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Full Length Research Paper

A molecular phylogeny of selected species of genus *Prunus* L. (Rosaceae) from Pakistan using the internal transcribed spacer (ITS) spacer DNA

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Prunus **is found in all four provinces of Pakistan, that is, Punjab, NWFP, Sindh and Baluchistan including Azad Kashmir region. Studies on the family** ***Rosaceae*** **is scanty in the Flora of Pakistan and there is a lot of taxonomic work yet to be done, for the proper classification and placement of different genera under different sub-families. In the present study, the genus** ***Prunus*** **was studied in detail to find out the phylogenetic relationship among the 23 species of** ***Prunus,*** **selected from different regions of Pakistan and GenBank using maximum parsimony analysis of sequence polymorphism in nuclear ITS-9 and ITS-6 spacer DNA. The results for the internal transcribed spacer (ITS)- 9 and ITS- 6 primers confirm the work done by early phylogenetists with additions of new species from Pakistan including** ***Prunus bokhariensis, Prunus dulcis*** **(Mill.) D.A. Webb. (Syn.** ***Prunus amygdalus*****) and** ***Prunus cornuta*** **(Wall. ex. Royle) Steudel. These are indigenous to Pakistan. In the ITS strict consensus results for example, the clade consisting of** ***Laurocerasus, Padus*** **and** ***Cerasus*** **subgenera are sister to the rest of the clades in the phylogenetic tree.**

Key words: Phylogeny, *Prunus*, Pakistan, molecular phylogeny, nuclear primers.

INTRODUCTION

Rosaceae is a family of about 100 genera and 3,000 species (Judd et al., 1999). According to Rehder (1940) *Prunus* has nearly 200 species, mostly in temperate zone. Many species cultivated for their edible fruits and few for their edible seeds. *Prunus* is divided into following sub-genera *Pranophora*, *Amygdalus, Padus, Cerasus* and *Laurocerasus.* Subgenus *Prunophora* have sections *Euprunus, Prunocerasus*, and *Armeniaca*; *Amygdalus* have sections *Euamygdalus* and *Chamaeamygdalus*; *Cerasus* having sections *Microcerasus, Pseudocerasus, Lobopetalum, Eucerasus, Mahaleb, Phyllocerasus* and *Phyllomahaleb*; while *Padus* and *Laurocerasus* have no sections (Rehder, 1940).

This study focuses on the Genus *Prunus* belonging to the subfamily *Amygdaloideae*. The subfamily *Amygdaloideae* belongs to the family *Rosaceae*. The largest genus in the sub-family *Amygdaloideae* is *Prunus,* which is distinct because of its fruit types that is, Drupe. The chromosome number of *Amygdaloideae* is x = 8 (Potter, 2003). The genus *Prunus* L. has more than 200 species of shrubs and trees (Bortiri et al., 2006).

Prunus is found in all four provinces of Pakistan that is Punjab, NWFP, Sindh and Baluchistan including the Azad Kashmir region in Pakistan. This study was undertaken at the Department of Plant Sciences, Quaid-I-Azam University Islamabad and the Department of Plant Sciences, Wickson Hall University of California Davis USA, where the phylogenetic analysis using the primers including the ITS- 9 and ITS- 6 (Potter et al., 2007) (Table 2)

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Abbreviations: ITS, Internal transcribed spacer; **PCR,** polymerase chain reaction; **CTAB,** cetyl trimethylammonium bromide.

were conducted as nuclear primers.

The family *Rosaceae* is not yet published in Flora of Pakistan and there is a lot of taxonomic work yet to be done for the proper classification and placement of the different genera under different subfamilies. The main objective of this research work is to create a phylogeny of the genus *Prunus* in Pakistan.

Phylogenetic relationship was studied among the 23 species of *Prunus* selected from different regions of Pakistan and GenBank, using the maximum parsimony analysis of sequence polymorphism in the nuclear internal transcribed spacer (ITS) spacer DNA. The results for the ITS primers confirms the work done by early phylogenetists including Potter and Bortiri with additions of new species from Pakistan including *Prunus bokhariensis, Prunus dulcis* (Mill.) D.A. Webb. (Syn. *Prunus amygdalus*) and *Prunus cornuta* (Wall. ex. Royle) Steudel. These are indigenous to Pakistan. In the ITS strict consensus results, for example, the clade consisting of *Laurocerasus, Padus* and *Cerasus* subgenera are sister to the rest of the clades in the phylogenetic tree.

MATERIAL AND METHODS

Study area

Pakistan is located on the North Western side of South Asia. Their geographical extension lies between 24° and 37° North and longitude 61° and 78° East. The area of Pakistan is about 7, 93, 000 square km and it is the second largest nation in South Asia, India being the largest (Bano et al., 1995).

Twenty-three (23) different species of *Prunus* were included in this study representing all the subgenera and important sections of the genus. The voucher specimens were sent to the herbarium of the Quaid-I-Azam University Islamabad and that of the University of California Davis CA. The fresh samples of the *Prunus* were collected from different parts of Pakistan along with the herbarium samples from the herbarium of the Quaid-I-Azam University Islamabad and the University of California Davis CA.

DNA extraction

DNA was extracted from fresh and herbarium samples of *Prunus* by the method of Doyle and Doyle (1987). Annealing temperature was 52 - 56°C with the primers ITS-9 and ITS-6 or Trn-L and Trn-F. Polymerase chain reaction (PCR) was carried out using ABI. Applied Biosystems 2720 Thermal cycler and Eppendorf Master Cycler. The PCR product was separated in 0.8% agarose gel. The bands were separated and cut and purified with QIA quick-Gel Extraction kit (250) (QIAGEN Inc). Sequencing of the purified product was done at the Plant genetics facility, University of California Davis with ABI/Prism 377 automated sequencer.

DNA isolation using cetyl trimethylammonium bromide (CTAB)

50 ml 2 × CTAB stock was placed in a small bottle and 100 µl β-mecrraptoethanol was added. With the liquid nitrogen, few leaves were crush to powder in a mortar. Leaves were not allowed to thaw. 1 ml of 2 × CTAB buffer was then added (with βmecrraptoethanol) as the grinding continues. Leaf extract was poured into the first labeled 1.5 ml tube (about 600 µl) and incubated at 65°C in water bath using a float for 45 min. The tubes were inverted to mix every 10 min. After incubation, 400 µl of chloroform/isoamyl alcohol (24:1) was added. The tubes were then inverted to create an emulsion and centrifuged at 12,000 rpm for 2 min. The supernatant (aqueous layer) obtained was carefully transferred to a second labeled tube. After the repetition of the above steps, transfer into a third tube was carried out. 700 µl of ice cold isopropanol (or at least 1:1 volume to the aqueous layer) was added to the tube and placed in a -20°C freezer overnight. The next morning tube was centrifuge at 14,000 rpm for 10 min. Precipitated DNA formed pellet. Loose pellet were removed using a pipette to remove the supernatant. The pellet was wash with 0.8 ml 75% ice-cold ethanol, centrifuged at 14,000 rpm for 10 min and the supernatant carefully decanted. The pellet was dried by placing in an incubator at 50°C with lids open and Kim wipes placed over open lids. After drying, (no visible liquid in the tube and pellet looks like glass), pellet was re-suspend in 30 µl 10 mM Tris-HCl (pH8).

Alignment

Sequences were edited in Sequencer 4.8 (Build-3768) (Gene Code Corporation). The alignment was done by Clustlax. Binary characters were also used in the missing data while working in PAUP. The aligned ITS-9 and ITS-6 sequences were submitted to the Genbank (Table. 1).

Phylogenetic reconstruction and primers used

The phylogenetic analysis was done in PAUP 4.0b10. The primers were used for the nuclear and chloroplast DNA that is ITS-9 and ITS-6 (Potter et al., 2007) (Table 2).

Softwares used

The software used include: Sequencer 4.8-build 3768. Reg. No. 9612040, 1991 – 2007, Clustal X (1.8), Se-Al v-2.0 a 11 (1996-2002, Andrew Rambaut) and PAUP Version 4.0 b 10 for Mac.

RESULTS

Out groups

For the out groups, *Sorbaria sorbifolia* and *Spiraea cantoniensis*, were selected which have been proposed as the sister to *Prunus* in past studies e.g. *Spiraea* and *Sorbaria* which were supported by data from PGIP and Mat-K, separately and combined (Potter et al., 1999).

ITS analysis

The aligned ITS sequence resulted in: Total characters: 716; parsimony informative characters: 87; parsimony uninformative characters: 124; maximum parsimony analysis of ITS showed tree length: 365; consistency index (CI): 0.7589; homoplasy index (HI): 0.2411 and retention index (RI): 0.7241. A total of 100 parsimonious trees (MPT) were produced. The results of the bootstrap for the

Table 1. Genbank accession numbers of *Prunus* species using ITS - 9 and ITS - 6.

S/N	Taxon	Locality	Source/voucher	Gene bank accessions ITS
1	*Prunus persica*	GenBank	Cultivar-548-455.EB69 gi/19032452/gb/AF348560.1	AF318741
2	*Prunus armeniaca*	GenBank	PI 128556. EB99 gi/149391858/emb/AM282691.1	AF318756
3	*Prunus avium*	GenBank	Cultivar-var. No voucher gi/15991342/gb/AF327586.1	AF318737
4	*Prunus cerasifera*	GenBank	DPRU-563-EB.79 gi/149391812/emb/AM282665.1	AF318755
5	*Prunus cerasus*	GenBank	Gi/135752991/gb/EF211080.1 Gi/1181847041/gb/EF010970.1	EF211080
6	*Prunus domestica*	GenBank	PI 131179. EB. 97 Gi/14939182/emb AM282672	AF318713
7	*Prunus mahaleb*	GenBank	DPRU 1488.5 JSH 966	AF318747
8	*Prunus tomentosa*	GenBank	No voucher	AF179500
9	*Prunus mexicana*	GenBank	UCDA 90.0690.EB 71	AF318734
10	*Prunus laurocerasus*	GenBank	UCDA T0140.EB 88	AF318724
11	*Prunus avium*	Skardu/Skardu/Northern areas (Pakistan)	Gil-31-ITS	GQ179664
12	*Prunus cornuta*	Murree/Rawalpindi/Punjab (Pakistan)	Gil-38-ITS	GQ179666 GQ179667
13	*Prunus bokhariensis*	Skardu/Skardu/Northern areas (Pakistan)	Gil-24-ITS	GQ179663 GQ179665
14	*Prunus padus*	GenBank	DPRU-1075.2	AF318726
15	*Prunus avium*	Skardu/Skardu/Northern areas (Pakistan)	Gil-31-ITS	GQ179664
16	*Prunus fruiticosa*	GenBank	DPRU 385.11	AF318738
17	*Prunus mume*	GenBank	PI.418552	AF318728
18	*Prunus besseyi*	GenBank	DPRU 389.1	AF318732
19	*Prunus simonii*	GenBank	DPRU 545.EB 81	AF318720
20	*Prunus bucharica*	GenBank	DPRU 192.2	AF318719
21	*Prunus prostrate*	GenBank	No voucher	AF492415
22	*Prunus microcarpa*	GenBank	No voucher	AF492416
23	*Prunus fasciculata*	GenBank	No voucher	EU669086
24	*Prunus jacquemontii*	GenBank	No voucher	AF492417
25	*Sorbaria sorbifoila*	GenBank	UCBG 83.0529	AF318758
26	*Spiraea cantoniensis*	GenBank	UCDA No voucher	AF318722

Genbank accession numbers of *Prunus* species for ITS-9 and ITS-6.

Table 2. ITS-9 and ITS-6 primer sequences.

S/N	Primer	Primer Sequence
1	ITS 9 (Forward)	CCGCTTATTGATATGCTTAAAC
2	ITS6 (Reverse)	TCGTAACAAGGTTTCCGTAGGTGA

ITS-9 and ITS-6 primers details for the forward and reverse primer sequences.

ITS are presented in strict consensus tree (Figure 1). In the ITS bootstrap tree, the subgenera *Laurocerasus, Padus* and *Cerasus* form a monophyletic group.

These subgenera are sister to the rest of the clades, having good support (86%). The species in this clade include *Prunus laurocerasus, P. cornuta, Prunus padus, Prunus avium, Prunus fruiticosa* and *Prunus mahaleb*. *P. cornuta* is the new addition in this study (as it is not reported in the work by Bortiri et al. (2001). The second clade with strong support is subgenus *Amygdalus* (93 %) but relationship with in this clade are less resolved as compared to the *Laurocerasus, Padus* and *Cerasus* clade. The species included in this clade consists of *Prunus besseyi, Prunus persica, Prunus bucharica* and *P. dulcis*. The sub-genus *Prunus* has also relatively good support (81%) including *Prunus cerasiferea, Prunus domestica, Prunus simonii* and *Prunus jacquemontii*, as well as *P. bokhariensis* as a new addition. The other species which include the sections *Microcerasus* and *Prunocerasus* are less resolved clades. The section *Prunocerasus* includes *Prunus mexicana, Prunus microcarpa* and *Prunus fasciculata*. The section *Armeniaca* under subgenus *Prunus* has also less support (56%) and includes *Prunus armeniaca* and *Prunus mume*.

Species of *Prunus* under different subgenus and sections

Prunus has been divided into six subgenera including *Cerasus, Prunus, Amygdalus, Laurocerasus Emplectocladus* and *Padus*. The first four of these have been divided into sections. The species in our study include *P. armeniaca* and *P. mume* from section *Armeniaca* of subgenus *Prunus*. *P. mexicana*, *P. microcarpa* and *P. fasciculata* from section *Prunocerasus* of subgenus *Prunus. P. cerasifera, P. domestica, P. bukhariensis, P. simonii* and *P. jacquemontii* from section *Prunus* of subgenus *Prunus*. *P. besseyi, P. persica, P. bucharica* and *P. dulcis* from subgenus *Amygdalus*. *P. laurocerasus* from subgenus *Laurocerasus*, *P. padus* and *P. cornuta* from subgenus *Padus*. *Prunus cerasus*, *P. avium*, and *P. fruiticosa* from section *Cerasus* of subgenus *Cerasus. Prunus tomentosa* from section *Microcerasus* of subgenus *Cerasus. P. mahaleb* from section *Mahaleb* of the subgenus *Cerasus. P. fasciculata* from subgenus *Emplectocladus, Prunus mexicana* from section *Prunocerasus* of subgenus *Prunus.*

DISCUSSION

This work is based on the phylogenetic work done by Bortiri et al. (2001) on the phylogenetic analysis of *Prunus* using ITS nuclear primers. The main objective was to reconstruct the phylogeny of the *Prunus* with reference to the Bortiri's work to describe and review the previous taxonomic relationship of *Prunus* to provide the basis for the morphological evolution in *Prunus*.

Rehder (1940) classification was used as a base for the research work as Rehder described *Prunus* under different subgenera including subgenus *Prunophora* having sections *Euprunus; Cerasus* having sections *Microcerasus, Pseudocerasus, Lobopetalum, Eucerasus, Mahaleb, Phyllocerasus* and *Phyllomahaleb*; the other subgenus *Padus* and the *Laurocerasus* having no sections.

The main subgenus of *Prunus* consists of *Cerasus, Padus, Prunus, Amygdalus, Emplectocladus* and *Laurocerasus*. The sections consist *of Lobopetalum, Peeudocerasus, Cerasus, Mahaleb, Prunocerasus, Prunus, Penarmeniaca,* and *Microcerasus.* Here the species *Prunus armeniaca* and *Prunus mume* lies under the section *Armeniaca* of Subgenus *Prunus*. The species *P. mexicana*, *P. microcarpa* and *P. fasciculata* lie under the section *Prunocerasus* under the sub-genus *Prunus.* The species *Prunus cerasifera, P. domestica, P. bokhariensis, P. simonii* and *P. jacquemontii* lie under the section *Prunus* of subgenus *Prunus*. The species *P. besseyi, P. persica, P. bucharica* and *P. dulcis* lie under the subgenus *Amygdalus*. The species *Prunus laurocerasus* lies under the subgenus *Laurocerasus* and the species *P. padus* and *P. cornuta* lies under the subgenus *Padus*. The species *P. cerasus*, *P. avium*, and *P. fruiticosa* lie under the section *Cerasus* of the subgenus *Cerasus*. The species *P. tomentosa* lies under the section *Microcerasus* of the subgenus *Cerasus* and the species *P. mahaleb* lies under the section *Mahaleb* of the subgenus *Cerasus.* The species *P. fasciculata* lies under the subgennus *Emplectocladus* and the species *P. mexicana* lies under the section *Prunocerasus* of the subgenus *Prunus.*

For the out groups, *S. sorbifolia* and *S. cantoniensis* were selected which have been proposed as the sister to the *Prunus* in past studies (Potter et al., 1999).

The aligned ITS sequences resulted in 505 constant, 124 parsimony un-informative and 87 parsimony informative characters out of a total of 716 characters. The maximum parsimony analysis of the ITS showed the tree length = 365, with consistency index (CI) = 0.758,

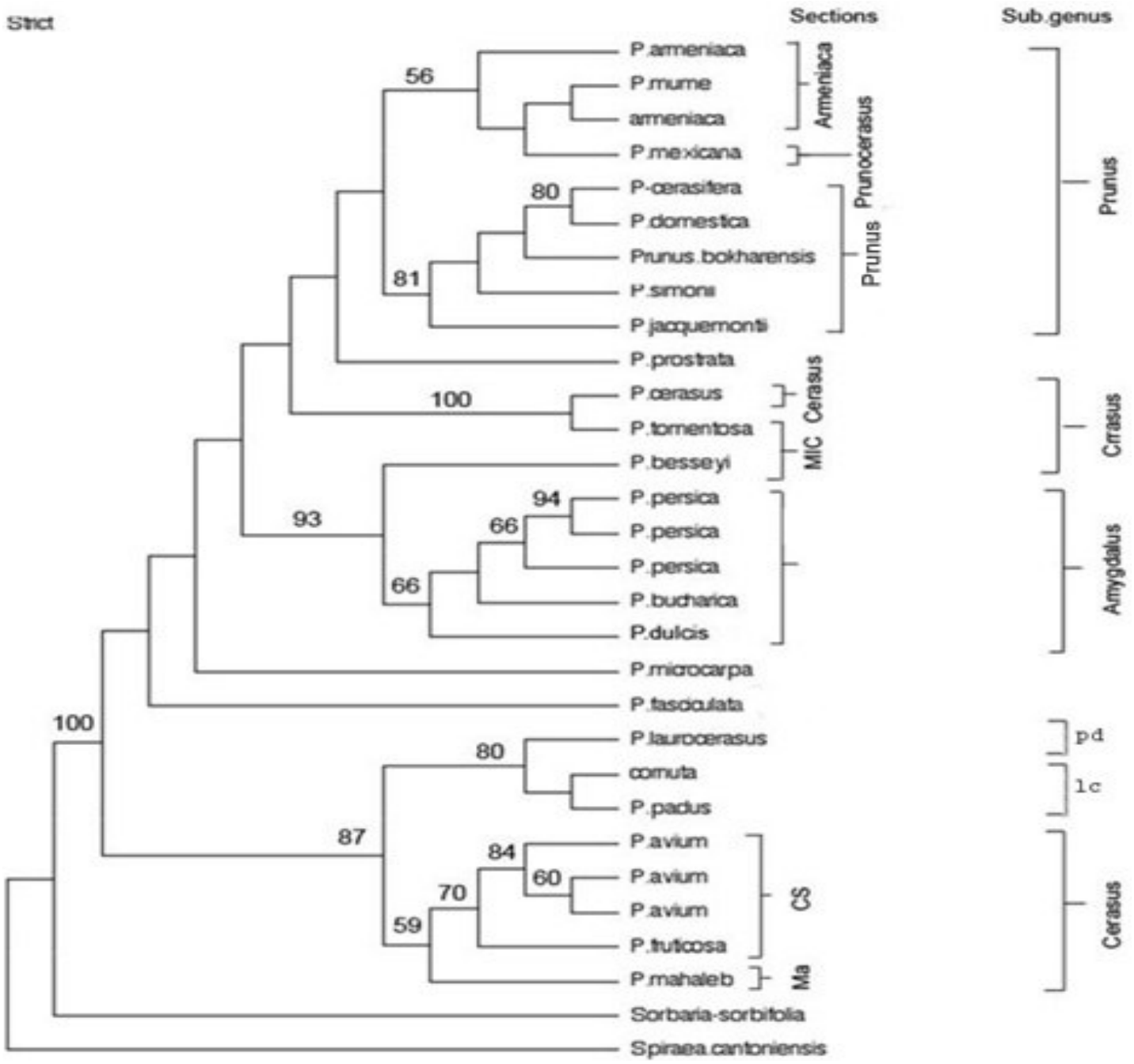

Figure 1. Strict consensus tree for ITS-9 and ITS-6. The strict consensus tree of the ITS primers (ITS - 9 and ITS - 6) having the bootstrap values with the subgenera *Amygdalus, Cerasus, Prunus, Padus* and *Laurocerasus*. Pd = *Padus*, Lc = *laurocerasus*. The sections under the subgenera are *Armeniaca, Prunocerasus, Prunus, Cerasus*, and *Mahaleb* where as Mic = *Microcerasus*, Cs = *Cerasus* and Ma = *Mahaleb*. The outgroups are *Spiraea cantoniensis* and *Sorbaria sorbifolia*.

homoplasy index (HI) = 0.241 and retention index (RI) = 0.724. The strict consensus tree contains the clades containing subgenus *Prunus, Cerasus*, *Amygdalus, Laurocerasus, Padus* and *Cerasus* while the sections contains *Armeniaca, Prunoceraus, Prunus, Cerasus, Microcerasus, Cerasus, Prunocerasus* and *Mahaleb*. The strict consensus tree contains the clades consisting subgenera *Laurocerasus, Padus* and *Cerasus* as strongly supported clades at 87% (bootstrap value) and is considered as monophyletic clade. *P. cornuta* is a new addition from Pakistan in the work already done by Bortiri and others. The second clade with high support is subgenus *Amyg-*

dalus at 93% support (bootstrap value) but it is less resolved as compared to the *Laurocerasus*, *Cerasus* and *Padus* clade. The third clade with relatively better support is subgenus *Prunus* at 81% support, this clade has new addition, *P. bokhariensis* to the work already done. The sections *Microcerasus* and *Prunocerasus* are less and weakly resolved clades. The section *Armeniaca* is also less resolved clade having less support at 56% and includes *P. armeniaca* and *P. mume*. Bortiri et al. (2001) also placed *P. persica* under the subgenus *Amygdalus*. In this study, the ITS results, *P. dulcis* and *P. persica* are also placed under the subgenus *Amygdalus* clade at 93% support. This clade is paraphylatic with *P. bucharica* along with *P. dulcis* and *P. persica*. This clade is less resolved as compared to *Laurocerasus, Padus* and *Cerasus* clade.

Lee and Wen (2001) used the parsimony analysis, distance analysis and maximum likelihood analysis of the ITS data. They found support for two main clades with in *Prunus,* one clade including the species classified in subgenera *Amygdalus* and *Prunus* and the other clade consist of species from subgenera *Cerasus, Padus* and *Laurocerasus*. None of the individuals were supported as monophyletic.

Bortiri et al. (2001) used the ITS and Trn-L and Trn-F primers. There were some differences in the first clade, which may be because of differences in the sampling of the taxon. The tree based on the Trn-L and Trn-F data alone placed species of subgenus *Cerasus* in the *Amygdalus* and *Prunus* clade. This research work support the work of Bortiri et al. (2001) as in the results obtained from ITS data, mainly two major clades were found one comprising the *Cerasus, Padus* and *Laurocerasus* and the other comprising the *Amygdalus* and *Prunus*, with one species of subgenus *Cerasus* that is, *P. cerasus* and other species from section *Microcerasus* that is, *P. tomentosa* and *P. besseyi*. The *Laurocerasus, Padus* and *Cerasus* clade is the sister to the rest of the clades in the ITS tree. None of the subgenera are monophyletic. *Prunus* itself as a whole, is monophyletic and it is divided into two major clades as described previously.

Conclusion

With reference to this research and previous work done by Bortiri et al. (2001) and other researchers, it is concluded that *Prunus* is treated as a single genus in the broader aspect rather than dividing and segregating into several different genera.

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