2296	0.3390
P. pictum	145	97	66.90	0.2205	0.3047
A. venetum and *P. pictum*	165	141	85.45	0.3044	0.4540

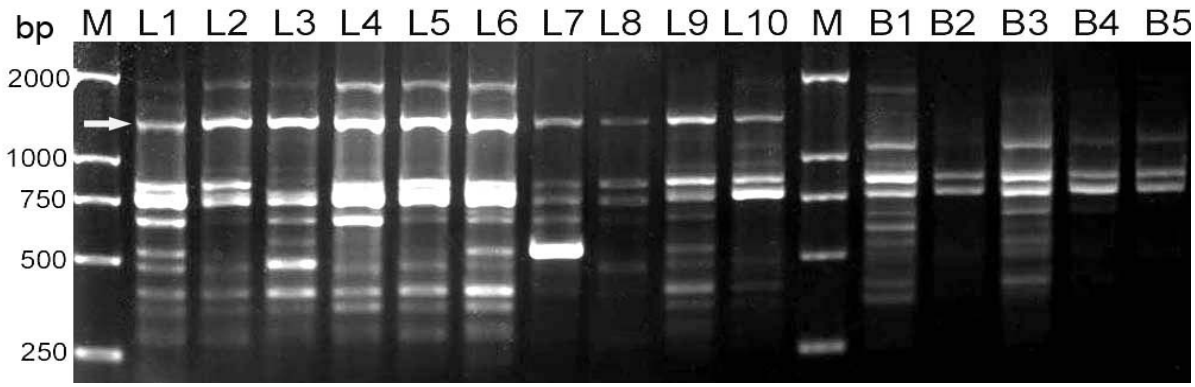

Fig. 1. RAPD pattern amplified with B03 primer. M: DNA marker; L1~L10: samples of *A. venetum*; B1~B5: samples of *P. pictum*. Their voucher numbers were 200547, 200536, 200578, 200567, 200564, 200596, 2005106, 200574, 2005102, 200552, 200542, 200587, 200556 and 200593 respectively. Their collection localities were showed in table 1. The white arrow showed the different bands between *A. venetum* and *A. pictum*.

Table 6 shows the genetic distances (under the diagonal) and genetic similarities (above the diagonal) among 10 populations of *A. venetum* and 5 populations of *P. pictum*. The genetic distances between populations of *A. venetum* and *P. pictum* (0.6375 on average) were far higher than the genetic distances among populations of *A. venetum* (0.2806 on average) and among populations of *P. pictum* (0.3203 on average). On the contrary, the genetic similarities between *A. venetum* and *P. pictum* (0.5872 on average) was lower than the genetic similarities among populations of *A. venetum* (0.7576 on average) and among populations of *P. pictum* (0.7284 on average).

Fig. 2 showed a cluster plot of 15 populations of *A. venetum* and *P. pictum* constructed according to their genetic similarity. The 15 populations were divided into 2 branches at the similarity 0.5872, and *A. venetum* and *P. pictum* were not mixed.

Table 6. Nei's genetic distance (below the diagonal) and genetic similarity (above the diagonal) among the populations of *A.venetum* and *P. pictum.*

Code	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	B1	B2	B3	B4	B5
L1		0.8667	0.8061	0.7697	0.7939	0.7394	0.7636	0.7132	0.6788	0.7394	0.6242	0.5879	0.5879	0.5273	0.4545
L2	0.1431		0.8061	0.7697	0.7455	0.7273	0.7152	0.6788	0.6303	0.6909	0.5879	0.5636	0.5515	0.5273	0.5030
L3	0.2156	0.2156		0.7818	0.7576	0.7152	0.7152	0.6909	0.6424	0.7030	0.6121	0.5394	0.5394	0.4788	0.4909
L4	0.2618	0.2618	0.2461		0.8061	0.7394	0.7273	0.7030	0.6545	0.7394	0.6242	0.6121	0.6364	0.5758	0.5030
L5	0.2307	0.2938	0.2776	0.2156		0.7879	0.8364	0.8727	0.7394	0.8485	0.6606	0.6485	0.6485	0.5758	0.5394
L6	0.3109	0.3185	0.3353	0.3019	0.2384		0.7818	0.7697	0.7697	0.7576	0.6545	0.5697	0.5939	0.5455	0.5212
L7	0.2697	0.3353	0.3353	0.3185	0.1787	0.2461		0.7939	0.8061	0.8424	0.6909	0.6182	0.6424	0.5697	0.5576
L8	0.3185	0.3874	0.3679	0.3524	0.1361	0.2618	0.2307		0.7697	0.9394	0.6303	0.6303	0.6424	0.5818	0.5818
L9	0.3874	0.4646	0.4425	0.4238	0.3019	0.2618	0.2156	0.2618		0.7687	0.6414	0.6061	0.6303	0.5697	0.5576
L10	0.3019	0.3697	0.3524	0.3019	0.1643	0.2776	0.1715	0.0625	0.2618		0.6182	0.6545	0.6424	0.6061	0.6061
B1	0.4712	0.5312	0.4908	0.4712	0.4146	0.4238	0.3697	0.4616	0.4425	0.4810		0.7333	0.7212	0.6848	0.6000
B2	0.5312	0.5733	0.6173	0.4908	0.4331	0.5627	0.4810	0.4616	0.5008	0.4238	0.3102		0.8303	0.7818	0.6970
B3	0.5312	0.5951	0.6173	0.4520	0.4331	0.5210	0.4425	0.4425	0.4616	0.4425	0.3268	0.1860		0.7687	0.7091
B4	0.6400	0.6400	0.7365	0.5521	0.5521	0.6061	0.5416	0.5416	0.5627	0.5008	0.3786	0.2461	0.2618		0.7576
B5	0.7885	0.6871	0.7115	0.6173	0.6871	0.6516	0.5416	0.5416	0.5842	0.5008	0.5108	0.3610	0.3438	0.2776	

Discussion

Apocynum L. and *Poacynum* Baill. were recognized 2 separate genera according to the differences of phyllotaxy and flower-shape (Baillon, 1888; Woodson, 1938; Pobedmova, 1952; Jiang & Li, 1977). But Nilsson *et al*., (1993) found that they were similar in floral structure and pollen morphology, and recommended that they represented one genus. As to *A. venetum* and *P. pictum*, they also were found similar in characters of stomata (Chen & Li, 1991), seeds (Ma *et al*., 2000) and anatomical structures of leaves and stems (Su *et al*., 1997; Tanaka *et al*., 1993), and were proposed to be grouped into one genus (Chen & Li, 1991). However, above inferences were from qualitative researches on morphology with a small number of samples. Researches on *A. cannabinum* showed that the variation in morphological traits was due to variations of environment and genetics (Ransom *et al*., 1998a and 1998b). Therefore, the taxonomic ralationship between *A. venetum* and *P. pictum* still need more evidence from more samples, especially the evidence from the DNA level, as also reported by many scientists (Gilani *et al*., 2002; Shinwari and Shinwari, 2010).

Among the commonly used molecular markers for taxonomy, regions of ITS, *trn*L intron and *trn*L-*trn*F IGS are often used to compare genetic relationships within genera or among populations within species (Chaw *et al*., 2005; Kojoma *et al*., 2002). Although some references showed the possibility of the same ITS sequence from

different plant species in the same genus (Roth *et al*., 1998; Hageskal *et al*., 2006), no reference up to date showed that plant species from different genus had the same ITS sequence. ITS sequences from all samples of *A. venetum* and *P. pictum* were fully consistent. The genetic distance between *A. venetum* and *P. pictum*, calculated upon ITS sequences, was zero which was much lower than the ordinary corresponding genetic distance among genera (0.096-0.288) and among species (0.012-0.102) of angiosperm (Qu & Chen 1999). These demonstrated that *A. venetum* and *P. pictum* were very closely related.

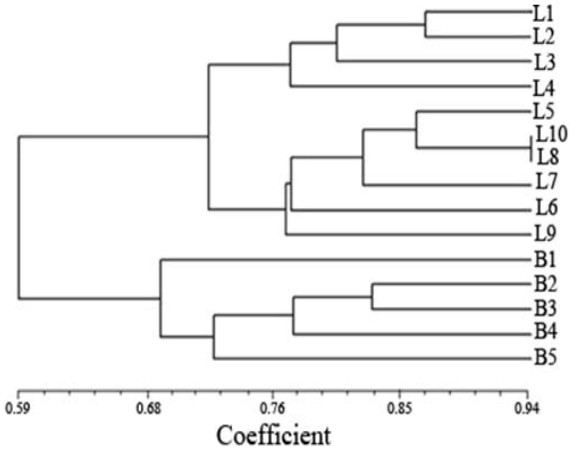

Fig. 2. Dendrogram derived from a UPGMA cluster analysis of RAPD marker using genetic similarity coefficients of *A.venetum* and *P. pictum* .

Minor but stable sequence differences between *A. venetum* and *P. pictum* (Table 3) in the regions of *trn*L intron and *trn*L-*trn*F IGS indicated that although *A. venetum* and *P. pictum* were similar, there were stable genetic difference between them. The genetic distance of 0.04 was lower than the genetic distance calculated upon the same regions among genera and among species within one genus, but was equivalent to the level among populations within one species (Emshwiller & Doyle, 1998; Roth *et al*., 1998; Qu & Chen, 1999; Hageskal *et al*., 2006). This demonstrated further that *A. venetum* and *P. pictum* were close related.

RAPD markers have been successively used for phylogenetic studies in many plant species (Kumar *et al*., 2008; Ransom *et al*., 1998b, Mahmood *et al*., 2010; Jan *et al*., 2011; Ream & El-Bakatoushi, 2011). In the present study, RAPD polymorphism analysis on indexes of PPB, Nei′s gene diversity, Shannon′s diversity and genetic distance *etc*. among populations of *A. venetum* and *P. pictum* indicated that the diversity among all the populations of *A. venetum* and *P. pictum* were higher than those among populations of *A. venetum* and among populations of *P. pictum* (Table 5 and Table 6). Specific different bands were found between *A. venetum* and *P. pictum* (Fig. 1). These results demonstrated that the genetic difference between *A. venetum* and *P. pictum* was greater than the difference among populations within each species.

Hamrick & Godt (1989) and Szczecińska *et al*., (2006) believed that the genetic similarity index of defining a biological species should be lower than 0.6. The genetic similarity index among populations within species of *A. venetum* and *P. pictum* were comparatively high, being 0.7576 and 0.7284 respectively, and the genetic similarity index between species of *A. venetum* and *P. pictum* was 0.5872. Cluster analysis showed that the 2 belong to 2 different branches. These signified that the similarity of *A. venetum* and *P. pictum* was close to but below the level of species.

It has been documented that there was another plant species, which was first named as *A. hendersonii* Hook. f.(Hook, 1873), and then renamed *P. hendersonii* (Hook. f.) (Woodson, 1930). This species was considered phenotypically intermediate between *A. venetum* and *P. pictum* (Liu *et al*., 2009). Many researchers believed that it was a transitional species or hybrid between *A. venetum* and *P. pictum*, or just a variety (Liu *et al*., 2009) or a synonym of *P. pictum* (Wu *et al*., 1995). Unfortunately, over the past 20 years, we were unable to find it living in China. Sequencing result of ITS, *trn*L intron and *trn*L-*trn*F IGS regions from a specimen collected 20 years ago showed that all the DNA sequences detected were fully consistent with those from *P. pictum* (Zhang *et al*., 2007). Since *P. pict*um could not be collected by our team, the analysis in this article was limited to *A. venetum* and *P. pictum*.

From above observations on the DNA level, it can be concluded that there are high similarity and stable difference between *A. venetum* and *P. pictum*. This was in agreement with previous morphological studies on the relationship between *A. venetum* and *P. pictum* (Chen & Li, 1991; Su *et al*., 1997; Ma *et al*., 2000). The present paper re-affirms the notion that *A. venetum* and *P. pictum* are close related species and they should belong to one genus. More molecular studies of other closely related species of the family to support the findings was done by several researchers in the past (Shinwari, 2000 and 2002).

Acknowledgement

Thanks for the financial assistance of Chinese "Tenth Five-Year" National Science and Technology Research Fund (2004 BA502B10) and High-tech Research Projects of Jiangsu Province (BG2006318).

References

Anonymous. 2005. Chinese Pharmacopoeia Commission. *Chinese Pharmacopoeia*, Chemical Industry Press, Beijing, pp. 147.

Baillon, H.E. 1888. Poacynum. *Bull. Mens. Soc. Linn. Paris*, 1: 757.

Chaw, S.M., W.W. Terrence, C.C. Chang, S.H. Hu and S.H. Chen. 2005. A phylogeny of cycads (Cycadales) inferred from chloroplast *matK* gene, *trnK* intron and nuclear rDNA ITS region. *Mol. Phylogenet. Evol.,* 37: 214-234.

Chen, X.M. and B.T. Li. 1991. An observation on the structure of leaf stomata of *Apocynum* and *Poacynum* with notes on the taxonomical treatment of *Poacynum. Guihaia,* 11(2): 146-148.

Emshwiller E. and J.J. Doyle. 1998. Origins of domestication and polyploidy in oca (*Oxalis tuberosa*: Oxalidaceae): nrDNA ITS data. *Am. J. Bot.,* 5(7): 975-985.

Gilani, S.S., M.A. Khan, Z.K. Shinwari and Z. Yousuf. 2002. Leaf epidermal anatomy of selected digiteria species, tribe Paniceae, family Poaceae of Pakistan. *Pak. J. Bot.*, 34(3): 257-273.

Hageskal, G., A.K. Knutsen, P. Gaustad, G.S. de Hoog and I. Skaar. 2006. Diversity and significance of mold species in Norwegian drinking water. *Appl. Environ. Microb.,* 72(12): 586-7593.

Hamrick, J.L. and M.J.W. Godt. 1989. Allozyme diversity in plant species. In: *Plant population genetics, breeding, and genetic resources*. (Eds.): A.H.D. Brown, M.T. Clegg, A.L. Kahler and B.S. Weir. Sinauer Associates, Inc., Sunderland. pp. 43-63.

Hook, f. 1873. *Apocynum hendersonii*. In: *Annals of the Missouri Botanical Garden*. (Eds.): G. Henderson and A.O. Hume. Lahore to Yarkand, pp. 327.

Jan, H.U., M.A. Rabbani and Z. K. Shinwari. 2011. Assessment of genetic diversity of indigenous turmeric (*Curcuma longa* L.) germplasm from Pakistan using RAPD markers. *Journal of Medicinal Plants Research*, 5(5): 823-830.

Jeanmougin, F., J.D. Thompson, M. Gouy, D.G. Higgins and T.J. Gibson. 1998. Multiple sequence alignment with Clustal X. *Trends Biochem. Sci.,* 23(10): 403-405.

Jiang, Y. and B. Li. 1977. *Apocynum*. In: *Flora of China*, Vol. 63. Science Press, Beijing. pp. 157-161.

Kamata, K., S. Seo and J. Nakajima. 2008. Constituents from leaves of *Apocynum venetum* L. *Nat. Med.*, 62(2): 60-163.

Kojoma, M., K. Kurihara, K. Yamada, S. Sekita, M. Satake and O. Iida. 2002. Genetic identification of cinnamon (*Cinnamomum spp*.) based on the trnL-trnF chloroplast DNA. *Planta Med.,* 68(1): 94-96.

Kumar, S., K. Tamura and M. Nei. 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform.,* 5: 150-163.

Kumar, S.A., J. Sudisha and H.L. Creenath. 2008. Genetic relation of *Coffea* and Indian *Psilanthus* species as revealed through RAPD and ISSR markers. *Int. J. Integr. Biol.,* 3(3): 150-158.

Liu, Q., Z. Xiao, W. Zhang, C. Lu and G. Zhang. 2009. Research on the ecogeographic characteristics of *Apocynum venetum* L. and *Apocynum pictum* Hook. f. *Chin. Wild Plant Res.,* 28(2): 13-16(20).

Ma, J., J. Li and X. Liu. 2000. Micromorphological characters of the seeds of *Apocynum venetum* and *Poacynum pictum*. *Acta Bot. Boreal-Occident Sin.,* 20(3): 476-479.

Mahmood, T., S. Muhammad and Z.K. Shinwari. 2010. Molecular and morphological characterization of *Caralluma* species. *Pak. J. Bot*., 42(2): 1163-1171.

Nilsson, S. , M.E. Endress and E. Grafström. 1993. On the relationship of the Apocynaceae and Periplocaceae. *Grana Suppl.,* 2: 3-20.

Pérez-Barros, P., M.E. D'Amato. N.V. Guzmán and G.A. Lovrich. 2008. Taxonomic status of two South American sympatric squat lobsters, *Munida gregaria* and *Munida subrugosa* (Crustacea: Decapoda: Galatheidae), challenged by DNA sequence information. *Biol. J. Linn. Soci.,* 94: 421-434.

Pobedmova, E.G. 1952. Apocynaceae. In: *Flora of the USSR*. (Ed.): V.L. Komarov, Vol. 18, Academy of Sciences of the U.S.S.R. Moscou, pp. 660-661.

Qu, L. and Y. Chen. 1999. Key to molecular taxonomy-principles and methods. *Acta Scientiarum Naturalium Universitatis Sunyatseni,* 38(1): 1-6.

Ransom, C.V., D.S. Douches and J.J. Kells. 1998b. Isozyme and RAPD variation among and within hemp dogbane (*Apocynum cannabinum*) populations. *Weed Sci.,* 46(4): 408-413.

Ransom, C.V., J.J. Kells, L.M. Wax and M.S. Orfanedes. 1998a. Morphological variation among hemp dogbane (*Apocynum cannabinum*) populations. *Weed Sci.,* 46(1): 71-75.

Ream, I.M. and R. El-Bakatoushi. 2011. Assessment of relocation of *Trigonella cylindraceae* L. and *T. polyceratia* (L.) Trautv. to genus *Medicago* as inferred by RAPD and RFLP analyses. *Pak. J. Bot*., 43(5): 2289-2294.

Rohlf, F.J. 1993. NTSYS-pc numerical taxanomy and multivariate analysis system. Version 1.8. Exeter Publisher, Setauket, New York.

Roth, A., M. Fischer, M.E. Hamid, S. Michalke, W. Ludwi and H. Mauch. 1998. Differentiation of phylogenetically related slowly growing mycobacteria based on 16s-23s RNA gene internal transcribed spacer sequences. *J. Clin. Microbiol.,* 36(1): 139-147.

Schrenk, S.L. 1844. *Apocynum pictum* Schrenk. Bull. Cl. Phys.-Math. Acad. Imp. Sci. Saint-Pétersbourg, sér. 2. 2: 115

Shinwari, Z.K. 1995 Congruence between morphology and molecular phylogenetics in Prosartes (Liliaceae). *Pak. J. Bot.,* 27(2): 361-369.

Shinwari, Z.K. 2000. Chloroplast DNA variation in Polygonatae (Liliaceae). *Pak. J. Bot.,* 32(1): 7-14.

Shinwari, Z.K. 2002. Sequence divergence of rbcL gene and phylogenetic relationships in Liliales. *Pak. J. Bot.,* 34(2): 191-204

Shinwari, Z.K. and S. Shinwari. 2010. Molecular data and phylogeny of family Smilacaceae. *Pak. J. Bot.,* Special Issue (S.I. Ali Festschrift), 42: 111-116.

Shinwari, Z.K., R. Terauchi and S. Kawano. 1994b. Phylogenetic relationships among genera in the Liliaceae-Asparagoideae-Polygonatae sensu lato inferred from rbcL gene sequence data. *PI. Systematic & Evolution,* 192: 263-277.

Shinwari, Z.K., R. Terauchi, F.H. Utech and S. Kawano. 1994a. Recognition of the new world disporum section Prosartes as Prosartes (Liliaceae) based on the sequence data of the rbcL gene. *Taxon*, 43(3): 353-366.

Su, H., M. Ma and X. Li. 1997. A comparative anatomy of difference populations on *Apocynum venetum* and *Poacynum pictum*. *Acta Bot. Boreal-Occident Sin.,* 17(3): 348-354.

Szczecińska, M., J. Sawick, K. Polok, C. Hołdyński and R. Zieliński. 2006. Comparison of three Polygonatum species from Poland based on DNA Markers. *Ann. Bot. Fenn.,* 43: 379-388.

Taberlet, P., L. Gielly, G. Pauton and J. Bouvet. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Mol. Biol.,* 17: 1105-1109.

Tanaka, T., E. Sakai, S. Yoshimi, S. Nishibe and H. Takemura. 1993. Phamacogonostical studies of *Apocynum* (2): Comparative anatomy of the leaves of *Apocynum* and *Poacynum spp. Shoyakugaku Zasshi*, 47(4): 388-395.

White, T.J., T. Bruns, S. Lee and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A guide to methods and application*. (Eds.): M. Innis, D. Gelfand, J. Sninsky and T. White. Academic Press, San Diego. pp. 315-322.

Woodson, R.E. 1930. A revision of the genus *Poacynum*. *Annuals of the Missouri botanical garden*, 17: 167.

Woodson, R.E. 1938. *Poacynum*. In: *North American Flora,* Vol. 29, Pt. 2. New York Botanical Garden, New York, pp. 103-192.

Wu, Z., P.H. Raven, S.L. Chen, S.C. Chen, H. Cui, L. Dai, B. Bartholomew, D.E. Boufford, N.R. Morin and W. Tai. (Eds.) 1995. Apocynum. In: *Flora of China* (English edition). Vol. 16. Science Press and Missouri Botanical Garden Press, Beijing & St. Louis. Online at EFloras.org, pp. 181.

Yeh, F.C. and R.C. Yang. 1999. POPGENE Version 1.31. University of Albert and center for international research, Edmonton.

Zhang, W., X. Peng, C. Lu, M. Wan and G. Gu. 2007. A taxonomic study of 'Luobuma' based on sequence data of 3 non-coding DNA regions. *Acta Bot. Boreal-Occident Sin,* 27(5): 0931-0937.

(Reeived for publication 26 August 2011)