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SYSTEMATICS AND MOLECULAR PHYLOGENETIC ANALYSIS OF ERECT SPECIES OF *CEROPEGIA* SECTION *BUPRESTIS* (APOCYNACEAE: ASCLEPIADOIDEAE), WITH TWO NEW SPECIES FROM INDIA

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ABSTRACT

Erect species of *Ceropegia* section *Buprestis* are revised based on morphology, palynology and molecular study, and a key to all species presented. Two new species, *C. karulensis* and *C. maharashtrensis* are described. The status of the systematically ambiguous *C. lawii is* addressed. In the molecular analysis of erect species using ISSR markers, taxa belonging to section *Buprestis* were found to be distinct from species of section *Indopegia* used as outgroup. The phylogenetic analysis of 20 Indian species of *Ceropegia* representing different sections and their congeners using nuclear ribosomal internal transcribed spacer (ITS) and non-coding chloroplast DNA (cpDNA) sequences shows that the erect species of section *Buprestis* form a separate clade along with section *Indopegia*. This supports Huber's hypothesis (1957) that the section *Buprestis* must have evolved from section *Indopegia*.

Keywords : *Ceropegia* section *Buprestis*, *Ceropegia karulensis*, *Ceropegia maharashtrensis*, India, molecular phylogeny, systematics.

INTRODUCTION

Ceropegia L. of the tribe Ceropegieae Decne. ex Orb. and subtribe Stapeliinae G. Don (Meve & Liede, 2004) comprises c. 200 species (Bruyns, 2003; Surveswaran & al., 2009) distributed in Arabia, warm Africa including Canary Islands to Australia. Ansari (1984) had recognized 44 species in his treatise on Indian *Ceropegia* and later Jagtap & Singh (1999) reported 45 species and 3 varieties from India. Over the years many new taxa from Andaman Islands, Eastern Ghats and Western Ghats were added (Swarupanandan & Mangaly, 1992; Sreekumar & al., 1997; Daniel & Umamaheshwari, 2001; Yadav & al., 2004, 2008; Malpure & al., 2006; Punekar & al., 2006; Yadav & Shendage, 2010; Diwakar & Singh, 2011; Kambale & al., 2012; Kullayiswamy & al., 2012 and Rahangdale & Rahangdale, 2012). Recently Karthikeyan & al., (2009) enumerated 55 species, 2 subspecies and 3 varieties from India. However, as of now, there are 67 taxa (60 species, 2 subspecies and 5 varieties) out of which 44 taxa are endemic to India. Huber (1957) in his treatise on *Ceropegia* classified the genus into 21 sections. Huber's classification is highly artificial as it was solely based on floral morphology, with recent molecular studies confirming this (Meve & Liede, 2007; Surveswaran & al., 2009). It is important to point out artificial nature of Huber's classification. He proposed 19 new sections including *Buprestis* and 22 new series. Under section *Buprestis*, Huber treated 8 taxa (6 species, 1 subspecies and 1 variety) out of which 7 are from western and southern India namely *C. ciliata* Wight, *C. ciliata* subsp. *ensifolia* (Bedd.) H. Huber (presently known as *C. ensifolia* Bedd.), *C. evansii* McCann, *C. evansii* var. *media* H. Huber (presently known as *C. media* (H. Huber) Ansari), *C. lawii* Hook.f. (including *C. panchganiensis* Blatt. & McCann), *C. odorata* Nimmo *ex* Hook.f., *C. omissa* Huber and 1 from the Yunnan province of China namely *C. muliensis* W. W. Sm. Subsequently various workers (Ansari, 1969, 1982b; Hemadri, 1969; Ansari & Kulkarni, 1971; Wadhwa & Ansari, 1969) added many new species to this section namely *C. huberi* Ansari, *C. maccannii* Ansari, *C. rollae* Hemadri, *C. sahyadrica* Ansari & B. G. Kulk. and *C. santapaui* Wadhwa & Ansari. All the species (except *C. lawii)* treated by Huber under this section are twiners, while *C. lawii* is the only erect species. At present, this section comprises 9 twining species and 7 erect species. Section *Buprestis* is characterized in having tuberous

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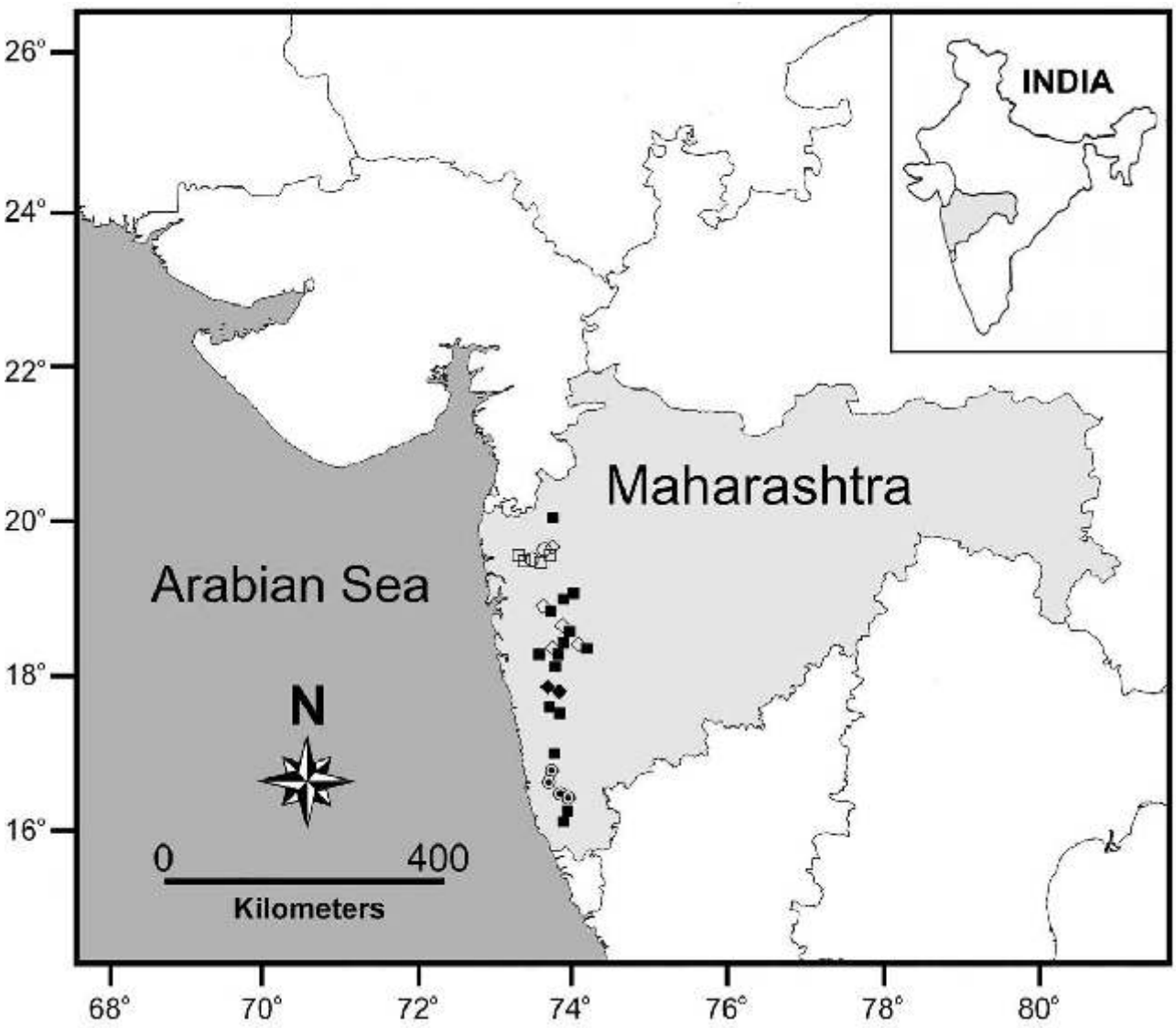

Fig. 1 : Distribution of erect species from *Ceropegia* section *Buprestis* in Maharashtra.

Symbols: ◉ *Ceropegia karulensis;* ◇ *Ceropegia macannii;* ○ *Ceropegia maharashtrensis;* ◆ *Ceropegia panchganiensis;* □ *Ceropegia rollae;* ■ *Ceropegia sahyadrica.*

rootstock; tubers globose or subglobose; stem twining or erect; leaves hairy or glabrous, not fleshy, petiolate; inflorescence generally pedunculate, subumbellate, many-flowered; peduncles patently hirsute, rarely glabrous or pilose in one row; corolla generally glabrous, sometimes pilose inside; tube slightly dilated at base; corolla lobes generally connate at apex; outer corona lobes bifid or entire at apex, sometimes extremely short; gynostegium long or extremely short, pilose or glabrous; inner corona lobes distinctly longer than outer, tip usually erect or sometimes diverted.

Apart from *C. muliensis,* all the species in the section *Buprestis* are endemic to Peninsular India, particularly to the Western Ghats, which is one of the possible loci of origin of *Ceropegia* (Punekar & al., 2006). The erect species of this section namely *C. karulensis*, *C. lawii*, *C. maccannii*, *C. maharashtrensis*, *C. panchganiensis*, *C. rollae* and *C. sahyadrica* are restricted to the high altitudes of north Western Ghats of Maharashtra suggesting that the stock of erect species is probably monophyletic in origin. (Fig. 1)

In the present paper, Indian erect species of *Ceropegia* under section *Buprestis* were studied using morphological, palynological and molecular aspects. Further to these studies, two species were found to be quite distinct from other known species and have been described as new species namely *C. karulensis* and

Fig. 2 : *Ceropegia lawii*. **A**. Holotype deposited at Kew herbarium; **B.** Enlarged view of illustration of corona drawn on the holotype, note the outer cupular corona and inner linear corona; **C.** Photograph of specimen collected from South Concan by Law "25 *Ceropegia*" deposited at Kew herbarium. **© The Board of Trustees of the Royal Botanic Gardens, Kew.** "Reproduced with kind permission of the Director and the Board of Trustees, Royal Botanic Gardens, Kew".

C. maharashtrensis from north Western Ghats based on comparison with herbarium collections at AHMA, BLAT, BM, BSI, CAL, G, K, L and our own field observations. A key to all the species under *Ceropegia* section *Buprestis* is presented. Further, using DNA analysis, phylogenetic relationship among the Indian erect species of *Buprestis* has been addressed.

MATERIALS & METHODS

Morphological & ecological examination : The species were described primarily based on morphological characters, followed by relevant data on flowering and fruiting. The ecological details such as frequency, habitat, microhabitat and associated species were also recorded. The details on specimens studied or collected are provided under materials examined. Special observations and nomenclatural information if any are given under notes. A key to the identification of different species is given to facilitate their identity.

Voucher specimens are deposited at Central National Herbarium, Howrah (CAL) and Agharkar Herbarium of Maharashtra Association, Pune (AHMA). Voucher material for pollen studies is deposited at the repository of Paleobiology Group, Agharkar Research Institute, Pune (MACSG).

Palynological studies : Pollinarium for scanning electron microscopy (SEM) study were collected from fresh flowers and processed using the method described by Juniper & al. (1970) with slight modifications.

Fig. 3 : *Ceropegia lawii* specimen collected from Bombay by Dalzell *Ceropegia* 25 deposited in general Kew herbarium.

Pollinarium were fixed with 2% glutaraldehyde, rinsed in distilled water and then dehydrated through an acetone series. This was followed by critical point drying, gold coating (JFC-1600 Auto Fine Coater), and examination under a JEOL-6360A analytical scanning electron microscope (SEM) in the Department of Physics, University of Pune. For describing pollinarium, terminology of Erdtman (1966) and Kremp (1968) was followed.

Plant material for DNA analysis : Leaf samples from healthy plants of *Ceropegia* species were collected from different localities of north Western Ghats of India and stored in saline NaCl-CTAB solution till their use for DNA extraction (Rogstad, 1992).

ISSR marker analysis : For genomic DNA extraction, the leaves were washed with sterile distilled water to remove the traces of NaCl-CTAB. DNA extractions were carried out following the modified CTAB method as

Table 1. : Source of material examined for DNA analyses and morphological studies and GenBank accession numbers for ITS and trnL sequences.

Taxon & Locality	Voucher	GenBank acc. no. (ITS)	(trnL)
Ceropegia karulensis Punekar & al. India: Maharashtra, Ratnagiri Dt., Karul ghat, 800-900m	*S.A. Punekar* 365	HQ154105	HQ154112
Ceropegia maccannii M. Y. Ansari India: Maharashtra, Pune, Sinhagad, 1200-1400m	*S.A. Punekar & A. Raut* 367	HQ154108	HQ154115
Ceropegia maharashtrensis Punekar & al. India: Maharashtra, Pune, Junnar, Dhakeshwar, 900-1100m	*S.A. Punekar & A. Raut* 357	HQ154104	HQ154111
Ceropegia panchganiensis Blatt. & McCann India: Maharashtra, Satara, Mahabaleshwar, Kate's Point, 1400m	*S.A. Punekar* 355	HQ154107	HQ154114
Ceropegia rollae Hemadri India: Maharashtra, Pune, Junnar, Durgakilla, 800-1000m	*S.A. Punekar & A. Raut* 580	HQ154109	HQ154116
Ceropegia sahyadrica M. Y. Ansari & B. G. Kulk. India: Maharashtra, Sindhudurg, Amboli, 1200-1400m	*S.A. Punekar* 579	HQ154106	HQ154113

Table 2. : Accession numbers of DNA sequences obtained from GenBank/EMBL databases (data of Meve & Liede, 2007 & Surveswaran & al., 2009)

Section	Species	ITS	trnL	Reference
Buprestis	*Ceropegia evansii*	EU106680	EU120003	Surveswaran & al., 2009
	Ceropegia huberii	EU106694	EU120004	Surveswaran & al., 2009
	Ceropegia media	EU106696	EU120009	Surveswaran & al., 2009
	Ceropegia odorata	EU106701	EU119993	Surveswaran & al., 2009
	Ceropegia santapaui	EU106695	EU119991	Surveswaran & al., 2009
Ceropegia	*Ceropegia bulbosa*	EU106687	EU120007	Surveswaran & al., 2009
Chionopegia	*Ceropegia pubescens*	AM493280	AM490379	Meve & Liede, 2007
Indopegia	*Ceropegia fantastica*	EU312083	EU120006	Surveswaran & al., 2009
	C.oculata var. *satpudensis*	HQ154110	HQ154117	Present data
Janthina	*Ceropegia elegans*	EU106677	-	Surveswaran & al., 2009
Lysanthe	*Ceropegia attenuata*	EU106700	EU119992	Surveswaran & al., 2009
Oreopegia	*Ceropegia longifolia*	AM493283	AM491572	Meve & Liede, 2007
Phalaena	*Ceropegia juncea*	EU106685	EU120008	Surveswaran & al., 2009
Outgroup	*Brachystelma edulis*	EU106702	EU120013	Surveswaran & al., 2009

described by Rogers & Bendich (1985). The isolated DNA was processed, quantified by agarose gel electrophoresis and used for PCR amplifications. ISSR-PCR amplifications were performed in reaction volume of 25m l, using selected primers from UBC primer set # 9 (University of British Columbia, Vancouver, Canada). The reaction mixture contained 1.5 mM $MgCl_2$, 0.5 U Taq DNA polymerase, 0.1 mM of each dNTP, 0.4 mM of spermidine, 2% formamide, 0.3m M of primer and 12.5 ng DNA. Thermal cycling was carried out in PTC 200 thermal cycler (MJ Research Inc, www.bio-rad.com) as per the protocol of Nagaoka & Ogihara (1997). The PCR reactions were repeated at least two times for each primer to ensure reproducibility of the band pattern. The

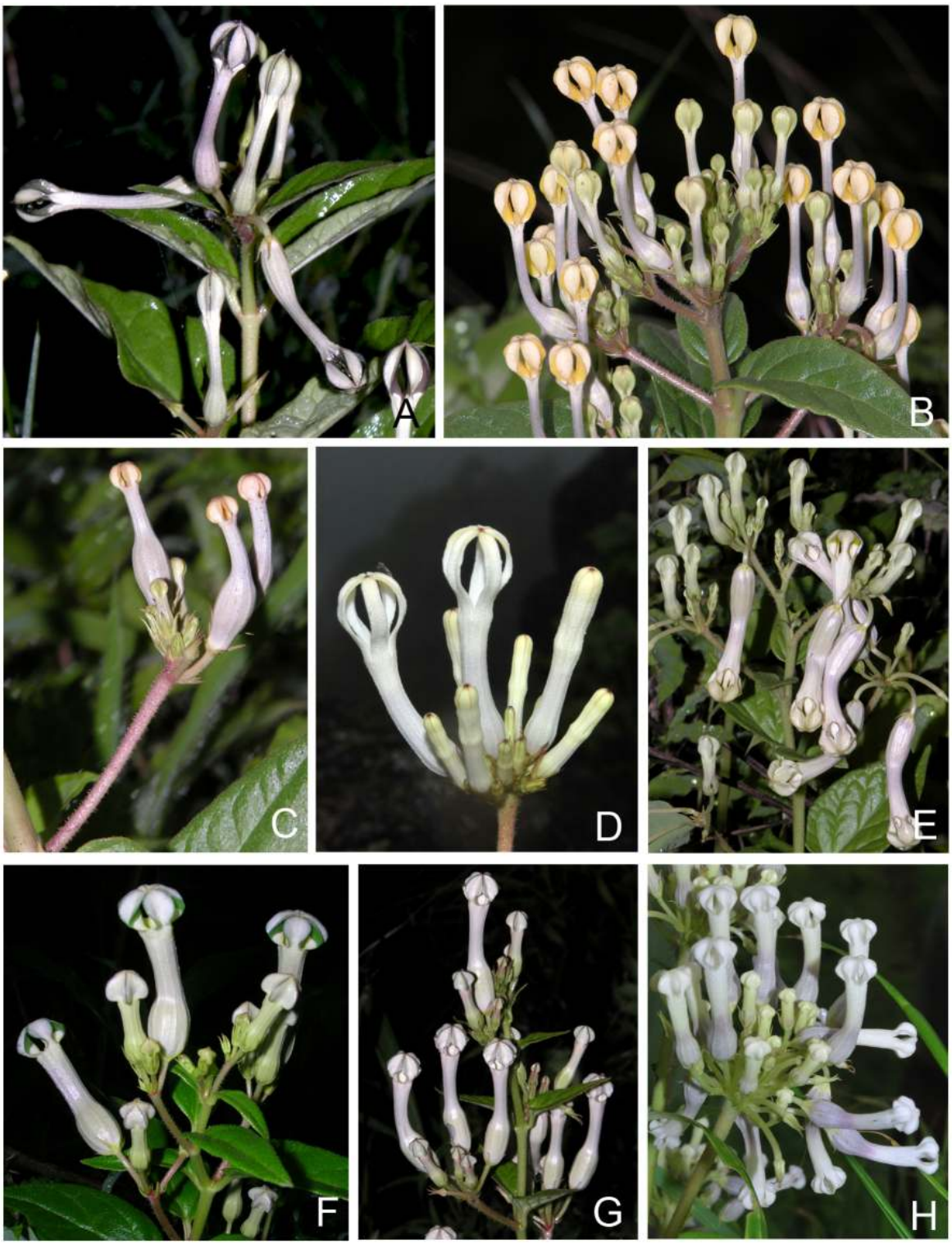

Fig. 4 : Inflorescence. **A.** *Ceropegia maharashtrensis;* **B.** *C. panchganiensis;* **C**. *C. maccannii;* **D**. *C. rollae*; **E.** *C. sahyadrica* - Sinhagadh population; **F.** *C. sahyadrica* - Amboli population; **G.** *C. sahyadrica* - Pabe ghat population; **H**. *C. karulensis*. Photos by Sachin A. Punekar.

amplification products were separated on 1.5% agarose gel in 0.5X Tris-acetic acid-EDTA (TAE) buffer. The gels were stained with ethidium bromide and documented using UVP GDS 8000 system.

The bands in amplification profiles were scored as discrete variables, using 1 to indicate presence and 0 for absence. The binary matrix was used to generate pair-wise similarities between the accessions using Dice coefficient. Similarity matrix was subjected to cluster analysis. The dendrogram was derived using UPGMA in SAHN module of NTSYS pc 2.1.

Sequence data analysis : Nuclear ribosomal DNA region containing 18S rRNA gene (partial), 5.8S rRNA gene, 26S rRNA gene (partial) and ITS1 and 2 was amplified with primers ITS4 and ITS5 (White & al., 1990). Chloroplast trnL-intron region were amplified using primer pairs c/d (Taberlet & al., 1991).

PCR DNA amplifications were performed in a 25 µl volume containing 20 ng genomic DNA template, 1 µl (10 pM/µl) of both forward and reverse primers, 0.2 mM of each dNTPs, 0.5 U Taq DNA polymerase, 2.5 mM $MgCl_2$, 10x PCR buffer containing 50 mM of KCl and 10 mM of Tris-HCl. PCR was performed on a Peltier Thermal Cycler (PTC-200, MJ Research, MA, USA) with an initial 3 min at 94^0 C followed by 35 cycles of 94^0C for 30 s, 50^0C for 30 s, 72^0C for 1 min and finally at 72^0C for 5 min. For trnL-intron amplifications the annealing was carried out at 60^0C and the extension time was 1.5 min. The sizes of the amplified products were checked by electrophoresis on 1.5% agarose gels in 0.5x TAE buffer and staining with ethidium bromide. The PCR products were purified by PEG precipitation and sequenced on ABI 3100-Avant Genetic Analyzer. Sequencing reactions were performed with the Big Dye Terminator kit v3.1 (Applied Biosystems, www.invitrogen.com) as per manufacturer's protocol. Both the strands were sequenced for each region.

Phylogenetic and molecular evolutionary analyses of the sequence data were conducted using MEGA version 4 (Tamura & al., 2007). The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Jukes-Cantor method (1969) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). The bootstrap test (1000 replicates) was also conducted.

RESULTS & DISCUSSION

Genetic relationships based on ISSR markers

PCR-based DNA markers provide powerful tools for genetic analysis mainly because of their simplicity and ease of handling. Among various PCR based markers, the inter simple sequence repeat amplification (Zietkiewicz & al., 1994) is a simple, quick and reliable technique used in various species for detecting polymorphism. The ISSR markers have been used extensively for phylogenetic studies, the evaluation of genetic diversity as well as for cultivar identification in a number of plant species (Wolfe, 2005). Six taxa belonging to *Ceropegia* section *Buprestis*, *C. karulensis*, *C. maccannii*, *C. maharashtrensis*, *C. panchganiensis*, *C. rollae* and *C. sahyadrica*, were therefore analyzed using ISSR markers. *Ceropegia oculata* var. *satupudensis* from section *Indopegia* was used as an outgroup. Total 114 bands obtained from 13 primers were scored and the similarity matrix was generated using Dice coefficient. The similarity coefficient ranged from 0.42 to 0.66. In cluster analysis using UPGMA algorithm, *C. oculata* var. *satupudensis* which was used as outgroup species, separated from the rest of the erect species which belong to section *Buprestis*. In this clade, two clusters were observed viz., first cluster consisted of *C. rollae*, *C. sahyadrica* and *C. karulensis* and the second of *C. maharashtrensis*, *C. panchganiensis* and *C. maccannii.* (Fig. 8)

The grouping of the species as observed in the present analysis was compared with the morphological data. Taxa from section *Buprestis* with erect habit formed separate clade and were distinct from outgroup taxon from section *Indopegia* which has twining habit. The two clusters observed in the clade of species with erect habit are also in agreement with the relationships observed based on morphological characters. All the three species in the first cluster viz., *C. maharashtrensis*, *C. panchganiensis* and *C. maccannii* are characterized by a distinctly patterned light windows or windowpanes either in the form of vertical stripes, ring or small circular blotches in the inflated portion of tube or tube in general, cupular outer corona and clavate, subclavate or spathulate inner corona. *C. maharashtrensis* which is separated in this cluster has light windows or windowpanes in inflated

Fig. 5 : L.S. of flower showing windows and corona. **A**. *Ceropegia maharashtrensis;* **B-C.** *C. panchganiensis*; **D.** *C. maccannii;* **E**. *C. rollae*; **F**. *C. sahyadrica* - Sinhagadh population; **G**. *C. sahyadrica* - Amboli population; **H**. *C. sahyadrica* - Pabe ghat population; **I**. *C. karulensis*. Photos by Sachin A. Punekar.

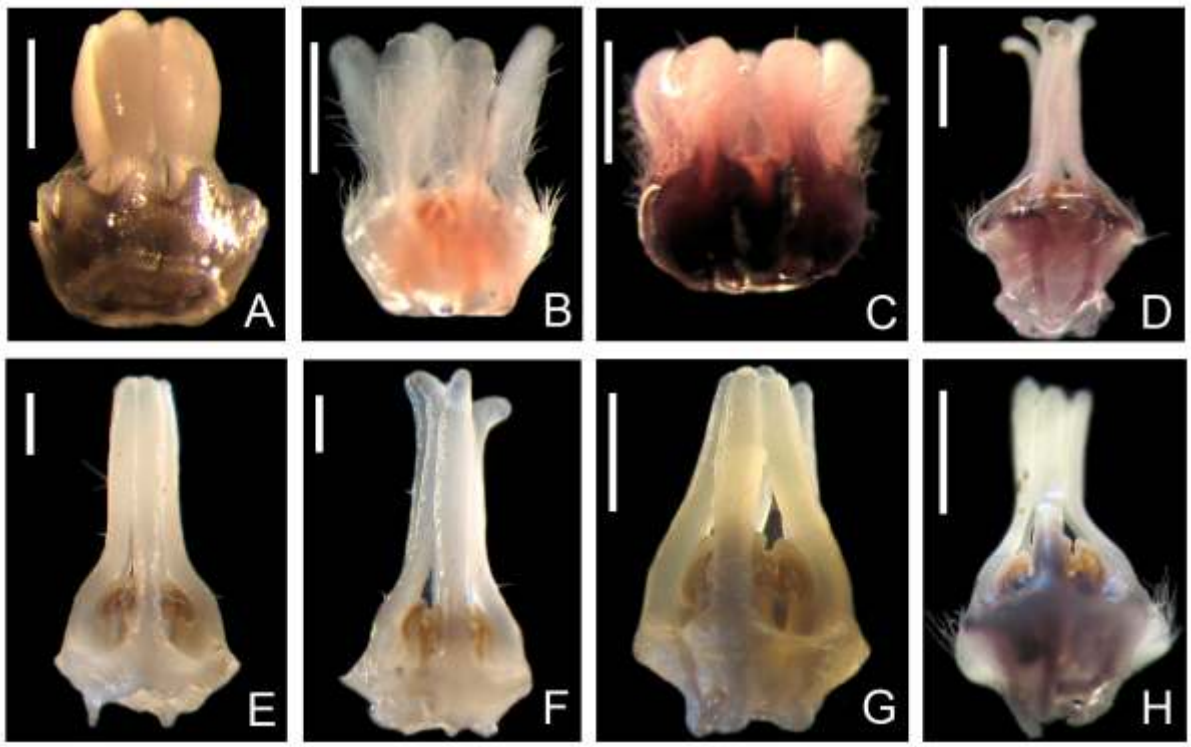

Fig. 6 : Corona. **A**. *Ceropegia maharashtrensis;* **B**. *C. panchganiensis*; **C**. *C. maccannii;* **D**. *C. rollae*; **E**. *C. sahyadrica* - Sinhagadh population; **F**. *C. sahyadrica* - Pabe ghat population; **G**. *C. sahyadrica* - Amboli population; **H**. *C. karulensis*. Scale bar = 1mm. Photos by Sachin A. Punekar.

portion with small circular blotches and inner corona lobes convergent, glabrous or sparsely hairy; while both *C. maccannii* and *C. panchganiensis* which are grouped together have light windows or windowpanes in inflated portion with vertical stripes and densely hairy, divergent inner corona lobes. The species in second cluster viz., *C. rollae*, *C. sahyadrica* and *C. karulensis* are characterized by uniformly dark purple or reddish colouration inside the tube without light windows or windowpanes, saucer-shaped outer corona and linear inner corona. *C. rollae* which is separate in second cluster has oblong corolla lobe and corolla tube slightly inflated at base. Whereas the remaining two taxa grouped together in second cluster viz., *C. sahyadrica* and *C. karulensis* are similar in having distinctly inflated corolla tube at base, oblate to obconic corolla head and corolla lobes broadly ovate. The results show that DNA analysis using molecular markers is efficient and a reliable tool for studying *Ceropegia* species and is complementary to the morphological observations. Morphologically, the erect species belonging to section *Buprestis* have been many times erroneously treated under a certain species; therefore, study of the genetic relationships among them will be very important to resolve such errors.

Sequence data and phylogenetic relationships

Sequence data from nuclear ribosomal DNA and trnL (UAA) intron regions of all the erect species of section *Buprestis* was separately used to derive phylogenetic relationships. The ITS amplification product was ~700 bp in length while chloroplast trnL-intron region was ~550 bp long.

The aligned dataset containing 20 taxa included sequences of erect species of section *Buprestis* studied presently and sequences of other taxa representing the sections within genus *Ceropegia* taken from database (Table 1 & 2). Based on aligned sequence data (627 bp) from ITS region (Fig. 9), all the erect species of section *Buprestis* including two new species (*C. karulensis*, *C. maccannii*, *C. maharashtrensis*, *C. panchganiensis*, *C. rollae* and *C. sahyadrica*) along with 3 twining species (*C. evansii*, *C. fantastica* and *C. oculata* var. *satpudensis*) formed a single clade characterized by broad leaves, which further separated into two. First consisted of 4 species with erect habit while other consisted of 2 species from *Buprestis* with erect habit grouped together along with 3 twining species belong to sections *Indopegia* (*C. fantastica* and *C. oculata* var. *satpudensis*) and *Buprestis* (*C. evansii*). Grouping of *Indopegia* along with erect species of section *Buprestis* suggests close relationship which in turn

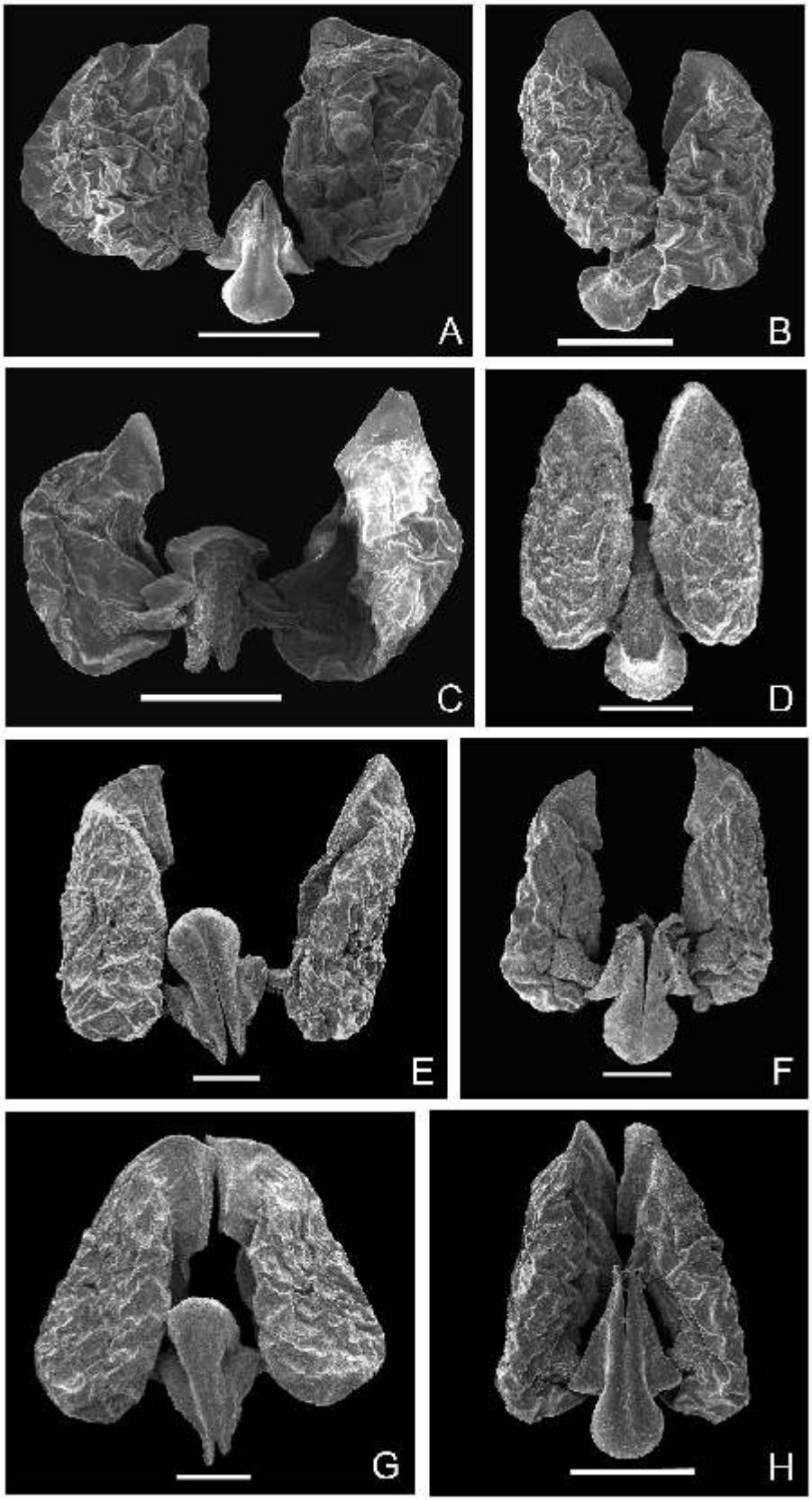

Fig.7 : Pollinarium (Scanning Electron Microscopic photographs). **A.** *Ceropegia maharashtrensis*; **B.** *C. panchganiensis;* **C.** *C. maccannii;* **D.** *C. rollae;* **E.** *C. sahyadrica* - Sinhagadh population; **F.** *C. sahyadrica* - Amboli population; **G.** *C. sahyadrica* - Pabe population; **H.** *C. karulensis.* Scale bar = 100µm.