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

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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 a Internal Transcribed Spacer (ITS) of nuclear ribosomal region and a plastid gene (*mat*K). 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, *mat*K, 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 (Pfosser & 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 (*trn*L intron, *trn*L-F spacer, *rbc*L and *mat*K) 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 (*mat*K) 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

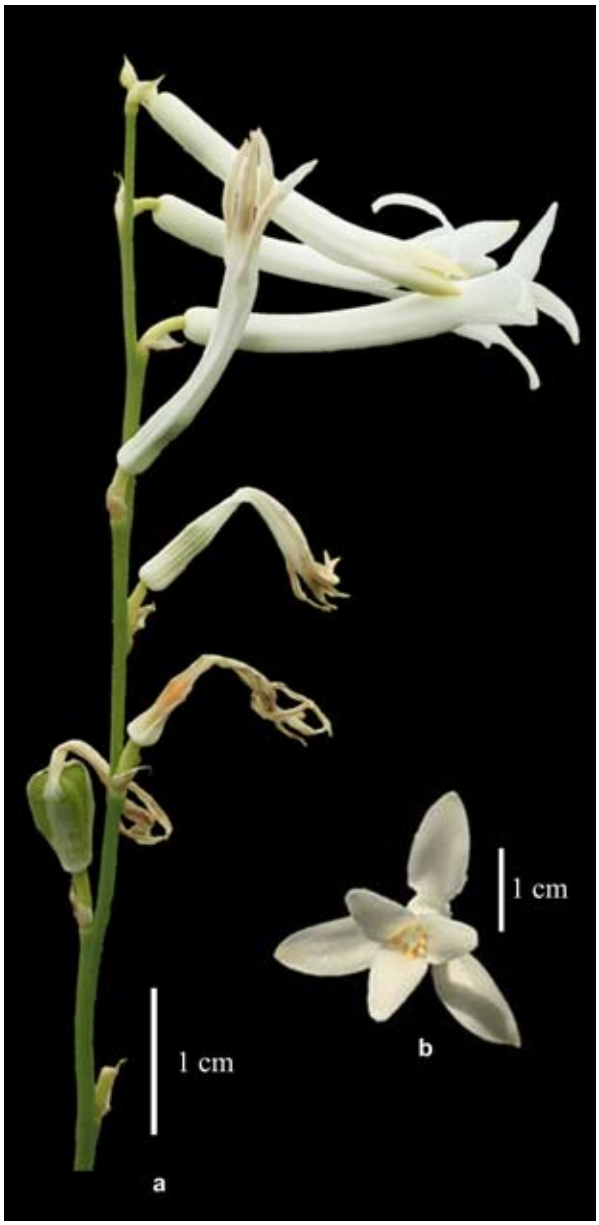

Fig. 1. *Dipcadi concanense* (Dalz.) Baker: **a.** Inflorescence; **b.** Single flower.

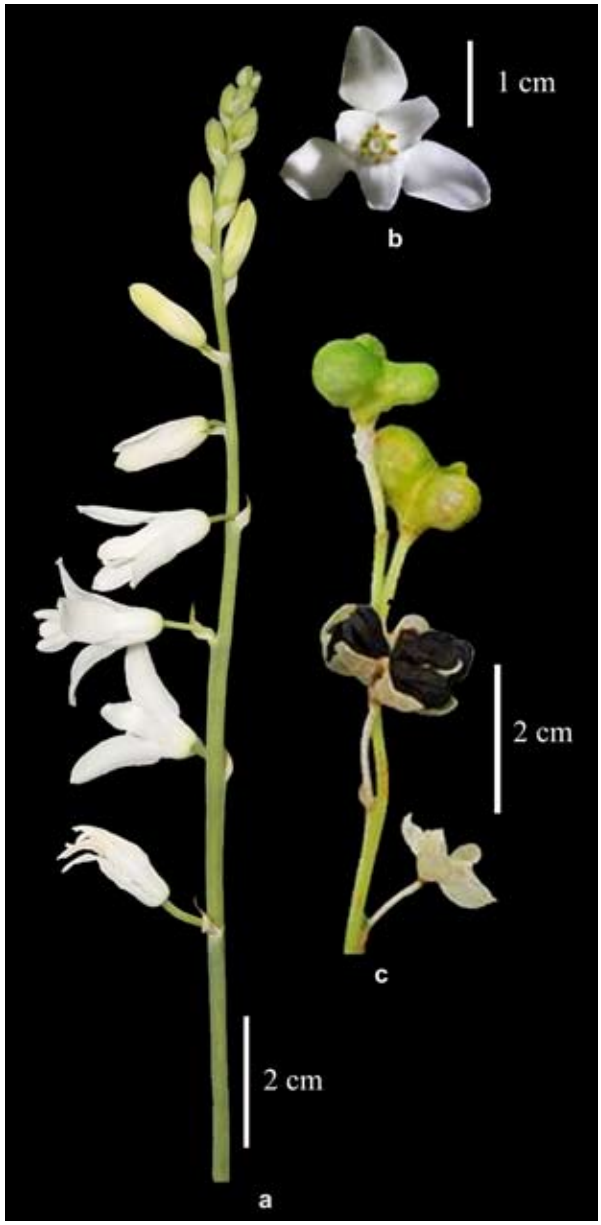

Fig. 2. *Dipcadi goaense* Prabhug. *et al.*: **a.** Inflorescence; **b.** Single flower; **c.** Infructescence.

(**Table 2, 3**). Sequencing of PCR products was carried out in Gene Amp PCR system 9700. All these were carried out from Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala, India. Obtained DNA sequences were submitted to NCBI (**Table 1**).

Table 1: Collection details of *Dipcadi* species and Voucher numbers of DNA sequences submitted to NCBI.

Plant Name	Location	Geographical coordinates	Voucher number	*mat*K accession no.	ITS accession no.
D. concanense	Kurdhe, Ratnagiri, Maharashtra	N 16.84723° E 79.32078°	GUH 5037	KP645194	KF871334
D. goaense	Rivona, Kevan, Goa	N 15.14282° E 74.13435°	GUH 5166	KP645195	KF871335

Table 2: Primers used for sequencing of *Dipcadi* species.

Target gene	Primer Name	Direction	Sequence (5′➔3′)	Reference/Remarks
matK	*matK*-390F	Forward	CGATCTATTCATTCAATATTTC	CBOL Plant Working Group (http://www.barcoding.si.edu /plant_working_group.html)
	matK-1326R	Reverse	TCTAGCACACGAAAGTCGAAGT	
ITS	*ITS*-F	Forward	AATGGTCCGGTGAAGTGTTC	New primers designed
	ITS-R	Reverse	CTCGCCGTTACTAGGGGAAT	

Phylogenetic analysis

Highly similar gene sequences of *mat*K 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, *mat*K were constructed using the combined sequence data (ITS + *mat*K). 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 *mat*K 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, *mat*K 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 *mat*K 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

Step	Temp.(°C)		Time (min.)		Cycles	
	*mat*K	ITS	*mat*K	ITS	*mat*K	ITS
Initial Denature	95	95	5.00	5.00	1	1
Denature	95	95	0.30	0.30	40	40
Annealing	48	58	0.40	0.40	40	40
Extension	72	72	1.00	0.40	40	40
Final Extension	72	72	5.00	7.00	1	1
Hold	4	4	∞	∞	-	-

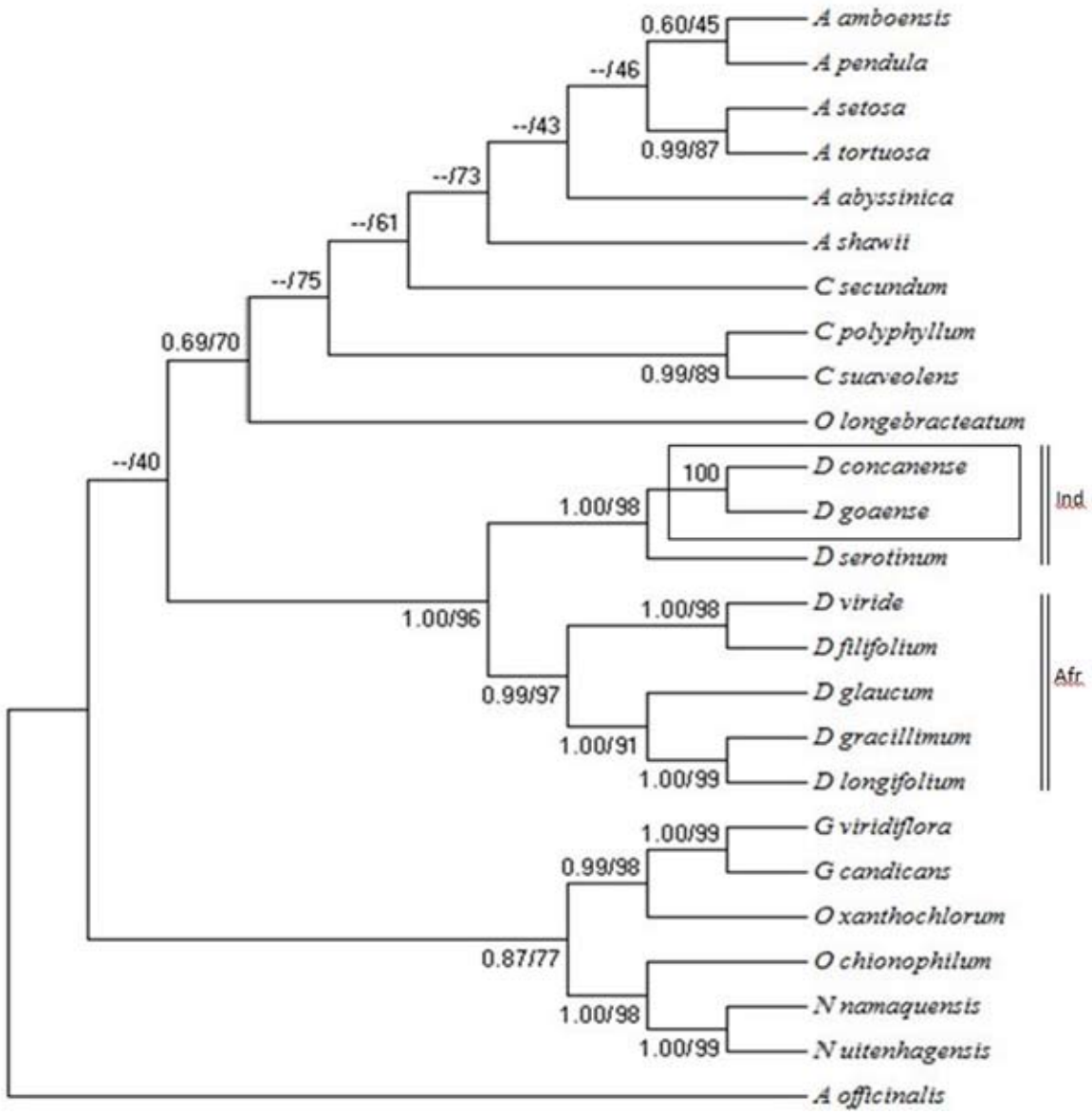

Fig. 3. The ML tree obtained from the phylogenetic analysis based on combined data of ITS and *mat* KDNA sequences. The posterior probability and bootstrap percentage have shown below or obove the branches (pp/bs%). [Abbreviations: Ind, clade of Indian *Dipcadi* species; Afr, clade of African and Mediterranean *Dipcadi* species. Absence of posterior probabilities indicates the different topologies in Bayesian analysis].

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, Coilonox, 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.

*mat*K 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 *mat*K 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 2*n*=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* [2*n*=8 (Corsi *et al.*, 1996; Goldblatt & Manning, 2011); 2*n*=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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