*Full Length Research Paper*

# A molecular phylogeny of selected species of Genus *Prunus* L. (Rosaceae) from Pakistan using the TRN-L & TRN-F spacer DNA

**Syed Aneel Gilani[1,3]\*, Rizwana A. Qureshi[1], Amir M. Khan[1], Faizan Ullah[1], Zarqa Nawaz[1], Ijaz Ahmad[1] and Dan Potter[2]**

[1]Department of Plant Sciences, Quaid-i-Azam University, Islamabad Pakistan.
[2]Department of Plant Sciences, University of California, Davis 95616, USA.
[3]Botanical Sciences Division, Pakistan Museum of Natural History, Islamabad, Pakistan.

The genus *Prunus* L. is an important plant for fruit production and it includes plums, apricots, cherries, almonds and peaches that are sources of food for the local people. The family *Rosaceae* is not yet published in Flora of Pakistan and there is a lot of taxonomic work that is yet to be done for the proper classification and placement of different genera under different sub-families. *Prunus* is found in almost all the four provinces of Pakistan including Punjab, Khyber Pakhtunkhwa (former NWFP), Sindh and Baluchistan which includes Azad Kashmir region. In the present study, the genus *Prunus* was studied in detail to find out the phylogenetic relationship among the 12 species of *Prunus* selected from different regions of Pakistan and GenBank using the maximum parsimony analysis of sequence polymorphism in chloroplast TRN-L and TRN-F spacer DNA. The results for the TRN-L and TRN-F primers confirm the work done by early phylogenetists including Potter and Bortiri with additions to new species from Pakistan including *Prunus dulcis* (Mill.) D.A. Webb. (Syn. *Prunus amygdalus*) and *Prunus cornuta* (Wall. ex. Royle) Steudel. which are indigenous to Pakistan.

**Key Words:** *Prunus*, chloroplast, TRN-L, TRN-F, Pakistan.

## INTRODUCTION

*Rosaceae* is a family of about 100 genera and 3,000 species. Members of *Rosaceae* occur in a variety of habitats throughout the world but the family is best developed in the Northern Hemisphere (Judd et al., 2002) where it is also of tremendous economic importance.

The vast majority of fruits of north temperate regions including species of *Malus* Mill. (Apples), *Pyrus* L. (pears), *Fragaria* L. (strawberries), *Rubus* L. (raspberries and blackberries) and *Prunus* L. (peaches, plums, cherries,

apricots and almonds), are produced by species of *Rosaceae*. The family also includes many ornamentals, cultivated primarily or their beautiful flowers, such as species of *Rosa* L. (roses), *Potentilla* L. (chinquefoil), *Sorbus* L. (mountain ash) and *Spiraea* L. (bridal wreath) (Potter, 2003).

According to Rehder (1940), *Prunus* has nearly 200 species, mostly in temperate zone. Many species are in cultivation for their edible fruits and few for their edible seeds. *Prunus* is divided into the following subgenera: *Prunophora*, *Amygdalus*, *Padus*, *Cerasus* and *Laurocerasus*. Subgenus includes: *Prunophora* (having sections *Euprunus*, *Prunocerasus* and *Armeniaca*), other subgenus: *Amygdalus* (having sections *Euamygdalus* and *Chamaeamygdalus*), other sub-genus *Cerasus* having (sections *Microcerasus*, *Pseudocerasus*, *Lobopetalum*, *Eucerasus*, *Mahaleb*, *Phyllocerasus* and *Phyllomahaleb*), other sub-genus: *Padus* (having no sections), and the other subgenus (having no sections)

---

*Corresponding author. E-mail: aneelgilani@gmail.com. Tel. 92-321-5850175.

**Abbraviations: CTAB,** Cetyl trimethyl ammonium bromide; **PCR,** polymerase chain reaction; **L,** tree length; **CI,** consistency index; **RI,** retention index; **HI,** homoplasy index; **MPA,** maximum parsimony analysis; **MPT,** most parsimonious trees; **ITS,** internal transcribed spacer.

(Rehder, 1940).

Stewart (1972) listed a total of 26 genotypes including 23 species, 2 subspecies and 1 variety from West Pakistan. *Prunus* has both wild and cultivated species. *Prunus* is found in tropical to temperate and sometimes in moist temperate regions. A lot of work needs to be done on the taxonomic as well as phylogeny, palynology and other aspects of the family *Rosaceae* and the taxa included in it. There is a lot of confusion about the listing of species by different taxonomists and there has been so far no revision of the subfamily *Amygdaloideae* in Pakistan. *Prunus* is found in almost all the four provinces: Punjab, Khyber Pakhtunkhwa (former NWFP), Sindh and Baluchistan including the Azad Kashmir region in Pakistan. The family *Rosaceae* is not yet published in Flora of Pakistan and there is a lot of taxonomical work that is yet to be done for the proper classification and placement of different genera under different sub-families. The main objective of this research work is to create a phylogeny of the genus *Prunus* in Pakistan.

## MATERIALS AND METHODS

This study was undertaken at the Department of Plant Sciences, Quaid-i-Azam University, Islamabad and Department of Plant Sciences, Wickson Hall University of California, Davis, USA, to conduct the phylogenetic analysis using the primers including the TRN-L and TRN-F (Potter et al., 2007) (Table 1) as chloroplast primers.

Phylogenetic relationship was studied among the 12 species of *Prunus* selected from different regions of Pakistan and GenBank (Table 2), using the maximum parsimony analysis of sequence polymorphism in chloroplast TRN-L and TRN-F spacer DNA. The results for the TRN primers confirm the work done by early phylogenetists including Potter and Bortiri with additions to new species from Pakistan including *Prunus dulcis* (Mill.) D.A. Webb. (Syn. *Prunus amygdalus*) and *Prunus cornuta* (Wall. ex. Royle) Steudel. which are indigenous to Pakistan.

The bootstrap majority-rule consensus tree (Figure 1) for TRN contain two main clades, the first one include members of subgenus *Laurocerasus* and *Padus* and the other consists of the rest of the subgenera and sections, that is they include the subgenera *Amygdalus, Cerasus* and *Prunus*, this clade is less resolved subclades and within it are weakly supported.

Twelve different species of *Prunus* were included in this study and they represent all the subgenera and important sections of the genus. The fresh samples of the *Prunus* were collected from the different parts of Pakistan along with the herbarium samples from the Herbarium of the Quaid-i-Azam University, Islamabad and Herbarium of the University of California, Davis, CA.

Silica gel is mostly used to dry the fresh leaves as silica gel is inexpensive and reliable to preserve field-collected leaves for molecular studies of variation in DNA (Chase and Hills, 1991).

### Protocol for DNA extraction using the cetyl trimethyl ammonium bromide (CTAB)

DNA was extracted from the fresh and herbarium samples of *Prunus* by the method of Doyle and Doyle (1987). For the polymerase chain reaction (PCR) analysis, annealing temperature was 52 to 56°C with the Primers TRN-L and TRN-F. PCR used was ABI, Applied Biosystems 2720 Thermal c ycler and Eppendorf

Master Cycler. The PCR product was separated in 0.8% agarose gel. The bands were cutted and purified with QIA quick-Gel Extraction kit (250) (QIAGEN Inc). Sequencing of the 1 purified product was done at the plant genetics facility; University of California Davis with ABI/Prism 377 automated sequencer.

Before beginning, 1.5 ml tubes were labeled for each sample and placed in a rack. The water bath was set to 65°C. 50 ml 2X CTAB stock was placed in a small bottle and 100 μl B-mecrraptoethanol was added. With the liquid nitrogen, few leaves (3 to 5 g) were crushed to powder in a mortar. One milliliter of 2X CTAB buffer (with B-mecrraptoethanol) was added and grinding was continued. The leaf extraction slurry was poured into the first labeled 1.5 ml tube (about 600 μl) and incubated at 65°C in the 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 tube was inverted to create an emulsion and centrifuged at 12,000 rpm for 2 min. The supernatant (aqueous layer) was transferred to the second labeled tube, avoiding pipetting up of any of the interface. These steps were repeated for the third labeled tube. 700 μl of ice-cold isopropanol (or at least 1:1 volume to the aqueous layer) was added to the tube and placed in -20°C freezer overnight. The next morning, the tube was centrifuged at 14,000 rpm for 10 min. Precipitated DNA formed a pellet. The supernatant was poured off. The pellet was washed with 0.8 ml 75% ice-cold ethanol. The tube was centrifuged at 14,000 rpm for 10 min and the supernatant was poured off. The pellet was dried by placing it in incubator at 50°C with lids open and Kim wipes were placed over open lids. When pellet was dried (no visible liquid in the tube and pellet looked like glass), it was re-suspended in 30 μl 10 mM Tris-HCL (pH 8).

### PCR conditions and sequencing

For the PCR analysis, annealing temperature was 52-56°C with the Primers TRN-L and TRN-F. PCR used was ABI, Applied Biosystems 2720 Thermal cycler and Eppendorf Master Cycler. The PCR product was separated in 0.8% agarose gel. The bands were separated, 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.

### Alignment

Sequences were edited in Sequencer 4.8 (Build-3768. Reg. No. 9612040, 1991-2007) (Gene Code Corporation). The alignment was done by Clustlax (1.8). Binary characters were also used in the missing data while working in PAUP Version 4.0 b 10 for Mac.

### Phylogenetic reconstruction and primers used

The phylogenetic analysis was done in PAUP 4.0b10. The primers were used for the chloroplast DNA, that is, TRN-L and TRN-F (Potter et al., 2007) (Table 1)

## RESULTS

The aligned TRN-L and TRN-F sequences resulted in 541 constant, 25 parsimony un-informative and 18 parsimony informative characters from a total of 584 characters. Maximum parsimony analysis of the TRN-L and TRN-F showed that tree length (L) = 45, consistency index (CI) = 0.977, retention index (RI) = 0.977 and homo-

**Table 1.** TRN-L and TRN-F primer sequences.

| S/N | Primer | Primer Sequence |
|-----|--------|-----------------|
| 1 | TRN-L (Forward) | CGAAATCGGTAGACGCTACG |
| 2 | TRN-F (Reverse) | ATTTGAACTGGTGACACGAG |

**Table 2.** GenBank accession numbers.

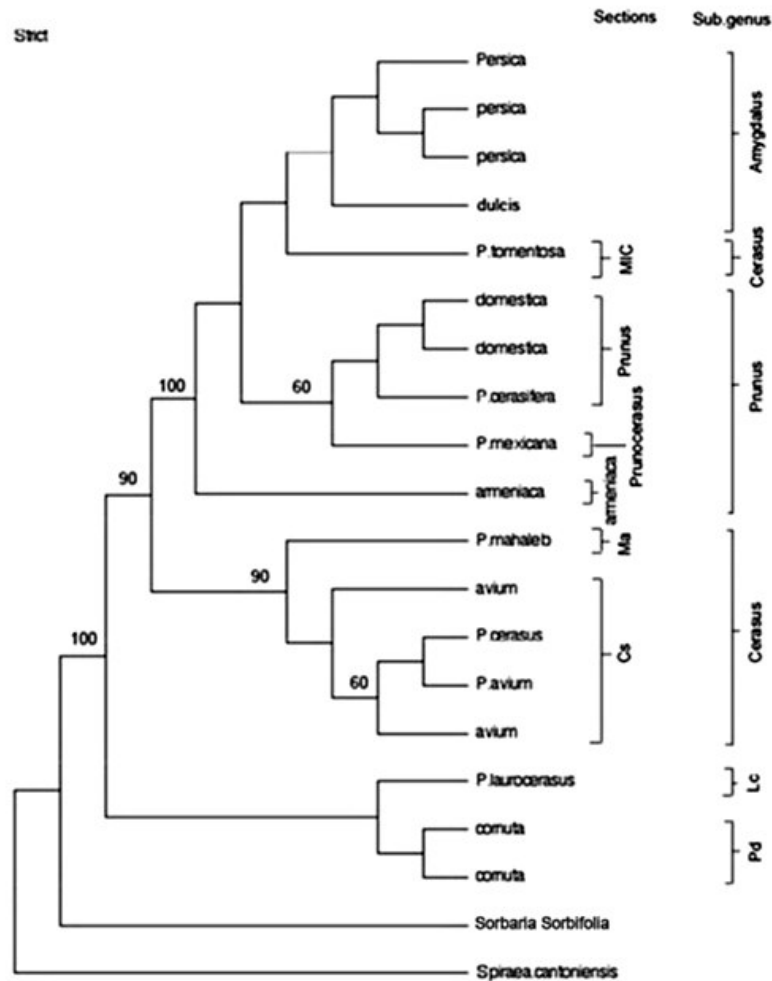| S/N | Taxon | Locality | Source/voucher | GenBank accessions |
|-----|-------|----------|----------------|--------------------|
| 1 | *Prunus persica* | GenBank | Cultivar-548-455.EB69 gi/19032452/gb/AF348560.1 | AF348560 |
| 2 | *Prunus armeniaca* | GenBank | PI 128556. EB99 gi/149391858/emb/AM282691.1 | AM282691 |
| 3 | *Prunus avium* | GenBank | Cultivarr-var. No voucher gi/15991342/gb/AF327586.1 | AF327586 |
| 4 | *Prunus cerasifera* | GenBank | DPRU-563-EB.79 gi/149391812/emb/AM282665.1 | AM282665 |
| 5 | *Prunus cerasus* | GenBank | Gi/135752991/gb/EF211080.1 Gi/1181847041/gb/EF010970.1 | EF010970 |
| 6 | *Prunus domestica* | GenBank | PI 131179. EB. 97 Gi/14939182/emb AM282672 | AM282672 |
| 7 | *Prunus mahaleb* | GenBank | DPRU 1488.5 JSH 966 | AY500761 |
| 8 | *Prunus tomentosa* | GenBank | | AM282689 |
| 9 | *Prunus mexicana* | GenBank | UCDA 90.0690.EB 71 | AY500747 |
| 10 | *Prunus laurocerasus* | GenBank | UCDA T0140.EB 88 | AF348559 |
| 11 | *Prunus avium* | Skardu/Northern areas (Pakistan) | Gil-41-TRN | GQ179671 |
| 12 | *Prunus persica* | Murree/Rawalpindi/Punjab /Pakistan | Gil-45-TRN | GQ179674 |
| | | Quaid-i-Azam University /Islamabad (Pakistan) | Gil-54-TRN | |
| 13 | *Prunus cornuta* | Murree/Rawalpindi (Pakistan) | Gil-43-TRN Gil-42-TRN | GQ179672 |
| 14 | *Prunus dulcis* | Skardu/Northern areas (Pakistan) | Gil-51-TRN | GQ179668 GQ179669 |
| 17 | *Prunus avium* | Skardu/Northern areas (Pakistan) | Gil-41-TRN | GQ179671 |
| | | Murree/Rawalpindi (Pakistan) | Gil-46-TRN | |
| 18 | *Prunus armeniaca* | Skardu/Northern areas (Pakistan) | NIL | GQ179670 |
| 19 | *Prunus domestica* | Murree/Rawalpindi (Pakistan) | Gil-40-TRN | GQ179673 |
| 20 | *Sorbaria sorbifoila* | GenBank | UCBG 83.0529 | AF348569 |
| 21 | *Spiraea cantoniensis* | GenBank | UCDA No voucher | DQ897578 |

**Figure 1.** The strict consensus tree of the TRN primers (TRN-L and TRN-F) 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,* whereas, Mic = *Microcerasus*, Cs = *Cerasus* and Ma = *Mahaleb.* The outgroups are *Spiraea cantoniensis* and *Sorbaria sorbifolia.*

homoplasy index (HI) = 0.222.

**Out groups**

For out groups, *Sorbaria sorbifolia* and *Spiraea cantoniensis* were selected. For the out groups, those genera 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, were selected (Potter et al., 1999).

**TRN-L and TRN-F results interpretation**

Maximum parsimony analysis (MPA) of the chloroplast

TRN-L and TRN-F region provided the total tree length, that is, L = 45, CI =0.977, RI =0.977 and HI =0.222. A total of 100 most parsimonious trees (MPT) were produced. The results of the bootstrap analysis are presented in Figure 1. The bootstrap majority-rule consensus tree contains two main clades: one including members of subgenus *Laurocerasus* and *Padus* and other consists of the rest of the subgenera and sections which includes the subgenera *Amygdalus, Cerasus* and *Prunus*; this clade is less resolved and subclades within it are weakly supported. The Laurocerasus and Padus clade includes the species *Prunus laurocerasus* and *P. cornuta* (*P. cornuta* is the new addition and not reported by the earlier workers). *P. cornuta* is present along with *P. laurocerasus*. This *Laurocerasus* and *Padus* clade is sister to the rest of the clades. Within the second main

clade, the sub-clades are *Cerasus* clade with support (90 %), while subgenus *Prunus* with support (60%), the section *Microcerasus* consists of species i.e. *Prunus tomentosa*, these clades are not well resolved as compared to *Laurocerasus* and *Padus* clades.

## DISCUSSION

The aligned TRN-L & TRN-F sequences resulted in 541 constant, 25 parsimony un-informative and 18 parsimony informative characters from total of 584 characters. The bootstrap majority – rule consensus tree contains two main clades one including members of subgenus *Laurocerasus* and *Padus* and the other consisting the rest of the subgenera and sections i.e. including the subgenera *Amygdalus, Cerasus,* and *Prunus,* this clade is less resolved subclades within it are weakly supported. The *Laurocerasus* and *Padus* clade includes the species *P. laurocerasus* and *P. cornuta* (*P. cornuta* is the new addition and not reported by the earlier workers). *P. cornuta* is present alone with *P. laurocerasus*. This *Laurocerasus* and *Padus* clade is sister to the rest of the clades. Within the second main clade, the sub-clades are *Cerasus* clade with support (90%), and subgenus *Prunus* with support (60%), the section *Microcerasus* consists of species: *P. tomentosa*, and these clades are not well resolved as compared to *Laurocerasus* and *Padus* clades.

Rehder (1940) divided the *Prunus* into the following subgenera which includes *Prunophora, Amygdalus, Padus*, *Cerasus* and *Laurocerasus*. According to him, *Prunus persica* and *P. dulcis* are placed under the subgenus *Amygdalus.* Bortiri et al. (2001) used the internal transcribed spacer (ITS) and TRN-L and TRN-F primers. There were some differences in the first clade, which may be as a result of the 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.

Bortiri et al. (2006) also worked on the morphological analysis of the *Prunus* with reference to phylogenetic studies. They took 25 morphological characters of 37 *Prunus* species. They examined the evolution of the vegetative and reproductive characters by using the parsimony reconstruction on the tree obtained from ITS, TRN-L, TRN-F, TRN-S and TRN-G primers. They also described the character evolution in the 37 *Prunus* and 8 other genera of family *Rosaceae.*

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 within *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.

The results obtained from the aligned data of TRN-L and TRN-F has the same results as described by Bortiri et al. (2006). It has the clade with subgenera *Padus* and *Laurocerasus* as sister to the rest of the clades in the tree with relatively weaker support, while the other clade has *Cerasus, Prunus* and *Amygdalus* with *Cerasus* having support at 90% and *Prunus* having relatively less support with 60%. *Amygdalus* also had less support.

## REFERENCES

Bortiri  E, Sang-Hun Oh, Jianguo Jiang, Scott Baggett, Andrew Granger, Clay Weeks, Megan Buckingham, Daniel Potter, Dan E. Parfitt (2001). Phylogeny and systematics of *Prunus* (Rosaceae) as determined by sequence analysis of ITS and the chloroplast trnL – trnF spacer DNA. Systematic Botany, 26(4): 797-807.

Bortiri E, Vanden H, Potter D (2006). Phylogenetic analysis of the morphology in *Prunus* reveals extensive homoplasy. Plant Systematics and Evolution, 259: 53-71.

Chase MW, Hills HH (1991). Silica Gel: An Ideal material for field preservation of leaf samples for DNA studies. Taxon, 40 (2): 215 - 220.

Doyle JJ, Doyle JL (1987). A rapid DNA isolation procedure for the small quantities of fresh leaf tissue. Phytochem. Bull. 19: 11-15.

Judd WS, Christopher SC, Elizabeth AK, Peter FS, Michael JD (2002). Plant Systematics. A Phylogenetic Approach. 2: 365-372. Sinauer Associates, Inc. Publishers Sunderland, Massachusetts U.S.A.

Lee S, Jun W (2001). A phylogenetic analysis of *Prunus* and the Amygdaloideae (Rosaceae) using the ITS sequences of Nuclear Ribosomal DNA. Am. J. Bot. 88(1): 150-160.

Potter D, Gao F, OH S, Baggett S (1999). Molecular phylogenetic studies in *Rosaceae*. In International Botanical Congress Abstract, p: 39.

Potter D, (2003). Molecular phylogenetic studies in *Rosaceae*. Pp. 319-351 in: Sharma AK,  Sharma A, eds., *Plant Genome: Biodiversity and Evolution, Vol. I, Pt. A: Phenarogams*. Science Publishers, Inc., Enfield (NH) USA & Plymouth, UK.

Potter D, Still SM, Grebenc T, Ballian D, Božič G, Franjiæ J, Kraigher H (2007). Phylogenetic relationships in tribe Spiraeae (Rosaceae) inferred from nucleotide sequence data. Plant Systematics and Evolution 266: 105-118.

Rehder A, (1940). Manual of cultivated trees and shrubs hardy in North America 2nd edition Macmillan Company New York, pp: 425-481.

Stewart RR, (1972). Flora of Pakistan, An annotated catalogue of the vascular plants of West Pakistan and Kashmir Fakhri Printing Press Karachi,  pp. 360-364.