Systematic position of two endemic *Dipcadi* spp. (Asparagaceae) from northern Western Ghats of India using molecular markers

Anup S. Deshpande, S. Krishnan and M.K. Janarthanam*
Department of Botany, Goa University, Goa 403 206, India
E-mail: jana@unigoa.ac.in

Abstract
Phylogenetic relationships of two endemic and critically endangered species viz. *Dipcadi concanense* (Dalz.) Baker and *D. goaense* Prabhug. *et al.*, from northern Western Ghats, were investigated using sequences from an Internal Transcribed Spacer (ITS) of nuclear ribosomal region and a plastid gene (*matK*). Maximum Likelihood (ML) and Bayesian analysis were carried out for that purpose. The phylogenetic tree resulting from ML as well as Bayesian analysis showed that *D. concanense* and *D. goaense* are closely related species. This analysis was significantly supported by bootstrap as well as posterior probability values. The resolution of *Dipcadi* clade is also explained by cytological data as well as distribution patterns.

Keywords: *Dipcadi*, endemic, ITS, *matK*, Phylogenetic analysis

Introduction
Genus *Dipcadi* Medik., belonging to the family Asparagaceae (earlier Hyacinthaceae subfamily Ornithogaloideae), is distributed from Africa, Mediterranean region to South West Asia (Mabberly, 1997) and comprises 41 species (The Plant List, 2013). In India, it is distributed from Western Himalayas to Peninsular India and is represented by ten species with four varieties. Two of the species, viz. *D. concanense* (Dalz.) Baker and *D. goaense* Prabhug. *et al.* are endemic to Western Ghats and are morphologically distinct with their pure white flowers. The former is restricted to Maharashtra state (Deb & Dasgupta, 1981; Mishra & Singh, 2001) and was considered “possibly extinct in the wild” by Dasgupta & Deb (1987). The latter species was recently described from Goa State and is only known from the type locality (Prabhugaonkar *et al.*, 2009) Fig. 1, 2

Phylogenetic studies of family Hyacinthaceae subfamily Ornithogaloideae were carried out comprehensively using molecular data (Flosser & Speta, 1999; Manning *et al.*, 2004, 2009; Martínez-Azorín *et al.*, 2011). The latest study in this subfamily by Martínez-Azorín *et al.*, (2011) accepts a total of nineteen monophyletic genera, including *Dipcadi*, based on a nuclear (ITS) and four plastid (*trnL* intron, *trnL*-F spacer, *rbcL* and *matK*) regions.

*D. goaense* is morphologically similar to *D. concanense* (Prabhug. *et al.*, 2009) and share the same chromosome number (Gosavi *et al.*, 2011) but both are allopatrically distributed. Considering their morphological distinctness, distribution and conservation concern, their systematic position and phylogenetic relationship have been investigated using a plastid (*matK*) and ITS sequences.

Materials and Methods

Plant Materials
The leaf samples of *D. concanense* and *D. goaense* were collected from the States of Maharashtra and Goa respectively and the collection details are provided in Table 1. Fresh leaf samples were placed in silica gel for drying and completely dried leaves were used for DNA extraction. The voucher specimens are deposited in Herbarium at the Department of Botany, Goa University, Goa, India.

DNA extraction and PCR amplification
The DNA extraction was carried out using GenElute Plant Genomic DNA Miniprep Kit (Sigma, manufacturer’s protocol) using 200 µg of silica dried leaf samples. PCR amplification was performed using forward and reverse primers
### Table 1: Collection details of Dipcadi species and Voucher numbers of DNA sequences submitted to NCBI.

<table>
<thead>
<tr>
<th>Plant Name</th>
<th>Location</th>
<th>Geographical coordinates</th>
<th>Voucher number</th>
<th>matK accession no.</th>
<th>ITS accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. concanense</em></td>
<td>Kurdhe, Ratnagiri, Maharashtra</td>
<td>N 16.84723° E 79.32078°</td>
<td>GUH 5037</td>
<td>KP645194</td>
<td>KF871334</td>
</tr>
<tr>
<td><em>D. goaense</em></td>
<td>Rivona, Kevan, Goa</td>
<td>N 15.14282° E 74.13435°</td>
<td>GUH 5166</td>
<td>KP645195</td>
<td>KF871335</td>
</tr>
</tbody>
</table>
Table 2: Primers used for sequencing of Dipcadi species.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer Name</th>
<th>Direction</th>
<th>Sequence (5’→3’)</th>
<th>Reference/Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>matK</td>
<td>matK-390F</td>
<td>Forward</td>
<td>CGATCTATTCAATTCAATATTC</td>
<td>CBOL Plant Working Group (<a href="http://www.barcoding.si.edu/plant_working_group.html">http://www.barcoding.si.edu/plant_working_group.html</a>)</td>
</tr>
<tr>
<td>matK</td>
<td>matK-1326R</td>
<td>Reverse</td>
<td>TCTAGCACACGAAAGTGAAGTGAATC</td>
<td></td>
</tr>
<tr>
<td>ITS</td>
<td>ITS-F</td>
<td>Forward</td>
<td>AATGGTCCGGAAGTGTGTC</td>
<td>New primers designed</td>
</tr>
<tr>
<td>ITS</td>
<td>ITS-R</td>
<td>Reverse</td>
<td>CTCGCCGTTACTAGGGGAAT</td>
<td></td>
</tr>
</tbody>
</table>

Phylogenetic analysis

Highly similar gene sequences of matK and ITS regions of DNA were obtained from GenBank (NCBI, MEGABLAST). Default parameters of ClustalX 2.012 software were used for alignment. Phylogenetic trees of ITS, matK were constructed using the combined sequence data (ITS + matK). The Maximum Likelihood (ML) method using MEGA5.2 (Tamura et al., 2011) and Bayesian analysis using MrBayes v3.1.2 software (Ronquist & Huelsenbeck, 2003) were employed for the construction of phylogenetic trees. Best-fit-model was determined using ML calculations in MEGA5.2. The Kimura 2-parameter model (Kimura, 1980) was selected with 5 gamma distribution categories (K2+G) for combined sequence data. For the analysis of matK and ITS regions independently, Kimura 2-parameter (Kimura, 1980) with 5 gamma distribution categories (K2+G) and Kimura 2-parameter (K2) (Kimura, 1980) respectively were selected. Bootstrap replicates were set to 1000 for all the methods.

The General Time Reversible model (GTR+G+I) was chosen for combined data for Bayesian analysis with invariable proportion of the sites with gamma distribution (GTR+I+G) after analysing the best-fit-model in JModelTest 2.1.3 software (Darriba et al., 2012). The Markov Chain Monte Carlo (mcmc) chains were run for 2 × 10^6 iterations and sampled every 100 iteration. First 25% of trees were discarded (‘burn-in’). Posterior probability distribution was compiled and consensus tree was constructed using remaining trees after ‘burn-in’. Consensus tree was extracted and edited using Mesquite v2.75 (Maddison & Maddison, 2011). Asparagus officinalis was chosen as an out-group taxon for all analyses.

Results and Discussion

Sequence characteristics

Phylogenetic analyses of ITS, matK and cumulative DNA sequences were performed by Maximum Likelihood method and Bayesian analysis. The tree construction using ITS sequence data was performed using 750 aligned base pairs and that of matK using 751 aligned base pairs. Analysis of combined data of D. concanense and D. goaense comprises 1557 aligned base pairs.

Phylogenetic relationship

ITS tree: Tree constructed using only ITS sequences included 62 species belonging to 4 genera. Three major clades were obtained in ML tree. First clade

Table 3: PCR Amplification Profile

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp.(°C) matK</th>
<th>Time (min.) matK</th>
<th>Cycles matK</th>
<th>Temp.(°C) ITS</th>
<th>Time (min.) ITS</th>
<th>Cycles ITS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denature</td>
<td>95</td>
<td>5.00</td>
<td>1</td>
<td>95</td>
<td>5.00</td>
<td>1</td>
</tr>
<tr>
<td>Denature</td>
<td>95</td>
<td>0.30</td>
<td>40</td>
<td>95</td>
<td>0.30</td>
<td>40</td>
</tr>
<tr>
<td>Annealing</td>
<td>48</td>
<td>0.40</td>
<td>40</td>
<td>58</td>
<td>0.40</td>
<td>40</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>1.00</td>
<td>40</td>
<td>72</td>
<td>0.40</td>
<td>40</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72</td>
<td>5.00</td>
<td>1</td>
<td>72</td>
<td>7.00</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>∞</td>
<td>∞</td>
<td>4</td>
<td>∞</td>
<td>∞</td>
</tr>
</tbody>
</table>
comprised of only *Ornithogalum fimbrimarginatum*. Second clade includes species of *Albuca* and *Dipcadi*. The third clade includes species of *Ornithogalum*. The species belonging to *Battandiera*, *Cathissa*, *Coi lonox*, *Elsiea*, *Galtonia*, *Honorius*, *Loncomelos*, *Melomphis*, *Neopatersonia* and *Stellarioides* were clustered either in *Ornithogalum* or *Albuca*. Also, two species belonging to *Ornithogalum* were synonymised under genus *Albuca* (Manning et al., 2009). Bayesian tree shows similar topology to that of ML tree at the terminal nodes except at some nodes. It also shows polytomy at some nodes.

*Dipcadi* clade is similar in both analyses.

matK tree: 47 species belonging to 4 genera were included for the analysis. Two major clades were obtained in ML tree. First clade includes some species of *Dipcadi*, while other clade includes *Pseudogaltonia*, *Ornithogalum*, *Albuca* and remaining species of *Dipcadi* that are mainly of Asian distribution. Other species are merged either in *Ornithogalum*, *Albuca* or *Drimia* (Manning et al., 2009). Bayesian tree showed different topology to the ML tree showing polytomy at almost all nodes.
Tree constructed based on only matK sequence is not adequate for determining exact positions of species.

Combined tree: The phylogenetic tree obtained from combined data included 24 species belonging to 3 genera. Three distinct clades were formed in combined ML tree. The first clade is represented by Albuca (incl. Coilonox and Ornithogalum longibracteatum). The second clade is represented by genus Dipcadi and the third clade includes Ornithogalum (incl. species of Galtonia and Neopatersonia).

The Dipcadi clade is strongly supported with the high posterior probabilities (1.0) as well as high bootstrap values (96%). The genus Dipcadi is morphologically distinct from other genera in having tubular flowers, quadrate, apically retuse capsules and large discoid seeds (Manning et al., 2009). One sub-clade out of two includes the Indian Dipcadi species, while other sub-clade includes African and Mediterranean species (Fig. 3). Positions of the Indian Dipcadi species in the tree can be explained by morphology and distribution. White coloured perianth and Western Ghats, endemic nature bring D. concanense and D. goaense closer, thus forming strongly supported clade (pp=1.0, bs%=100). Perianth is green to yellow turning brownish-pink during maturity in D. serotinum (Ghazanfar, 1996). This is closest to the Indian species in the tree which is also supported by its distribution from Northern India, Nepal, Pakistan to South Africa.

Chromosome number of D. concanense is reported as 2n=12 (Darlington & Janaki Ammal, 1945; Dixit et al., 1992), which is similar to D. goaense (Gosavi et al., 2011). Variations in chromosome number of D. serotinum [2n=8 (Corsi et al., 1996; Goldblatt & Manning, 2011); 2n=32 (Humphries et al., 1976; Goldblatt & Manning, 2011)] also indicated its adaptability to wide geographical areas.

Bayesian tree of combined analysis showed three main clades similar to ML tree. Dipcadi clade is highly supported by posterior probabilities values (1.00).

Conclusion

The present study revealed that D. concanense and D. goaense are morphologically as well as phylogenetically closely related species, supported by maximum likelihood as well as Bayesian analysis.

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Literature Cited


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