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RESEARCH ARTICLE

Phylogenetic analysis of *Prunus* (Rosaceae) from Northeast India based on ribosomal ITS sequence data

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Abstract

Prunus L., a diverse genus in the family *Rosaceae*, includes several economically significant species like apricot, almond, peach, plum and nectarines. The aim of the study is to elucidate the phylogenetic relationships of *Prunus* species collected from Northeast India. Molecular phylogenetic analysis was conducted using Internal Transcribed Spacer (ITS) sequences. A total of 32 accessions were analyzed in this study, comprising six voucher specimen of *Prunus cerasoides* and a single accession representing each of *Prunus campanulata*, *Prunus napaulensis* and *Prunus domestica*, along with 22 other specimens representing five subgenera according to Rehder and outgroup *Pyrus communis* (pear), was included from GenBank for comparative purposes. Phylogenetic relationships were inferred using a combination of Maximum Likelihood (ML) and Bayesian inference methods. The results revealed two distinct major clades: one comprising *Cerasus, Padus* and *Laurocerasus* and the other consisting of *Prunus* and *Amygdalus*, reflecting evolutionary divergence within the genus. This study provides phylogenetic analysis of *Prunus* species from the Northeast India region and the ITS sequence data will be helpful in future taxonomic and evolutionary studies as it adds up to the already available resources and enhancing the genetic resource base. The findings also contribute to the broader understanding of the evolutionary history of *Prunus* species in the region.

Keywords: Bayesian; internal transcribed spacers; phylogeny; *Prunus*; Rosaceae

Introduction

Prunus L. is a genus in Rosaceae comprising of trees and shrubs such as almond, apricot, cherry, peaches, plum and nectarines (1). Rosaceae typically exhibit pentamerous flowers, characterized by sepals with a valvate arrangement, five prominent petals that are clawed at the base and a large number of stamens that arise from a cup-shaped receptacle (2). *Prunus* is distinguished by its simple, alternate leaves, which are typically lanceolate. The flowers are generally white to pink, occasionally red and comprise five petals, five sepals and numerous stamens. Floral arrangement varies among species: flowers occur singly, clusters of two to six (umbels) and in racemes (3). *Prunus* fruits are distinctly recognized as drupes, featuring a succulent outer mesocarp and a tough, hardened endocarp that encases the seed (4). *Prunus* species, such as cherries and almonds, have evolved to endure cold winters by going dormant and developing resistance to freezing conditions and withstand summer droughts (5). Globally, the genus *Prunus* comprises around 430 accepted species, predominantly distributed across the temperate zones of the Northern Hemisphere. Within India, 36 species have been documented as part of its native and naturalized flora (6, 7). Of the 36 *Prunus* taxa reported in India, 18 are extensively cultivated and commercially valued for their edible fruits and kernels. The remaining 18 taxa are recognized for their wild occurrence and economic significance. Commercial cultivation is primarily concentrated in the northern hill states such as Himachal Pradesh, Jammu and Kashmir and Uttarkhand (8, 9). In the Himalayan region, 19 species of the genus *Prunus* are commonly found, whereas around 13 species have been documented in the Northeastern parts of India (10). *Prunus napaulensis*, *P. undulata* and *P. cerasoides* have been reported from the Khasi Hills region of Meghalaya (11).

ITS region of ribosomal DNA (rDNA), includes ITS1 spacer, 5.8S rRNA gene and ITS2 spacer (12, 13). The ITS sequences have high evolution rate and are useful for phylogenetic analysis among related species or among populations within a species. The ITS marker was proposed as a barcode for flowering plants (14). Genetic markers such as ITS, rbcL and matK have been widely utilized in previous molecular studies to examine the phylogenetic relationships within the *Prunus* genus (15, 16).

In Northeast India, previous research on the genus *Prunus* has predominantly focused on species distribution and chemical composition, with comparatively limited focus on resolving taxonomic ambiguities and elucidating evolutionary relationships.

To address these critical gaps, a preliminary investigation employing ITS sequence data was undertaken to assess the phylogenetic relationships among *Prunus* species. The study aimed to examine the genetic affinities between accessions collected from Northeast India and those previously deposited in the GenBank database. By integrating molecular data, the study seeks to clarify taxonomic boundaries, enhance the understanding of evolutionary patterns within the genus. These findings are expected to contribute to more informed and effective conservation strategies for *Prunus* species in Northeast region.

Materials and Methods

Sample collection and identification

Extensive botanical surveys were conducted between 2019 and 2021 across various districts of Manipur namely Senapati, Ukhrul, Tamenglong, Bishnupur and Chandel during the flowering and fruiting season (October to December) for the collection of *Prunus* germplasm. Additional specimens were also collected from other Northeastern states, including Nagaland, Meghalaya, Arunachal Pradesh and Sikkim, with further sampling extending to regions such as Tamil Nadu in southern India. Voucher specimens were prepared and deposited in IBSD (Institute of Bioresources and Sustainable Development) herbarium, where species identification was carried out using conventional morphological techniques. For confirmation, herbarium specimens were also cross-referenced with collections at the Botanical Survey of India (BSI), Kolkata. Nine samples were collected for the study and geographic coordinates of sample collection sites across Northeast India are provided in Table 1.

DNA isolation, amplification and sequencing

The dataset comprises nine accessions, including six voucher specimens of *Prunus cerasoides* collected from distinct locations, along with one accession each of *Prunus campanulata*, *Prunus domestica* and *Prunus napaulensis*. Species selection was based on geographic variation across sampling sites. Total genomic DNA was extracted using the Qiagen DNA mini kit and DNA quality and concentration were assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). PCR amplification of the ITS region was performed using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (17). Reactions were carried out in a Bio-Rad thermal cycler in a final volume of 20 µL, containing 1 µL of genomic DNA, 2 µL of 10× PCR buffer, 0.25 µL of 1 mM dNTPs, 1 µL each of 5 µM ITS1 and ITS4 primers, 0.2 µL of Taq polymerase (5 units/µL, Sigma-Aldrich) and 14.55 µL of nuclease-free water (Qiagen). The PCR protocol included an initial denaturation at (94 °C) for 5 min, followed by denaturation at (95 °C) for 30 sec, annealing at (58 °C) for 1 min and extension at (72 °C) for 2 min for a total of 35 cycles and a final extension at (72 °C) for 7 min. Reactions were then held at 4 °C. PCR products were resolved on 1.2 % (w/v) agarose gels using 1× TBE buffer, electrophoresed at 60 V for 65 min and stained with GelRed™ nucleic acid stain. Bands were visualized under UV illumination using a UV transilluminator (Bioglow) and imaged with a Bio-Rad GelDoc™ system. A 1 kb DNA ladder (Promega) was used as a size marker. Amplified products were purified using the Qiagen Gel/PCR purification kit as per the manufacturer's protocol (Valencia California USA). Purified amplicons were submitted to Bionivid Technologies Pvt. Ltd., Bangalore, for sanger sequencing. The same primers used for amplification were employed in the sequencing reactions.

Nucleotide sequence data and submission

The nucleotide sequences of the ITS region from all nine *Prunus* accessions analyzed in this study have been submitted to the GenBank database. Both newly generated and previously downloaded sequences are available through the National Center for Biotechnology Information (NCBI), with their corresponding accession numbers provided in Table 2.

Sequence alignment and analysis

The Basic Local Alignment Search Tool (BLAST) in the NCBI (https://blast,ncbi.nlm.nih.gov/Blast.cgi) was employed to identify the most homologous sequences available in the GenBank, NCBI database. Multiple sequence alignment was performed using MEGA X software version 10.1.8 to analyse sequence variation and evolutionary patterns. This analysis included the identification of conserved sites (CS), variable sites (VS), parsimony-informative sites (PIS), singleton sites (SS), as well as the calculation of transition/transversion ratios and the identification of specific transition and transversion substitutions (18). The obtained sequences were aligned with additional *Prunus* sequences retrieved from GenBank, representing taxa with previously established phylogenetic relationships. This comparative alignment facilitated a broader analysis of genetic relatedness within the genus (19). The aligned sequences were visually inspected to ensure accuracy and any poorly aligned or ambiguous sequences were excluded from further analysis. The remaining sequences were then re-aligned using the MUSCLE algorithm implemented in MEGA X ran using the provided tool in EMBL-EBI website (https://www.ebi.ac.uk/Tools/msa/muscle/) alongside the other amplicons, to confirm consistency and improve alignment quality.

Construction of phylogenetic tree

The dataset used for the phylogenetic analysis includes 31 ITS

Table 1. GPS co-ordinates of the *Prunus* species sampled in the present study across Northeast India and other regions

Species name	Location	Latitude	Longitude
1.Prunus domestica	Chandel, Manipur	24° 19' 34.32'' N	94° 0' 2.16'' E
2.Prunus cerasoides	NH-2 b/w khuzama and viswema (Nagaland)	25° 32' 82.4'' N	94° 07' 65.7'' E
3.Prunus napaulensis	Laitlyngkot, Meghalaya	25° 26' 53.83'' N	91°50' 34.13'' E
4.Prunus cerasoides	Shirui village, Ukhrul Manipur	25° 07' 46.1'' N	94° 0' 2.16'' E
5.Prunus cerasoides	Sikkim	25° 53' 30'' N	88° 51' 22'' E
6.Prunus campanulata	Mao, Manipur	25° 20' 25'' N	94° 31' 17'' E
7.Prunus cerasoides	Arunachal Pradesh, Ziro	27° 35' 41.89'' N	93° 50' 18.74'' E
8.Prunus cerasoides	Wards lake, Meghalaya.	25° 34' 48.8'' N	91° 53'26.7'' E
9.Prunus cerasoides	Kodaikanal	10° 13' 41.00'' N	77° 28'12.16'' E

Table 2. List of *Prunus* taxa and outgroup analyzed in this study and along with their corresponding GenBank accession numbers

Taxa and site of collection	Accession numbers of ITS sequences in GenBank
Prunus domestica Chandel, Manipur	MT811814
Prunus campanulata Mao,Manipur	MT811815
Prunus napaulensis Meghalaya	MT811816
Prunus cerasoides Meghalaya	MT811817
Prunus cerasoides Kodaikanal	MT811818
Prunus cerasoides Sikkim	MT811819
Prunus cerasoides Nagaland	MT811820
Prunus cerasoides Arunachal Pradesh	MT811821
Prunus cerasoides Ukhrul, Manipur	MT828305
Prunus cyclamina	AF179503.1
Prunus cerasoides var campanulata	AF179503.1
Prunus serrulata var spontanea	AH010149.2
Prunus verecunda	AF179522.1
Prunus maackii	AH010152.2
Prunus fordiana	AF179530.1
Prunus laurocerasus	AH010157.2
Prunus serotina	AH101542
Prunus ilicifolia subsp. lyonii	AH010155.2
Prunus tenella	AH010142.2
Prunus persica	AF179562.1
Prunus mume	AH009376.2
Prunus tomentosa	AF179500.1
Prunus domestica	AF179485.1
Prunus salicina	AH009373.2
Prunus armericana	AF179489.1
Prunus armericana	AF179488.1
Prunus augustifolia	AF179490.1
Prunus nigra	AH009374.2
Prunus mandshuria	AH009375.2
Prunus caroliniana	AF179540.1
Prunus grayana	AF179531.1
Pyrus communis	JQ392467.1

sequences, representing five subgenera of *Prunus*. This comprised 22 sequences retrieved from GenBank and 9 newly generated in the present study. The phylogenetic tree was re-rooted using the ITS sequence of *Pyrus communis* (NCBI accession no. JQ392467.1) as an outgroup. All sequence characteristics and phylogenetic analyses were conducted using MEGA X software. The phylogenetic trees were constructed using MEGA-X software. The sequences were aligned using the MUSCLE algorithm and trimmed. To determine the most appropriate substitution model for phylogenetic analysis, MEGA X software was used to calculate model parameters and the best -fitting model was selected based on the lowest Bayesian Information Criterion (BIC) score. The Kimura 2-parameter model with gamma distribution (K2+G) was identified as the best -fit model for the ITS nucleotide dataset. Phylogenetic relationships were inferred using both the Maximum Likelihood (ML) method implemented in MEGA X and the Bayesian inference method implemented in MrBayes version 3.2.7 (20). Maximum Likelihood (ML) analysis was performed using the Nearest-Neighbour Interchange (NNI) heuristic method (21). For Bayesian analysis, a Markov Chain Monte Carlo (MCMC) simulation was run for 1000000 generations, utilizing one cold and three heated chains. Trees were sampled every 200 generations, beginning from a random starting tree. Convergence of the MCMC run was confirmed when the average standard deviation of split frequencies dropped below 0.01. The initial 25 % of sampled trees were discarded as burn-in and the remaining trees were used to estimate posterior probabilities (PP) for clade support.

Results

PCR amplification of ITS and sequence analysis

PCR amplification and sequencing using universal primers ITS1 and ITS4 successfully amplified the nuclear ITS region across all *Prunus* accessions examined in this study. The resulting sequences were deposited in GenBank under accession numbers MT811814-MT811821 and MT828305. The length of the ITS1 region ranged from 226 to 256 base pairs (bp), the 5.8S region was consistently 102 bp across all taxa and the ITS2 region ranged from 232 to 305 bp (Table 3). The average GC content was calculated for each region, with ITS1 showing a mean of 59.24 %, 5.8S at 57.8 % and ITS2 at 57.81 %. Among the taxa analysed, *P. cerasoides* from Ukhrul exhibited the longest ITS sequence (633 bp), while *P. cerasoides* from Nagaland had the shortest (566 bp). The highest GC content (63.8 %) and lowest AT content (36.2 %) were recorded in *P. napaulensis*, whereas *P. cerasoides* from Arunachal Pradesh displayed the lowest GC content (53.20 %) and highest AT content (46.8 %). These patterns are consistent with previous findings on ITS GC content variation reported (19). Alignment of the complete ITS region required 10 insertion-deletion events (indels): 4 in ITS1, 1 in the 5.8S region and 5 in ITS2. Single nucleotide polymorphisms (SNPs) were also identified, with 2 in ITS1 and 10 in ITS2. Nucleotide diversity (π) was assessed using the Tajima neutrality test, yielding a value of 0.055852 (22). The analysis revealed 189 segregating sites (S) across a maximum of 484 nucleotide positions (N). The estimated transition/transversion bias (R) was 1.67, indicating a moderate preference for transitions over transversion in the ITS region of the analyzed taxa.

Molecular phylogeny analysis

Phylogenetic trees were constructed using ITS region dataset comprising 31 *Prunus* taxa and analyzed using both ML and Bayesian inference methods. The ITS sequence of *Pyrus communis* (GenBank accession no. JQ392467.1) was used as outgroup for rooting the phylogenetic tree. This study incorporates representatives from five subgenera of *Prunus*, as classified by Rehder: *Amygdalus*, *Prunus*, *Padus*, *Cerasus* and *Laurocerasus* (12). The ML tree inferred from the ITS sequences revealed two major clades. The first clade includes a *Cerasus*-

Table 3. Sizes and percent G+C content (%) of ITS 1, 5.8s and ITS 2 regions for each *Prunus* taxon included in the analysis

Species name (Taxon)/ accession number	Size (bp) ITS1	5.8s	ITS2	A	T	A+T	G	C	G+C	GC %	Total Length
Prunus domestica MT811814	233	102	234	113	118	231	190	176	366	61.3	569
Prunus napaulensis MT811816	233	102	243	109	108	217	194	189	383	63.8	578
Prunus cerasoides MT828305	226	102	305	146	142	288	175	174	349	54.8	633
Prunus campanulata MT811815	232	102	233	130	116	246	170	151	321	56.60	567
Prunus cerasoides MT811821	232	102	232	155	110	265	166	135	301	53.20	566
Prunus cerasoides MT811817	241	102	232	116	121	237	170	168	338	58.80	575
Prunus cerasoides MT811818	232	102	237	112	121	233	169	169	338	59.20	572
Prunus cerasoides MT811821	232	102	232	114	121	235	165	166	331	58.50	566
Prunus cerasoides MT811819	256	102	232	128	118	246	165	179	344	58.30	590

Padus-Laurocerasus complex, while the second comprises the *Amygdalus-Prunus* complex. Our analysis demonstrated that the subgenera *Cerasus* and *Prunus* each form distinct monophyletic clades. The newly sequenced accessions in this study clustered closely with other members of the subgenus *Cerasus*, indicating a strong genetic affinity. Within the ML tree, subgenera *Amygdalus*, *Laurocerasus* and *Padus* were positioned basally relative to the *Cerasus* clade. Additionally, the clade containing *Prunus caroliniana*, *P. grayana* and *P. napaulensis* appeared basal within the subgenus *Prunus*. The majority of taxa classified under *Cerasus* grouped together in a well-supported clade. The resulting ML tree, shown in Fig. 1, includes bootstrap values (BT) from the ML analysis and posterior probability (PP) values from the Bayesian inference. Branches with bootstrap support values below 50% were excluded from the final ML tree for clarity. The accession numbers of all taxa included in the phylogenetic analysis are provided within the tree diagram.

Discussion

Our analysis demonstrates that the universal primers ITS1 and ITS4 effectively amplified the nuclear ITS region across diverse *Prunus* accessions, supporting their broad applicability across plant, fungal and even animal genomes, as previously reported (23). The nuclear ribosomal ITS region, known for its high interspecific divergence and strong intraspecific conservation, remains a widely used genetic marker for species-level identification. It offers exceptional resolution at the infrageneric level, making it particularly valuable for phylogenetic studies in complex genera such as *Prunus* (24). In this study, molecular variation was examined across 32 ITS sequences representing five *Prunus* subgenera, with *Pyrus communis* (Rosaceae) serving as the outgroup. The total length of the trimmed ITS sequences, encompassing ITS1, 5.8S and ITS2 regions, ranged from 566 to 633 bp, consistent with earlier studies that reported lengths between 591 and 615 bp for *Prunus* species (19), suggesting limited variation in sequence length across taxa. Phylogenetic analysis using both ML and Bayesian inference approaches revealed two major clades: one representing the *Cerasus-Padus-Laurocerasus* complex and another comprising the *Amygdalus-Prunus* lineage. We confirmed that our study correlates with previous studies by forming well supported clades within the subgenera *Cerasus* and *Prunus* (19). Within the *Cerasus-Padus-Laurocerasus* complex, taxa classified under *Cerasus* formed a distinct cluster that included all seven individuals newly sampled in this study. Morphologically, *Cerasus* can be distinguished from the others by its axillary or umbel-shaped inflorescences, whereas *Padus* and *Laurocerasus* exhibit racemose types. Our findings do not support a clear separation between *Padus* and *Laurocerasus*, as species from both subgenera were found to cluster together in the ITS-based phylogeny. While *Padus* and *Laurocerasus* are morphologically distinct, the phylogenetic tree suggests a close relationship, forming a paraphyletic clade. This may reflect shared morphological traits, particularly their inflorescence structure (3). Interestingly, *P. tomentosa* section *Microcerasus*, traditionally placed within *Cerasus*, was nested within the *Amygdalus-Prunus* clade, consistent with previous findings (24). This phylogenetic clustering may be due to shared traits such as solitary or fascicled inflorescences, which *P. tomentosa* shares with *Amygdalus* and *Prunus*. This observation suggests that further sampling within *Cerasus*, particularly section *Microcerasus*, is necessary to clarify its

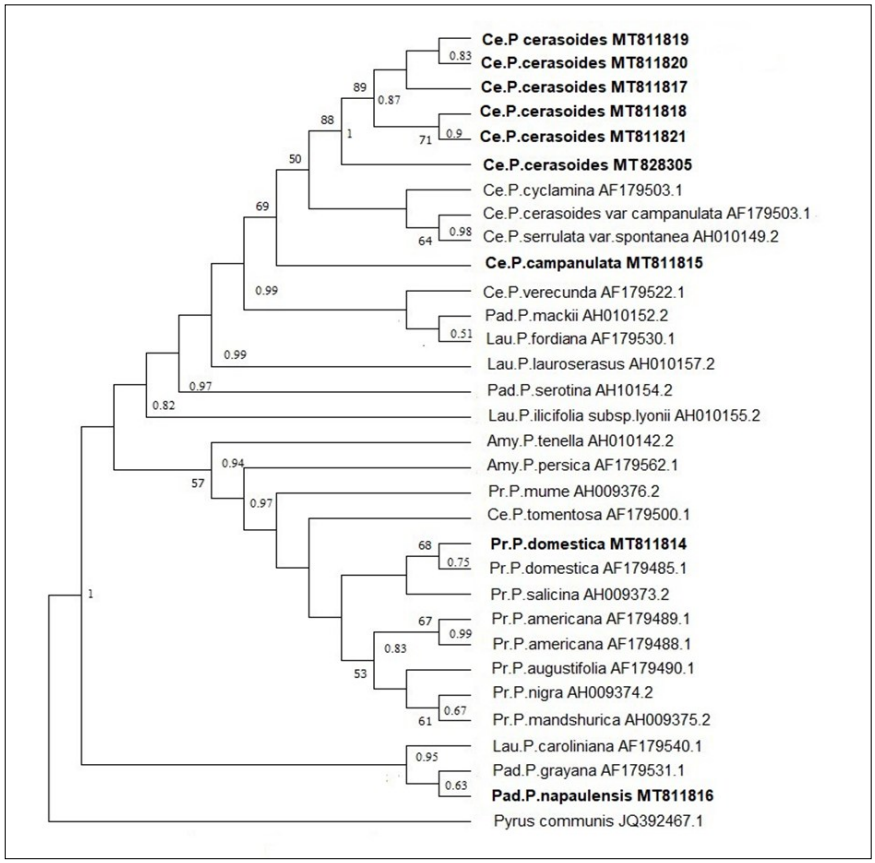

Fig. 1. ML phylogenetic tree from ITS sequences of *Prunus* species. Bootstrap values (only values > 50 % shown) above the branches and Bayesian posterior probabilities are indicated at the corresponding nodes. GenBank accession numbers follow the species names.

phylogenetic placement as our dataset may have affected clade resolution. Previous phylogenetic work in *Prunus* using ITS, *trnF* and other molecular markers such as *S6pdh* and *trnL-trnF* spacers has also highlighted the utility of ITS in resolving taxonomic relationships within Rosaceae (25, 26). The study finds that analyses based solely on ITS have yielded comparable or even superior resolution relative to combined datasets, likely due to the higher number of parsimony-informative sites in ITS.

Conclusion

This study demonstrates that the nuclear ITS region is a robust tool for resolving phylogenetic relationships within *Prunus*. The study was conducted to investigate the evolutionary relationships of *Prunus* species from the Northeast region using ITS sequences, in comparison with those available in GenBank. Our research works align with earlier reports (19), which also placed *P. tomentosa* near the *Amygdalus-Prunus* clade. Our findings also revealed paraphyletic groupings within the subgenera *Padus* and *Laurocerasus*. This suggests that the taxonomic boundaries between these subgenera remain unclear, as species from both groups were found to be intermixed in the phylogenetic analysis. For better resolving the subgeneric relationships particularly between *Padus* and *Laurocerasus* expanded sampling and incorporation of chloroplast markers is recommended. However, additional data will be necessary in the future to clarify the phylogenetic relationships among *Prunus* species and to support a more accurate taxonomic classification of the genus. To our knowledge, this is the first report of nine ITS sequences from *Prunus* species in Northeast India submitted to GenBank.

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Authors' contributions

BT and JNM planned and designed the study. JNM performed all the experiments. Data analysis was done by JNM. JNM wrote the manuscript with input from BT. BT checked and improved the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The authors declare there is no conflict of interest.

Ethical issues: None

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