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*Original Research Paper*

# Molecular characterization of *Apis florea* (Hymenoptera: Apidae), An unsung pollinator of major horticultural crops

**Ramasamy P.[1], Krishnasamy S.[2*], Balakrishnan U.[3], Ramiah N.[1], Palanisamy A. A. [2], Ramasamy A.[4] and Karuppannasamy A.[4,5]**

[1]Department of Agricultural Entomology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai- 625104, Tamil Nadu, India
[2]ICAR - Krishi Vigyan Kendra, Tamil Nadu Agricultural University, Madurai - 625104, Tamil Nadu, India
[3]ICAR - Krishi Vigyan Kendra, Tamil Nadu Agricultural University, Aruppukottai - 626107, Tamil Nadu, India
[4]ICAR-Indian Institute of Horticultural Research, Bengaluru - 560 089, India
[5]Tata Institute for Genetics and Society, Bengaluru, Karnataka, India
Corresponding author Email: suresh.k@tnau.ac.in

## ABSTRACT

*Apis florea* (Hymenoptera: Apidae), also known as the red dwarf honeybee due to its reddish-brown abdomen and small size, is the smallest known bee species. These bees play a vital role in pollinating wild and cultivated plants in their habitat. This study examines *A. florea* populations from Tamil Nadu, Karnataka, Maharashtra, and Gujarat, shedding light on their genetic diversity and evolutionary relationships. Gel electrophoresis revealed a common band of around 800 base pairs in all samples, indicating a shared genetic fragment. This consistency suggests conserved genetic traits across different groups. Molecular analysis showed high sequence similarity with reference sequences from the NCBI database, with slight regional variations. A phylogenetic tree, backed by high bootstrap values, demonstrated strong genetic similarity among the isolates, distinguishing them from *A. mellifera.* Pairwise nucleotide difference ranged from 370 to 402, indicating moderate genetic diversity. The closest genetic relationship was between the Tamil Nadu and Gujarat isolates, while the greatest differences were between Bangalore and Maharashtra. This points to distinct genetic lineages shaped by geographic variation. PCA and MDS analyses confirmed the genetic diversity, with the Bangalore isolate showing the most divergence. The Haplotype Network and Minimum Spanning Tree analyses further highlighted the unique genetic characteristics of the Bangalore isolate. Overall, the study underscore highlights both genetic uniformity and diversity within *A. florea,* reflecting their evolutionary dynamics and adaptation to different regions. These findings are important for the conservation and management of these species.

**Keywords**: *Apis florea*, pollinator, phylogeny, evolution, genetic diversity

## INTRODUCTION

*Apis florea* (Hymenoptera: Apidae) is the smallest known bee species. Also referred to as the red dwarf honeybees due to its reddish-brown abdomen and diminutive size, these bees play a key role in pollinating both wild and cultivated plants in their habitats (Oldroyd, 2021). Bees are critical to ecosystem health and global food security (Potts, 2016). Among the many bee species, *A. florea*, often called the red dwarf bee, serves as an important pollinator across a variety of environments, from deserts to urban settings (Oldroyd & Wongsiri, 2006; Deodikar, 2019). Unlike traditional bees that live in human-made hives, *A. florea* constructs nests in crevices and hollow twigs (Ali et al., 2023). The decline of bees and other pollinators is a growing concern, given their vital role in agriculture and ecosystem stability (Potts, 2016; Rader et al*.,* 2016). The importance of pollinators has gained global attention, stressing the need to protect this crucial ecological service (Biesmeijer et al., 2006). Bees, being highly sensitive to extinction, are often seen as indicators of environmental stress (Kevan et al., 1997; Kevan, 1999). One major challenge in studying bees and other insects is the lack of comprehensive taxonomic knowledge (Weeks et al., 1999). Correct identification of species or taxonomic units (morphospecies) is crucial for ecological research (Gotelli, 2004), but for bees, this knowledge is often incomplete. Genetic approaches like DNA barcoding,

particularly using the cytochrome *c* oxidase I (*cox1* or COI) gene, have proven useful in identifying difficult taxonomic groups (Hebert et al., 2003; Köhler, 2007) by recognizing molecular operational taxonomic units (Smith et al., 2005). These methods improve the accuracy of bee species identification, benefiting conservation and ecological studies. The genetic variation in the mitochondrial DNA of *A. florea* is lower compared to other honeybee species like *A. cerana indica* and *A. dorsata* (Rattanawannee et al., 2007; Takahashi et al., 2017). This study aims to analyse the nucleotide genetic diversity of *A. florea* populations from various Indian states by sequencing the mitochondrial CO1 gene. This molecular characterization will provide insights into the genetic diversity and evolutionary adaptations of *A. florea*, aiding in its conservation.

## MATERIALS AND METHODS

### Sample collection

The samples of *A. florea* were collected from various parts of India, such as Tamil Nadu (Agricultural college and Research Institute, Madurai, 9.9699° N, 78.2040° E), Karnataka (Gandhi Krishi Vigyan Kendra, 13.0767° N, 77.5776° E), Maharashtra (Pimpalgaon tarf, Khed Pune division 18°74'N, 73°94'E) and Gujarat (Navsari Agricultural University 20.9248° N, 72.9079° E). These samples are kept in 70% ethanol to guarantee the preservation of DNA for upcoming analysis. The samples were collected from across habitats like trees, bushes, fences, mainly during May to July. The goal of this large-scale survey was to offer a thorough grasp of the genetic diversity of *A. florea* in various habitats.

### DNA Extraction, PCR amplification and agarose gel electrophoresis

To extract genomic DNA, five adult worker bees of A. *florea* were crushed using a tiny pestle within an Eppendorf tube. A simple modified CTAB (mCTAB) protocol was employed for extracting the insect DNA. The yield and quality of extracted DNA were estimated using the Nanodrop™ 8000 Spectrophotometer. Samples were further analyzed by gel electrophoresis on a 1.2% agarose gel in 1× Tris/Acetic Acid/ Ethylenediaminetetraacetic Acid (TAE) Buffer (Bio-Rad, USA) and visualized using Ethidium bromide. The polymerase chain reaction was performed using gene specific forward primer LCO (F): 5' GGTCAACAAATCATAAAGATATTGG 3' and reverse primer HCO (R): 5' TAAACTTCAGGGTGA CCAAAAAATCA 3' targeting the mitochondrial gene. The PCR reaction mixture consisted of 5 µL of master mix, 1µL of each forward and reverse primer, 2 µL of template DNA and 1 µL of sterile water. The PCR protocol included an initial denaturation step at 94 °C for 4 minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 52 °C for 45 seconds, extension at 72 °C for 1 minute, and a final extension step at 72 °C for 10 minutes (Lynn et al., 2020). The PCR products were purified and sequenced at Biokart Pvt. Ltd. in Bangalore, Karnataka, India. Agarose gel (0.8% agarose gel was prepared using 1xTAE buffer and ethidium bromide (4 µl per 100 ml of gel solution) for DNA visualization under UV light in order to confirm the size of the amplified DNA fragments. Following electrophoresis, the gel was examined under UV fluorescent illumination with a Gel Doc TM device (Bio-Rad) to detect the presence of the anticipated PCR product band, which is approximately 800 bp in length.

### Phylogenetic characteristics of *A. florea*

The Neighbour-Joining approach was used to infer the evolutionary history (Saitou & Nei M, 1987; Felsenstein, 1985). The tree was performed using 1000 bootstrap support. The evolutionary distances are expressed in base substitutions per site and were calculated using the Maximum Composite Likelihood approach (Tamura et al., 2004). There were fourteen nucleotide sequences in this investigation. For every sequence pair, all unclear places were eliminated (pairwise deletion option). The final dataset contained 894 locations in total. In MEGA11, evolutionary analyses were carried out (Tamura et al., 2021).

### Genetic diversity Analysis of *A. florea*

In this study, Genetic diversity, relationships and potential evolutionary history of the *A. florea* isolates were analysed by Phylogenetic Tree (Cladogram or Phylogram), Principal Component Analysis (PCA), Multidimensional Scaling (MDS), Network Analysis (Haplotype Network) and Minimum Spanning Tree (MST**).** For Phylogenetic tree (Cladogram or Phylogram) were constructed using MEGA 11 software to infer evolutionary relationships based on genetic sequence data. PCA, MDS were used to visualize the similarity or dissimilarity of data. For genetic data, it can show the genetic distance between

**Table 1 : Genetic variability between *A. florea* isolates from Various Regions in India**

| Name of the *A. florea* isolates | Accession number query sequence | Isolates Matches NCBI | | | | |
|---|---|---|---|---|---|---|
| | | Query cover | E value | Percent identify | Accession length | Query sequence matching NCBI Accession number |
| *Apis florea* AF (TN) - 1PQ109720 | | 100% | 0.0 | 99.84% | 652 | PP725722.1 |
| *Apis florea* AF (BLR) - 2PQ110386 | | 100% | 0.0 | 99.82% | 652 | PP725722.1 |
| *Apis florea* AF (MR) - 3PQ109783 | | 100% | 0.0 | 100.00 % | 658 | KU737494.1 |
| *Apis florea* AF (GR) - 4PQ109784 | | 100% | 0.0 | 100.00% | 658 | KU737494.1 |

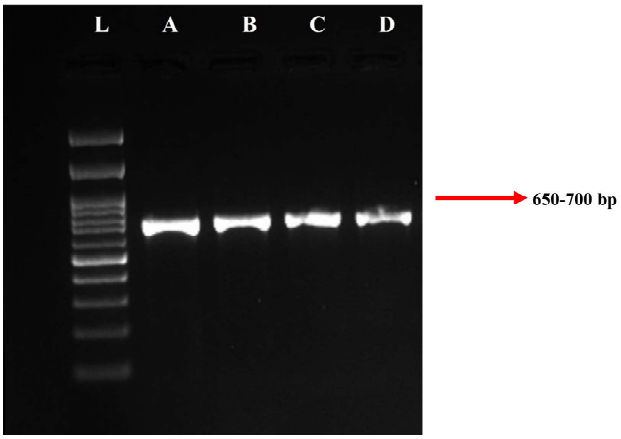
isolates, representing them in a space where the distance between points reflects genetic divergence. Haplotype Network for the four *A. florea* isolates sequences were analysed to identify unique haplotypes and the mutational steps between them. This was visualized in a network diagram where nodes represent haplotypes, and edges represent mutational differences. The MST connected all isolates with the minimum possible total edge weight, representing the shortest path to connect all nodes based on the genetic differences. Several analyses were performed in R software using packages suited for genetic data visualization to understand the genetic diversity and relationships among the four *A. florea* isolates.

## RESULTS AND DISCUSSION

### Molecular characterization of *A. florea*

To assess the genetic diversity of honey bee populations from different regions of India, samples from Tamil Nadu (PQ109720), Bangalore (PQ110386), Maharashtra (PQ109783), and Gujarat (PQ109784) were analysed. Electrophoresis results revealed a prominent DNA band between 650 and 700 base pairs (bp) in all samples (Fig.1), suggesting conserved genetic markers on traits among honeybees from these regions. This uniformity indicates that the honey bee populations may share evolutionary characteristics and genetic similarities. Similarly, Sheffield et al., (2009) observed genetic commonalities among *A. florea* populations in Egypt, Pakistan, and India. Molecular analysis of the samples from Tamil Nadu (TN), Bangalore (BLR), Maharashtra (MR), and Gujarat (GR) showed high sequence similarity with known reference sequences in the NCBI database (Table 1). Except for *A. florea* (MR), all samples had 100% query coverage and near 100% sequence identity with reference sequences. The slight difference in identity for *A. florea* (MR) (100%) compared to the other samples (99.82%-99.84%) suggests minimal genetic variation across the populations, indicating high genetic consistency in *A. florea* across these regions. Similar findings were reported by Ojha et al., (2016) when comparing populations from the Kaas Plateau, Western Ghats, with those from Iran and Pakistan using the cytochrome oxidase subunit I gene. The sequence length for *A. florea* (TN) and *A. florea* AF (BLR) was 652 bp, while for *A. florea* (MR) and *A. florea* (GR), it was 658 bp.

This variation in sequence length may be due to regional genetic differences or differences in the amplified genetic regions. Most of the samples matched with accession numbers PP725722.1 and KU737494.1 in the NCBI database, indicating that the genetic material of these bees aligns with previously documented *A. florea* sequences. The presence of these reference sequences in multiple regions supports the idea of a shared genetic lineage and relatively low genetic divergence across the sampled locations (Sheffield et al., 2009).




L – 100 bp Ladder, A – Tamil Nadu Af (TN -1),
B – Bangalore Af (BLR-2), C – Maharashtra Af (MR-3),
D – Gujarat Af (GR- 4) (Band at 650-700 bp).

Fig. 1 : Molecular characterization of *A. florea* isolates

**Table 2 : comparison of pairwise nucleotide differences among sequences PQ109720, PQ110386, PQ109783 and PQ109784**

| Sequence 1 | Sequence 2 | Pairwise nucleotide differences |
|---|---|---|
| PQ109720 | PQ110386 | 395 |
| PQ109720 | PQ109783 | 371 |
| PQ109720 | PQ109784 | 370 |
| PQ110386 | PQ109783 | 402 |
| PQ110386 | PQ109784 | 397 |
| PQ109783 | PQ109784 | 387 |

Note : The present study isolates are indicated with a red dot mark. Bootstrap values obtained from 1000 replicates are indicated at the nodes. *Apis mellifera* is used as outgroup.

## Phylogenetic relationship and genetic diversity of *A. florea*

The COX I gene has been widely used to identify various insect species, including honey bees (He et al., 2023; Sooraj et al., 2023; Babu et al., 2023). A phylogenetic tree generated using the Neighbour Joining method and the COX I gene showed that isolates PQ109720, PQ109784, PQ109783, and PQ110386 form a distinct and closely related cluster within the *A. florea* species. The grouping is supported by high bootstrap values (89-98), indicating strong genetic similarity among these isolates. The clear separation from the outgroup, *A. mellifera*, highlights the genetic uniqueness of *A. florea*, confirming their classification and evolutionary divergence (Fig. 2). Consistent results were obtained across different models used for analysis (Leelamanit et al., 2004). Phylogenetic analysis of the four *A. florea* isolates from Tamil Nadu (PQ109720), Bangalore (PQ110386), Maharashtra (PQ109783), and Gujarat (PQ109784) revealed moderate genetic diversity (Table 2). Pairwise nucleotide differences ranged from 370 to 402, indicating variability in genetic composition. The least genetic difference was observed between Tamil Nadu (PQ109720) and Gujarat (PQ109784) isolates (370), suggesting a close genetic relationship. Conversely, the largest genetic difference (402) was found between Bangalore (PQ110386) and Maharashtra (PQ109783), indicating greater genetic distance. Smith (1991) found that *A. florea* populations from India and Northern Thailand, and *A. andreniformis* from Malaysia and Borneo exhibited 5-7% sequence divergence. This study points to distinct genetic lineages within *A. florea*, potentially influenced by geographical diversity. The genetic variation observed, provides insights into the species' evolutionary dynamics and adaptations across regions.

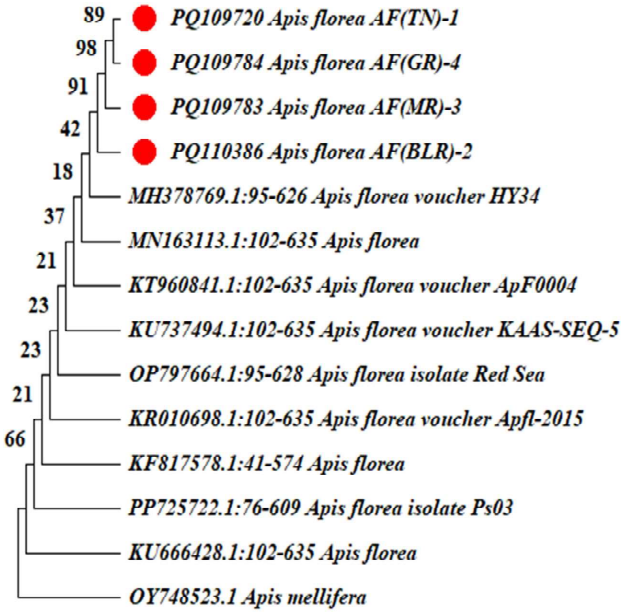



Fig. 2 : Phylogenetic tree generated based red sequences of COX 1 genes using the neighbour-joining method in Mega 11 software

Similarly, Alhissnawi et al., (2024) confirmed the identity of *A. florea* in the Eastern Region of Iraq based on nucleotide sequences. A heatmap showing pairwise nucleotide differences among the four *A. florea* isolates (PQ109720, PQ110386, PQ109783, and PQ109784) indicated moderate genetic diversity, with differences ranging from 370 to 402 nucleotides. The variation suggests that while the isolates are genetically similar, geographical or environmental factors may contribute to distinct differences. The heatmap uses a gradient from lighter to darker shades to visually represent the degree of divergence, with darker shades indicating more significant differences. This analysis sheds light on the genetic relationships and diversity among the *A. florea* isolates (Fig. 3).

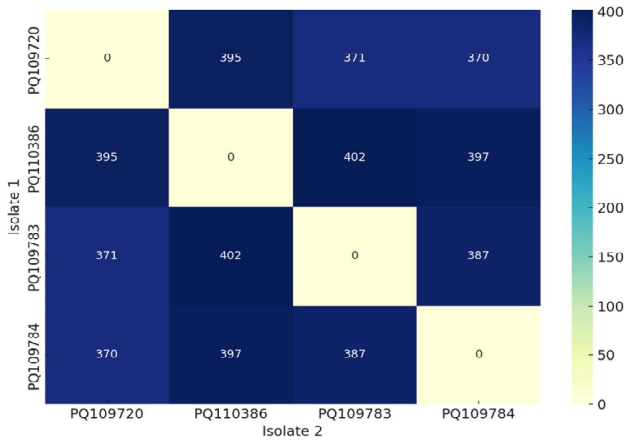



Fig. 3 : Pairwise nucleotide differences among *A. florea* isolates

## Principal Component Analysis (PCA) and Multidimensional Scaling (MDS) of *A. florea* isolates

The PCA and MDS analyses revealed varying degrees of genetic diversity among the four *A. florea* isolates (Table 3). In the PCA, the isolates were positioned differently across the first two principal components (PC1 and PC2), which captured most of the variance in the data. PQ109720 (Tamil Nadu) had coordinates (-8.95, -5.72), PQ110386 (Bangalore) was at (-1.22, 7.24), PQ109783 (Maharashtra) at (6.53, -1.15), and PQ109784 (Gujarat) at (3.64, -5.37). Similarly, the MDS analysis, which mapped genetic distances in a two-dimensional space, placed PQ109720 at (4.05, -2.21), PQ110386 at (-3.00, 2.69), PQ109783 at (-0.52, -6.92), and PQ109784 at (-1.56, 6.44). Among the isolates, PQ110386 (Bangalore) showed the greatest genetic diversity, as evidenced by its significant deviation from the centroid in both PCA and MDS plots. This distinct position highlights the unique genetic characteristics of the Bangalore isolate (Fig. 4).

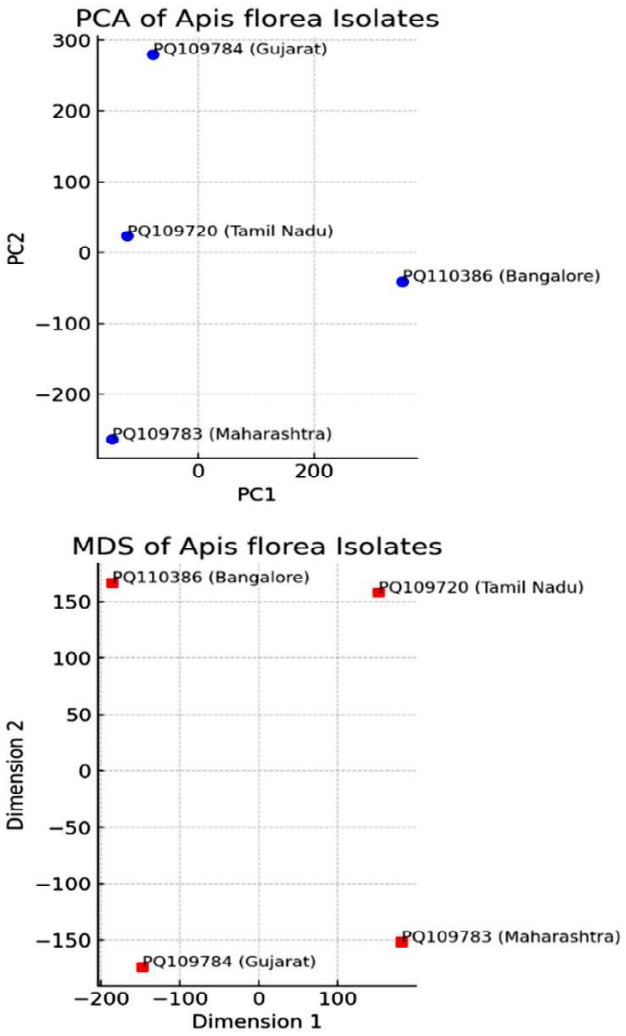



Fig. 4 : Principal Component Analysis (PCA) and Multidimensional Scaling (MDS) for *A. florea isolates*

## Haplotype Network of *A. florea* isolates

The Haplotype Network and previous analyses confirmed that PQ110386 (Bangalore) is the most genetically diverged isolate among the four *A. florea* isolates. Pairwise nucleotide differences between PQ110386 and the other isolates were: PQ109720 (Tamil Nadu) - 395 differences, PQ109783

**Table 3 : PCA and MDS of *A. florea isolates***

| Isolate | Location | PCA | | MDS | |
|---|---|---|---|---|---|
| | | PC 1 | PC 2 | Dimension 1 | Dimension 2 |
| PQ109720 isolates | Tamil Nadu | -8.95 | -5.72 | 4.05 | -2.21 |
| PQ110386 isolates | Bangalore | -1.22 | 7.24 | -3.00 | 2.69 |
| PQ109783 isolates | Maharashtra | 6.53 | -1.15 | -0.52 | -6.92 |
| PQ109784 isolates | Gujarat | 3.64 | -5.37 | -1.56 | 6.44 |

(Maharashtra) - 402 differences, and PQ109784 (Gujarat) - 397 differences. These values show that PQ110386 has the highest number of mutational steps compared to the others, emphasizing its significant genetic diversity. The Haplotype Network structure and mutational differences underline PQ110386's distinct genetic profile (Fig. 5). Similarly, Ali et al., (2023) found that the population of *A. florea* in Egypt was most closely related to the Indian population.

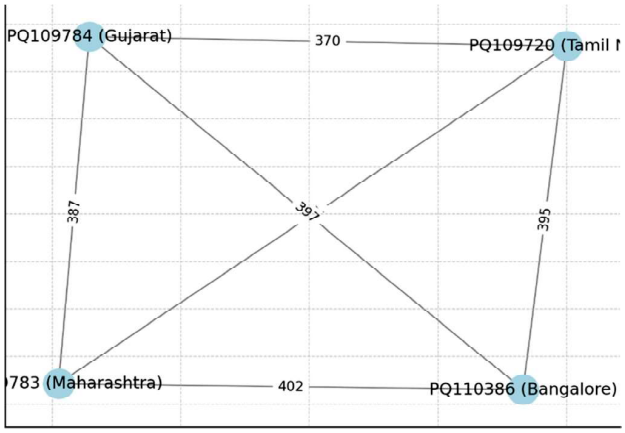



Fig. 5 : Haplotype Network of *A. florea* isolates

**Minimum Spanning Tree (MST) of *A. florea* isolates**

The Minimum Spanning Tree (MST) analysis mapped the shortest path connecting the four *A. florea* isolates based on genetic distances, minimizing mutational steps. The MST revealed the following connections: PQ110386 (Bangalore) to PQ109783 (Maharashtra) - 402 differences, PQ110386 (Bangalore) to PQ109784 (Gujarat) - 397 differences, and PQ109784 (Gujarat) to PQ109720 (Tamil Nadu) - 370 differences. The central position of PQ110386 (Bangalore) in the MST highlights its significant genetic divergence and unique role in connecting the other isolates (Fig. 6). Similar studies by Terzi et al., (2014) showed genetic variation within *A. florea* populations, with greater similarity within states than between states.

## CONCLUSION

The present study provides a deeper understanding of the genetic diversity and intraspecific variability of *A. florea*. The findings are crucial for developing effective conservation strategies and improving the *A. florea* populations for crop and ecological benefits. Future research should explore the genetic mechanisms driving these adaptations and their implications for the species' resilience to environmental challenges.

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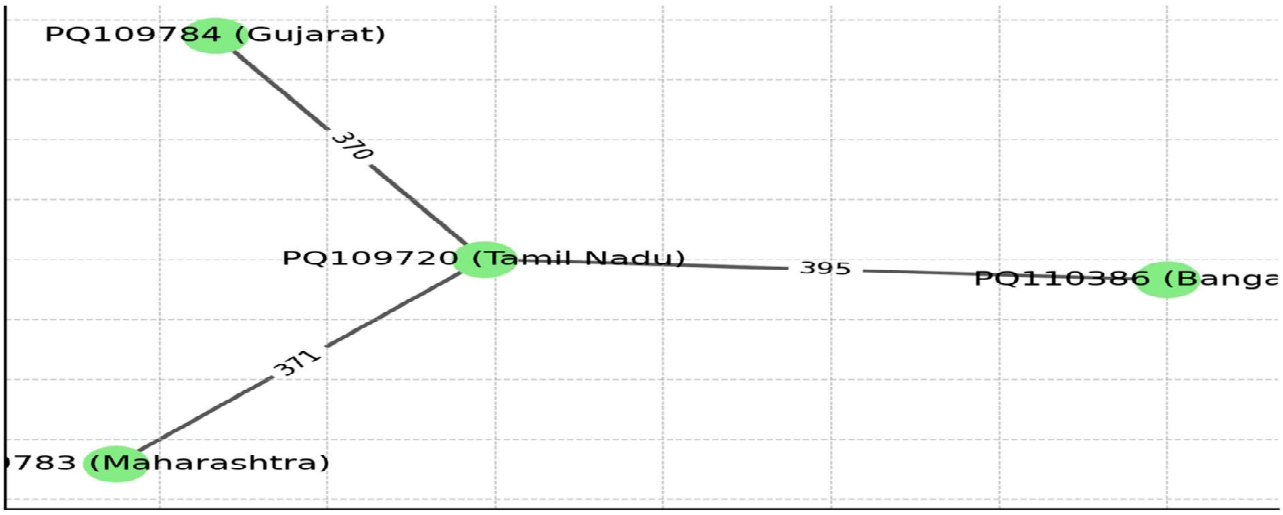


Fig. 6 : Minimum spanning Tree (MST) of *A. florea* isolates

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