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TAXONOMIC IMPLICATION OF AFLP FINGERPRINTING IN SELECTED POLYGONACEOUS SPECIES

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Abstract

Amplified Fragment Length Polymorphism (AFLP) was used for the first time to demonstrate the relationship among 28 accessions of 13 species belonging to 4 genera of Polygonaceae. Single primer was used to amplify AFLPs and fragments were separated in 6% denaturing acrylamide gels. A total of 131 fragments were analyzed. The AFLP knowledge was found to be sufficiently susceptible to identify small level of variations and could differentiate highly interrelated genotypes. According to present study, this marker system does not support the distinction of *Persicaria glabra* (Willd.) M. Gómes and *Persicaria hydropiper* (L.) Spach as a separate species of single genus. No ultimate genus level relationships was seen, owing to very low bootstrap support for branches connecting the different clusters in the tree, all four genera did not occupy distinct positions to form separate clusters and representatives of all genera are intermingled throughout the tree except the genus *Polygonum* L. Position of *Persicraia lapthifolia* (L.) S. F. Gray and *Persicaria barbata* (L.) Hara is also unresolved, as both of these did not show any relationship with other species. These results will form the base for further analysis of the family by AFLP marker system.

Introduction

Polygonaceae is a cosmopolitan family containing approximately 1,200 species in 48 genera (Freeman & Reveal 2005; Sanchez & Kron, 2008). In Pakistan it is represented by 19 genera and 103 species (Qaisar, 2001). Plants in this family are herbs, shrubs, small trees or woody vines geographically distributed from the tropics to the arctic although most species are concentrated in the northern temperate region (Heywood, 1978). Species in Polygonaceae are easily distinguished by simple leaves, stem with distinct nodes covered by ochrear stipules (with an exception in *Eriogonum*), unilocular ovary and endospermic seeds (Hutchinson & DaLziel, 1954; Brummitt, 1992). The family Polygonaceae is, however, one of the most diverse in morphology and difficult in taxonomy (Brandbyge, 1993). Since nineteenth century, a number of different classification systems have been proposed at the generic or higher taxonomic level. It has been divided variously into three subfamilies generally on the basis of morphological evidences (Dammer, 1893; Gross, 1913 and Roberty & Vautier, 1964). The most recent consensus is to recognize two subfamilies, Eriogonoideae and Polygonoideae (Jaretzky, 1925; Haraldson, 1978; Reveal, 1989; Brandbyge, 1993; Freeman & Reveal, 2005). Haraldson (1978) recognized two tribes, Eriogoneae and Pterostegieae in Eriogonoideae and five tribes, Polygoneae, Persicarieae, Rumicieae, Cocolobeae, and Triplareae in Polygonoideae, which has been one of the most generally accepted classification for a higher rank in Polygonaceae although modern molecular phylogenetic studies (Lamb Frye & Kron, 2003; Sanchez & Kron, 2008) are giving more different interpretations on

taxonomic rank and subdivision. For example, Sanchez & Kron (2008) proposed new definition of the subfamilies based on phylogeny inferred from the combined sequence data of chloroplast and nuclear *LEAFY* intron, where they proposed that the subfamily Eriogonoideae is composed of 6 genera (*Eriogonum, Antigonon, Brunnichia, Coccoloba, Ruprechtia,* and *Triplaris*) and Polygonoideae 11 genera (*Polygonum, Atraphaxis, Emex, Fagopyrum, Fallopia, Koenigia, Muehlenbeckia, Oxyria, Persicaria, Rheum* and *Rumex*).

Molecular markers have recently been employed to resolve some of the taxonomic and phylogenetic questions but very little attention has been given to the analysis at interspecific and intergeneric levels. During the present study, we used AFLPs instead of RAPDS because of the low reproducibility of RAPDs (Schierwater & Ender 1993) and non-homology across unrelated germplasm. We demonstrated the potential of AFLP for inferring the genetic variation and relationship in different species of Polygonaceae because AFLP is considered as a preferred method for analyzing the relationship between closely related taxa, where DNA sequencing shows hardly any difference (Hodkinson *et al*., 2000) and reproducible, therefore stand for a dominant technique for DNA analysis that has revolutionized fingerprinting and diversity studies (Vos *et al.,* 1995). AFLP data was used in a number of smaller studies demonstrating their use for detecting phylogenetic relationships among cultivated and natural populations of wild subspecies of *Fagopyrum tataricum* Gaert. from a wide geographical areas and proved to be more informative than RAPD markers because the RAPD analyses could not detect geographical groups in the wild subspecies populations (Tsuji & Ohnishi, 2001), Iwata *et al*., (2005) evaluated the genetic variation in *Fagopyrum esculentum* Moench., cultivars using AFLP and SSR markers and found that AFLP marker system is effective in the resolution of relationships among cultivars better than SSR. Konishi *et al*., 2005, used AFLP marker system to establish genetic relationship among cultivated and wild *Fagopyrum esculentum* Moench.

Previous studies on phylogenetic relationship among various species of Polygonaceae by using only chloroplast *rbcL* sequence data (Lamb Frye & Kron, 2003) or multiple gene regions (Sanchez & Kron, 2008) suggested that Polygonoideae is paraphyletic as nesting the monophyletic group, Eriogonoideae and proposed that many changes of classification for the broad sense of *Polygonum* L., are necessary. In a smaller scale, monophyletic relationship of Persicarieae was strongly supported by molecular phylogenetic analyses using additional chloroplast and nuclear gene regions (Kim & Donoghue, 2008 a).

However, the present step of AFLP studies on Polygonaceae is still a beginning to the final conclusion not only because we need more comprehensive samplings enough to overcome sampling bias but also because there must be more complicated evolutionary history in this heterogeneous group (*Polygonum s. lato*.) relating to hybridization and polyploidy as discussed in Kim & Donoghue (2008 b).

The objectives of the current study was to identify genetic variation by AFLP analysis, its taxonomic implication and at the same time assess the use of AFLP marker system to establish phylogenetic relationship among species of different genera in Polygonaceae.

Materials and Methods

A total of 28 samples belonging to 13 species of 4 genera of Polygonaceae were mainly collected from Pakistan for this study with an exception of two individuals of *Rumex senguineus* L., collected from Sussex University, England. Young leaves were collected and stored in sealed plastic bags with silica gel.

DNA extraction: Genomic DNA was extracted from the silica dried leaf samples (Chase & Hills 1991) by using DNeasy Plant Mini Kit, Qiagen according to the manufacturer's instructions. This involved the grinding of 1mg of silica dried leaves on dry ice for 30 – 50 seconds, so that the material was converted to powder form before the addition of extraction buffer. Then RNAse was added and incubation was done at 65°C. Sample was incubated on wet ice after the addition of AP2 buffer for the removal of detergents, proteins and polysaccharides. The material was centrifuged at full speed for 5 minutes and then subjected to column filtration. Dneasy mini spin column helped in binding of DNA which was washed with ethanol added buffer AW provided in the kit and eluted by using elution buffer AE. Quality and quantity of DNA was assessed by running on 1% agarose gel.

AFLP analysis: The AFLP analysis was carried out according to (Vos *et al.*, 1995) with some modifications. It involved following steps.

1. Preparation of *MseI* and *EcoRI* adaptors: 50µg of the two complementary single strands were mixed for each adaptor (MseA 5'-GACGATGAGTCCTGAG-3' and MseB 3'-TACTCAGGACTCAT-5',EcoA 5'-CTCGTAGACTGCGTACC-3' and EcoB 3'-CATCTGACGCATGGTTAA-5') + 100µl ddH_2O to make final volume of 200µl. The mixture was heated at 95°C in a heat block for 3 min and then cooled down slowly at room temperature. The adaptors were stored at -20°C. For the EcoRI adaptor a dilution of 1:10 was made.

2. Digestion/ligation: The digestion / ligation reaction was carried out by making reaction mixture for 30 reactions instead of 28 reactions that was little more than to be actually needed so that not to run out.It was made by taking 277.5 µl of ddH_2O, 37.5 µl of 10x ligase buffer, 7.5µl Msel adapter (50µM), 7.5µl EcoR1 adapter (5 µM), 7.5µl Msel enzyme (10u/µl), 7.5µl EcoR1 enzyme (20u/µl) and 15µl ligase (400u/µl). 12µl of this reaction mixture was taken in each tube, 0.5µl plant DNA was added and incubated at 37°C at room temperature for four hours (in hot block) with 10 µl oil overlay to stop from drying.

3. Preamplification (Cold PCR): For preamplification reaction mixture consisted of 349.5µl ddH_2O, 75 µl 10x PCR buffer, 112.5µl *MgCl2* (25 mM), 120µl dNTPs (1.25 mM each), 30µl EcoR1 primer (0.15 µg/ µl), 30µl Msel primer (0.15 µl/µl), 3µl taq polymerase (fermentas 5u/µ). Put 24µl of above in each PCR well to which was added 1µl of ligation product to make final volume of 25µl for preamplification. The PCR preamplification sketch was 50°C for two minute to get infill, 94°C for 30 s, then 30 cycles of 30 s at 94°C, 60 s at 48°C and 2 minutes at 72°C, followed by ultimate extension at 72°C for 7 min.

4. Labeling of the primer for the hot PCR: Before taking a start, pot of label was taken from freezer and put in radioactive cabinet for thawing, labelling was made a little more than to be needed so that not to run out. Selective primer was end-labeled with [γ-^{33}P] ATP. The recation mixture for 30 reactions consisted of 9.37µl ddH_2O, 1.5µl radiolabel (ICN 58404.2), 1.5µl 10x kinase buffer, 2.25µl EcoR1 primer (0.05µg/µl) and 0.37µl T4 polynucleotide kinase (10u/µl) before amplification. This mixture was incubated at 37°C for one hour, then 70°C for 10 minutes to stop reaction.

5. Selective amplification (Hot PCR): For selective amplification, each preamplification product was diluted 50 times with ddH_2O, of which 0.5µl was used as template for PCR amplification and mixed with 4.1µl water, 1µl 10x PCR buffer, 1.5 µl MgCl2, 1.6µl dNTPs (1.25 mM each), 0.3µl Msel primer (0.05 µg/µl), 0.5µl (5u/µl) taq polymerase and 0.5µl labeled primer so that to make final volume of 10µl for each reaction. The selective amplification PCR profile was 13 cycles of 30s at 94°C, 30s at 65°C followed by lowering the temperature of -0.7°C per cycle and 1 min at 72°C, followed by 27 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 60 s.

To each reaction an equal volume (10µl) of sequencing loading dye (98% formamide, 10mM EDTA pH 8.0 and bromophenol blue and was added and the samples were denatured at 80ºC for 5 minutes and put immediately on ice to inhibit renaturation of the DNA strands during the gel preparation.

6. 6% polyacrylamide gel electrophoresis and autoradiography: The 6% polyacrylamide urea stock solution (0.5xTBE) was prepared by adding 100ml of 40% acrylamide to 500ml of heat-dissolved urea (306g) and 66ml of 5xTBE. The stock solution was kept at room temperature in the dark. For 1 gel 90µl of fresh ammonium persulphate (25%) and 90µl of TEMED were added and mixed to 80ml of 6% gel solution. The denaturing polyacrylamide gel was thrown into glass plates and run into a Model S2 gel apparatus (Life technologies inc.) using 0.5xTBE as running buffer. The gel was pre run at 80W for 30 min and 3.2µl of each sample was loaded in each well. Electrophoresis was performed at 80W constant power for 1h 30 min. The gel was fixed for 30 min in 10% acetic acid and transferred to Whatman 3 MM chromatography paper, dried in a vacuum drier for 1 h at 80°C and exposed to X-ray film for 24 hours.

Computer analysis: AFLP markers were scored manually into a binary matrix, with `1' for the presence and `0' for the absence of a band at a particular position. Very faint bands were excluded and only the scorable, intense bands were used for further analysis. The neighbour joining tree was generated by Power Marker V3.0 (Liu & Muse, 2005) by frequency based distance using the algorithm of Nei (Nei & Takezaki, 1983). The bootstrap analysis was done using 1000 randomly generated trees (Felsenstein, 1985) and the values in the consensus tree relate to the number of these trees which contain a particular branching structure.

Results

AFLP analysis generated a large number of reproducible and clear-cut markers (Fig. 1) for fingerprinting the species of Polygonaceae used in the study. Across all the thirteen species evaluated (shown in table 1), only unambiguous, intense 131 bands were scored. The tree generated is shown in Figure 2. Robustness of clusters was estimated by means of the bootstrap approach (Felsenstein, 1985) with 1000 replicates.

The tree (Fig. 2) shows that 13 species belonging to 4 genera separate into 4 main clusters, generally with no genus specificity and 2 genetically distinct accessions of *R. nepalensis*. According to our results, majority of the accessions of same species collected from different localities are grouped together.

Present study shows that inter-relationship of the 4 clusters remain unclear due to low confidence support, however within the clusters various species show relationship supported by moderate to high confidence values. So out of four, three clusters are robust with confidence values, ranging from 50% to 76%.

Table 1. List of species used in the study, with accession numbers and locality.

No.	Species	Accession No.	Locality
	***Bistorta* Adans.**		
1.	*B. amplexicaulis* (D.Don) Green *Rumex* L.	125132	Swat, Northern areas, Pakistan
2.	*R. senguineus* L.	125133	Brighton, UK
3.	*R. senguineus*	125134	Brighton, UK
4.	*R. dentatus* L.	125135	Quaid-i-Azam University campus, Islamabad, Pakistan
5.	*R. dentatus*	125136	Fateh Jang, Pakistan
6.	*R. chalepensis* Mill.	125137	Quaid-i-Azam University campus Islamabad, Pakistan
7.	*R. chalepensis*	125138	Fateh Jang, Punjab, Pakistan
8.	*R. hastatus* D. Don	125139	Mall road, Murree, Punjab, Pakistan
9.	*R. hastatus*	125140	Mall Road, Murree, Punjab, Pakistan
10.	*R. nepalensis* Spreng.	125141	Matta, Swat, Northern areas, Pakistan
11.	*R. nepalensis*	125142	Mall road, Murree, Punjab, Pakistan
12.	*R, nepalensis*	125143	Near Ilyasi Mosque, Abbotabad, NWFP, Pakistan
	***Persicaria* Mill.**		
13.	*P. hydropiper* (L.) Spach	125144	Mall road, Murree, Punjab, Pakistan
14.	*P. hydropiper*	125145	Abbotabad, NWFP, Pakistan
15.	*P. glabra* (Willd.) M. Gómes	125146	Matta, Swat, Northern areas, Pakistan
16.	*P. glabra*	125147	Wah Gardens, Punjab, Pakistan
17.	*P. barbata* (L.) Hara	125148	Mir Pur, Azad Kashmir,Pakistan
18.	*P. barbata*	125149	Fateh Jang, Punjab, Pakistan
19.	*P. maculosa* S. F. Gay	125150	Chattar park, Islamabad, Pakistan
20.	*P. maculosa*	125151	Chattar park, Islamabad, Pakistan
21.	*P. maculosa*	125152	Chattar Park, Islamabad, Pakistan
22.	*P. maculosa*	125153	Chattar park, Islamabad, Pakistan
23.	*P. lapathifolia* (L.) S. F. Gray	125154	Mir Pur city, Azad Kashmir,Pakistan
24.	*P. lapathifolia* *Polygonum* L.	125155	Mir Pur city, Azad Kashmir, Pakistan
25.	*P. plebijum* R.Br.	125156	Quaid-i-Azam university campus, Islamabad, Pakistan
26.	*P. plebijum*	125157	Fateh Jang, Punjab, Pakistan
27.	*P. aviculare* L.	125158	Jhelum Cantt, Punjab, Pakistan
28.	*P. aviculare*	125159	Jhelum Cantt, Punjab, Pakistan

Cluster 1, a larger one with 5 species, consisting of 1 accession of *R. nepalensis*, 2 accessions of each of *P. glabra* and *P. hydropiper,* 2 accessions of *P. aviculare* and 2 accessions of *P. plebijum*. *P. glabra* and *P. hydropiper* are closely associated due to their short branch length; one accession of *P. hydropiper* and one accession of *P. glabra* are supported with 65% bootstrap value. These 2 accessions and another accession of *P. hydropiper* have 97% confidence value while these 3 accessions and second accession of *P. glabra* are in full confidence and their relationship is strongly supported by 100% bootstrap value at the node. The two accessions of *P. plebijum* are found genetically identical with each other, according to the AFLP marker system having full confidence value, while the two *P. aviculare* accessions possess some AFLP polymorphism due to the long branch length of one of the accession, although they showed 100% confidence value. One accession of *R. nepalensis* also present in this cluster which is in 66% confidence with all other accessions of the cluster.

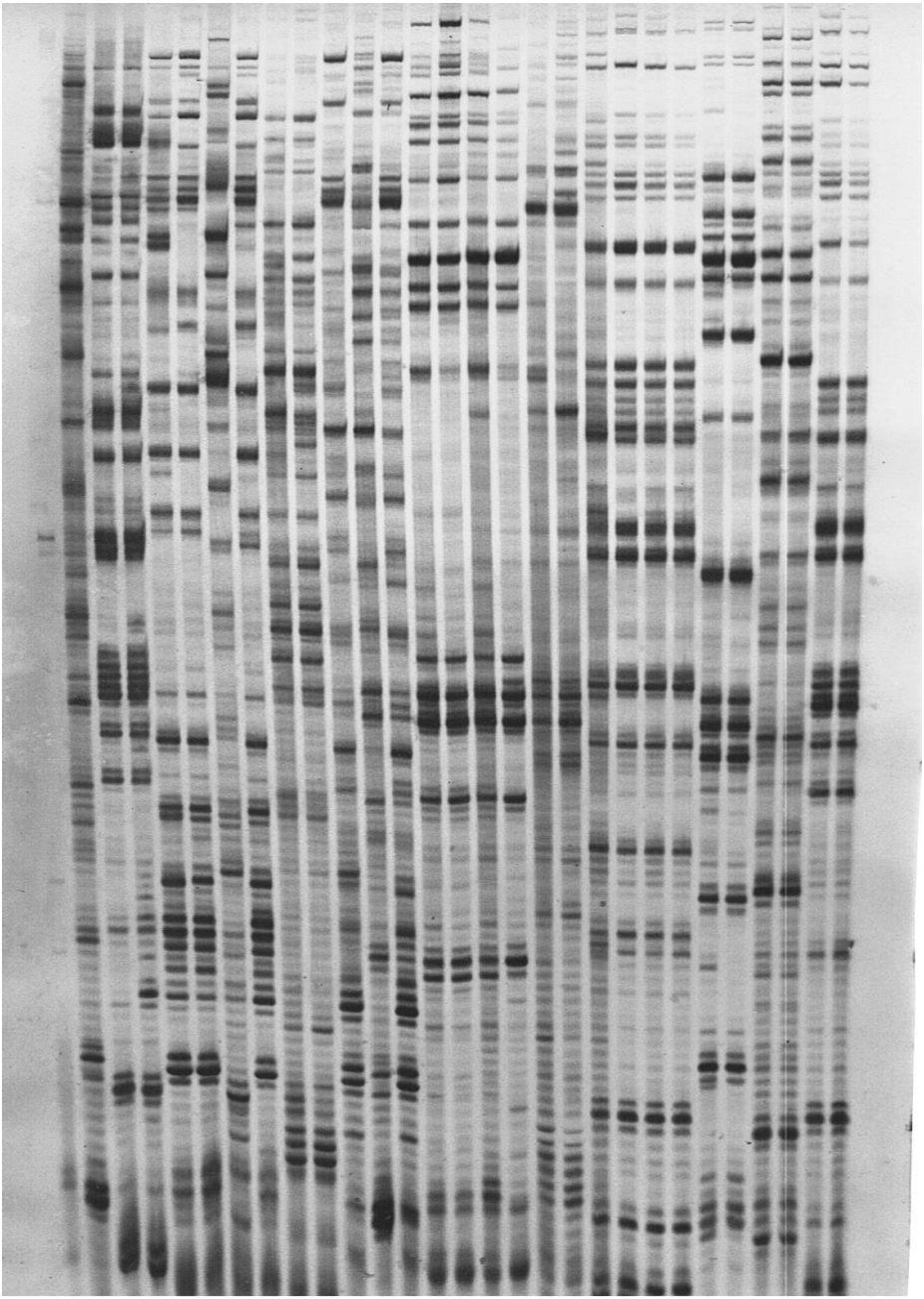

Fig. 1. AFLP profile showing 13 species of Polygonaceae.The samples are arranged from left to right in the order of (1) *B. amplexicaulis*, (2-3) *R. senguineus*, (4-5) *R. dentatus*, (6-7) *R. chalepensis*, (8-9) *P. aviculare*, (10-12) *R. nepalensis*, (13-14) *P. hydropiper*, (15-16) *P. glabra*, (17-18) *P. barbata*, (19-22) *P. maculosa*, (23-24) *P. lapathifolia*, (25-26) *P. plebijum*, (27-28) *R. hastatus*. Bracket on right side show the area of band counting.

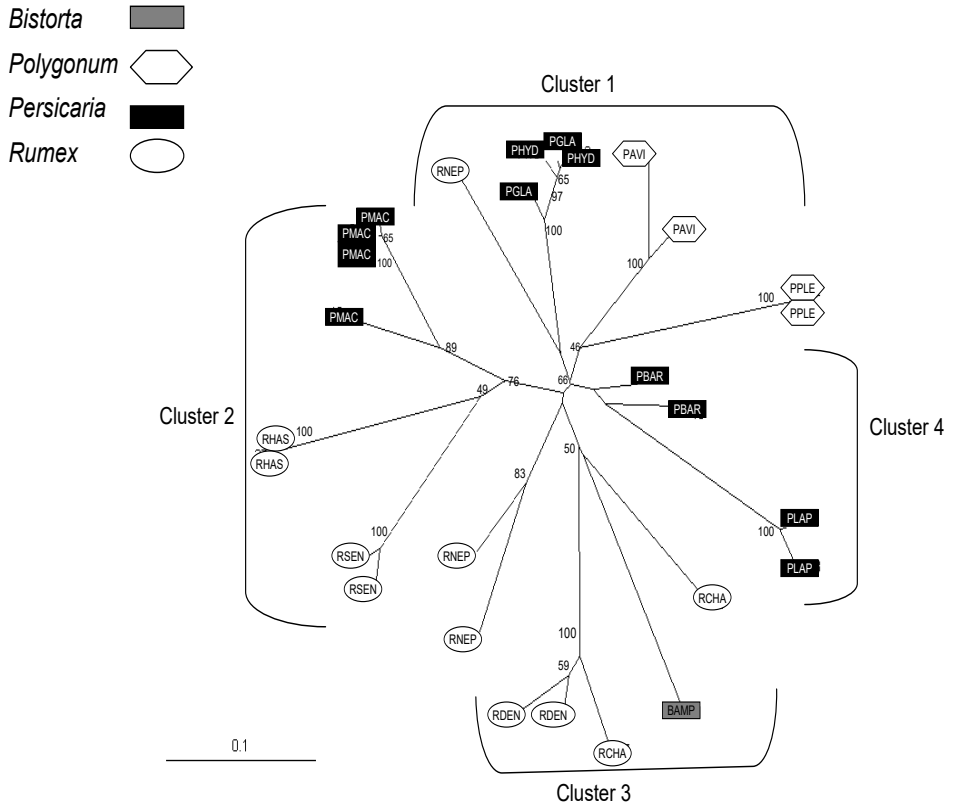

Fig. 2. Neighbour joining tree based on 1000 bootstraps of 13 Polygonaceae species.

1. PPLE: *Polygonum plebijum*, 2. PAVI: *Polygonum aviculare*, 3. BAMP: *Bistorta amplexicaulis*, 4. RDEN: *Rumex dentatus*, 5. RCHA: *Rumex chalepensis*, 6. RNEP: *Rumex nepalensis*, 7. RHAS: *Rumex hastatus*, 8. RCRI: *Rumex senguineus*, 9. PBAR: *Persicaria barbata*, 10. PMAC: *Persicaria maculosa*, 11. PGLA: *Persicaria glabra*, 12. PHYD: *Persicaria hydropiper*, 13. PLAP: *Persicaria lapathifolia*.

Cluster 2 is of four accessions of *P. maculosa* + two of *R. hastatus* + two of *R senguineus*, with relatively high bootstrap value of 76% forming a clade. Out of the four accessions of *P. maculosa*, two are with 65% confidence with each other, separated from each other by short branch, third accession is in full confidence with first two and all are found genetically alike while fourth one is separated from other three showing the confidence value of 89%. *R. hastatus* samples are genetically found to be identical and two accessions of *R. senguineus* are in full confidence with each other, possessing some level of polymorhism.

Cluster 3 consisting of two accessions of *R. dentatus*, two of *R. chalepensis* and one accession of *B. amplexicaulis. R. dentatus* samples show 59% bootsrap value, separated from each other by short branch, showing polymorphism with AFLP marker system. Two accessions of *R. chalepensis* are separated from each other, as one accession shows 100% confidence with *R. dentatus* while one accession is quite distinct although included in the same clade with 50% bootstrap value. One accession of *B. amplexicaulis* is nested within *Rumex* species, showing relationship with *Rumex*.

Cluster 4 of the tree consisting of two acccessions of *P. lapathifolia* separated from each other by short branch with 100% confidence value while two accessions of *P. barbata* arise independently on the tree and quite different from each other. According to AFLP marker system these two species have low bootstrap values and do not show any relationship with other species or even within themselves.

As our AFLP analysis comprises of three accessions of *R. nepalensis*, out of these three, one is grouped with *Polygonum* and *Persicaria* species, having moderate support but two accessions of *R. nepalensis* occupy a distinct position on the tree without showing any association with other species of the family. Both these accessions are separated from each other by a long branch length but showing 83% confidence value.

Discussion

Information on relationship of plant species of different genera of the same family is very important for plant taxonomist. It can be used to reveal the intensity of relatedness between different species. Taxonomic information based on morphological characters of the species would be advantageous but morphological characters are often under severe selection pressure which may result morphological differences between the same species. So, clear and detailed assessment of diversity within different taxa can be obtained by using molecular techniques for the identification of variation and phylogeny.

Among different PCR based molecular techniques, AFLP markers are better with a high degree of consistency and reproducibility, especially compared to other markers such as RAPDs or minisatellites. AFLP genotyping inaccuracy rates are generally < 2 % per AFLP band (Arens *et al*., 1998; Jones *et al*., 1997).

AFLP markers have been used to study a wide variety of taxa, from bacteria (Janssen *et al*., 1997; Siemer *et al*., 2004) and fungi (Gonzalez *et al*., 1998), to diverse groups of animals and plants (Ajmone-Marsan *et al*., 1997; Beardsley *et al*., 2003). It can be used in population and management genetics studies to understand population structuring, genetic diversity, hybridisation and detection of sex specific DNA (Arens *et al.,* 1998; Beismann *et al*., 1997). The use of AFLP for phylogenetic analysis is somewhat controversial while it appears to be most suitable for resolving interaction among closely related taxa at lower systematic levels (Mckinnon.*et al.,* 2008), even in species groups with a complex evolutionary history such as *Rosa* (Koopman *et al.*, 2008)

Molecular analyses that include some taxa of Polygonaceae are insufficient (Chase *et al*., 1993; Lledo *et al*., 1998) and only two published studies have focused on the broad phylogenetic relationship within the family (Lamb-Frye & Kron 2003; Sanchez & Kron, 2008) while particularly molecular phylogeny of *Persicaria* was studied by Kim & Donoghue (2008 a).

In the present work, AFLP technique has been used to analyse intra, interspecific and intergeneric relationship within the subfamily Polygonoideae (family Polygonaceae). Out of these 13 species, 9 species are never been considered for any molecular studies.

The general pattern of variation was found to be different from those of morphological studies. Variability of different species of the family, shown by morphological studies was confirmed by AFLP. In spite of the weakness with low bootstrap confidence for different clusters, several features of the data set are particularly striking and AFLP marker system can be used as a promising tool to identify relationships of different species. Though, the low bootstrap value is not amazing, while considering the composite origin of different species. In *rbcL* sequence analysis of Lamb Frye & Kron (2003), support value between *Rumex*, *Polygonum* and *Persicaria* was less than 50% while present AFLP analysis also suggests relationship among these genera is very weakly supported.

Interestingly in cluster 1, *P. plebijum* and *P. aviculare* were grouped together, forming monophyletic pair with support value of 66%. This finding is in accordance with Lamb Frye & Kron (2003) *rbcL* phylogeny and Kim & Donoghue (2008) molecular phylogeny of *Persicaria* where two species of *Polygonum* appeared with strongly supported relationship. Presence of two accession of each of *P. glabra* and *P. hydropiper* in the *Polygonum* cluster indicated that these *Persicaria* species shared common ancestor with *Polygonum*. At the genotypic level *P. glabra* and *P. hydropiper* were found to be closely related that they could not be considered as separate species due to their 100% support at the intermediate branching point. However morphologically they can easily be distinguished from each other, particularly by the shape of ochreae which is fully tubular in *P. glabra* while tubular below and shortly ciliate above in *P. hydropiper* (Qaisar, 2001). Such morphological differences may be due to environmental conditions or mutations which directly affect the developmental changes producing these differences.

Present AFLP data shows that most accessions of the same species collected from different localities group together, except three accessions of *R. nepalensis*, where one accession (13011) clustered with *Polygonum* and *Persicaria* with 66% confidence, while the position of other two accessions (13010 and 13012) is unresolved in the tree, with 83% of confidence for each other showing some polymorphism by the difference in their branch length. The plausible reason for the separation of the three samples may be that it is a polyphyletic species or the third accession may be another *Rumex* species, instead of *R. nepalensis* which is misidentified. For the clarification of its position, study on more accessions from different localities is required and this information will form the base for further analysis of the interaspecific variation.

The cluster consisting of *P. maculosa*, *R. hastatus* and *R. senguineus* show moderately high confidence value (76%), representing relationship in three species of two genera and their common ancestory. In *P. maculosa*, accession number 13021 and 13022 are with support value of 65%, separated from each other by a very short branch with some degree of polymorphism detected by AFLP marker system while accession number 13020 is in full confidence with 13021 and 13022. Its fourth accession (13019) separated from other three samples, having bootstrap value of 89%, although all these accessions were collected from same locality. Intraspecific variability is found between three accessions while fourth accession may represent its subspecies or variety.

In third cluster, two accessions of *R. dentatus* are with 59% support value for each other, possessing some extent of AFLP polymorphism, as shown by the difference in their branch length. One accession of *R. chalepensis* is in full confidence with two accessions of *R. dentatus*, while second accession (13006) is distant from the first one, showing value of 50% bootstrap with *R.dentatus* and first accession of *R .chalepensis*. This special position of one accession of *R.chalepensis* may perhaps be its misidentification so that it represents another *Rumex* species or a hybridization product with a *R. dentatus*. The first accession of *R. chalepensis* (13007) may be *R. dentatus*, as *R. dentatus* is highly variable species (Freeman and Reveal, 2005). These species of *Rumex* are identified by extensive branching in the upper half, basal leaves 2-3 times longer than broad, valves with 4-9 unequal teeth near to the base in *R. chalepensis* while *R. dentatus* with branching near to the base, panduriform leaves and 3-4 teeth at each margin of the valves (Rechinger, 2001). It was personally observed that both these species were found together in the same locality with different flowering period. Furthermore, Single accession of *B .amplexicaulis* is found to be in sister relationship with *Rumex* species. However close relationship between *Persicaria* and *Bistorta* was

presented based on anatomical (Haraldson, 1978), vegetative characters (Ronse Decraene & Akeroyd 1988) and in molecular phylogeny of *Persicaria* (Kim & Donoghue, 2008a). For more amplified results and to clear position, more samples of *B. amplexicaulis* from different areas and some more of *Bistorta* species are required.

Fourth cluster of two accessions of *P. lapathifolia* and two of *P. barbata* is weakly supported with confidence value less than 50%. These two species do not show any relationship with each other as well as with other species of the family. Two accessions of *P. lapathifolia* are genetically found to be similar with high support value while two accessions of *P. barbata* (13017 and 13018) are quite unrelated even with each other. On the basis of *rbcL*, *trnl-F*, *partial matK* with *trnK* intron, *psbA-trnH IGS* and from the nuclear ribosomal *ITS* region (Kim & Donoghue, 2008), *P. lapathifolia* and *P. hydropiper* are closely related to each other. Therefore, for their position justification more extensive studies are required.

Conclusion

Present NJ tree (Fig. 2) was appealing enough even though all clusters showed poor support at the base. Nevertheless, AFLP marker system proved to be a robustic and reliable method for evaluating genetic relationships at different taxonomic level. This, combined with generally low support above the species level in this tree lead us to avoid making overall conclusions; the key reason is that our genetic sample set is little so that AFLP results are questionable especially with reference to phylogeny because for phylogenetic relationship among different genera, more diverse species sampling of all the genera of the family is required. Our results show that if such investigations were extended to additional members of the Polygonaceae, particularly from various sources, it would be possible to get a comprehensive image of their genetic relationship.

In the present study using 1 – 4 accessions for each species, we investigated inter- and intraspecific relationships among the species. According to our analysis, separate species status of *P. glabra* and *P. hydrpiper* is doubtful. Position of *P. lapthifolia* and *P. barbata* is also unresolved, as both of these do not show any relationship with other species. There are also found some problems of misidentification of *R. chalepensis* and two accessions of *R. nepalensis* are not accommodated in any group

This research is the first one to report the use of the AFLP marker system for determining relationships, at inter, intraspecific and intergeneric level in the family Polygonaceae.

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