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REGULAR PAPER

Phylogenetic relationships of Malagasy Hyacinthaceae

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Background – Whereas subfamily Oziroeoideae of the petaloid monocot family Hyacinthaceae is restricted to South America, the three other subfamilies, Ornithogaloideae, Urgineoideae and Hyacinthoideae, have much larger primary distribution areas spanning the Mediterranean and Central Europe, Arabian Peninsula, Indian subcontinent, Far East (China and Japan) and Africa, with some members also in Madagascar. Based mainly on morphology, until recently, most of the Malagasy species have been included in genera found also outside this island. Morphological characters alone have been misleading in many cases, resulting in erroneous generic classifications.
Method – Analysis of plastid DNA sequences was used to reconstruct phylogenetic relationships among members of old world Hyacinthaceae.
Key results – Phylogenetic analysis based on multiple plastid DNA markers has changed our views substantially, leaving many of the Malagasy Hyacinthaceae taxa as monophyletic groups. All Malagasy members of Urgineoideae form a well-supported clade (*Rhodocodon*/*Drimia cryptopoda*) pointing to a single colonization from continental Africa. *Drimia cryptopoda* is a morphologically deviant species previously misplaced in *Hyacinthus*. The Urgineoideae from India do not appear to be directly related to African or Malagasy species, but show close relationships to the Mediterranean *Urginea* s. str. Two members of Hyacinthoideae are present in Madagascar. One of them, *Ledebouria* sp. ined., is related to South African species, whereas the other, *L. nossibeensis*, shows strong relationships to *L. hyacinthina* from India and to *L. grandifolia* from Socotra. *Dipcadi* (Ornithogaloideae) forms a well-supported monophyletic clade.
Conclusion – We presume a single colonization from mainland Africa followed by rapid radiation in different habitats in Madagascar. The close relationship of Indian *Dipcadi* with those of the Mediterranean points to a Northern Hemisphere migration route linking India and the Mediterranean and possibly involving also the Arabian Peninsula.

Key words – Hyacinthaceae, phylogeny, DNA sequence analysis.

INTRODUCTION

The division of the Hyacinthaceae into the four subfamilies Oziroeoideae, Urgineoideae, Ornithogaloideae, and Hyacinthoideae (Pfosser & Speta 1999, Manning et al. 2004, 2009, Wetschnig et al. 2007) is now well accepted. It is supported by morphological, chemotaxonomical, and molecular data alike. Hyacinthaceae occurs in South America (Oziroeoideae only), in Africa south and north of Sahara, in Madagascar, Arabian Peninsula, and India (Urgineoideae, Ornithogaloideae, and Hyacinthoideae). The latter three subfamilies share a secondary center of diversity spanning the Mediterranean and Eurasian floristic regions. A single isolated group of species is found in SE Asia (*Barnardia* of subfamily Hyacinthoideae; Speta 1998a, 1998b, Pfosser & Speta 1999, Pfosser et al. 2003).

It has been suggested that the involvement of species from South Africa, South America, and Madagascar in the basal branching patterns of subfamilies and tribes indicates a southern, probably Gondwanan, origin of Hyacinthaceae (Pfosser & Speta 1999). This hypothesis implies that major lineages diverged from each other in Gondwana and were separated later by continental break-up. Taxa rafting on the Palaeo-Indian plate as it moved northwards play a key role in this hypothesis. Similar northward transport via India

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has been inferred in plant families such as Crypteroniaceae (Conti et al. 2004, Rutschmann et al. 2004), Dipterocarpaceae (Ashton & Gunatilleke 1987, Ducousso et al. 2004), but also for animals such as acrodont lizards (Macey et al. 2000), ranid frogs (Biju & Bossuyt 2003), caecilian amphibians (Wilkinson et al. 2002), and ratite birds (Cooper et al. 2001). Alternatively, the present distribution of Hyacinthaceae could be explained by more recent long-distance dispersal across the Indian Ocean and/or by migration from the Mediterranean and Arabian regions towards India.

Historical biogeographic reconstruction based on phylogenetic analysis is an important way to understand the evolutionary history of organisms in space and time. In order to test the possible involvement of India as a raft for the northwards transport, we have sampled palaeotropic taxa present in southern Africa, Madagascar, India, Arabian Peninsula, and in the Mediterranean, with emphasis on *Drimia/Rhodocodon* (Urgineoideae), *Dipcadi* (Ornithogaloideae) and *Ledebouria* (Hyacinthoideae) because of their large distribution areas. In addition, we included representative members for each subfamily in order to position the target genera in a phylogenetic framework.

MATERIALS AND METHODS

Taxonomic sampling

Species and lineages sampled are listed in the electronic appendix. Sampling focused on lineages having representatives in South Africa, Madagascar and India, including their closest relatives from other areas. Information for this selection was taken from the literature (Pfosser & Speta 1999, Pfosser et al. 2003, 2006, Manning et al. 2004, 2009) and from own data.

Molecular techniques, DNA data generation

DNA was extracted from leaf tissue using either the DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA) or the CTAB method (Doyle & Doyle 1987) with modifications (Pfosser et al. 2006). The *trnC*^{GCA}-*ycf6* intergenic region was sequenced for this study. Primers used for amplification were *trnC*^{GCA}F (CCA GTT CRA ATC YGG GTG) (modified from Demesure et al. 1995) and *ycf6*R (GCC CAA GCR AGA CTT ACT ATA TCC AT) (Shaw et al. 2005) using standard thermal cycling conditions (95°C, 5 min; 35 cycles of 94°C, 20 sec; 50°C, 30 sec; 72°C, 1 min; final extension at 72°C, 10 min). PCR was performed using Hybaid thermal cyclers in 20 µL volumes with the following reaction components: 2 µL template DNA (10–100 ng), 2X DreamTaq ReadyMix PCR reaction mix (Fermentas) and 0.1 µmol/L each primer. Amplified double-stranded DNA fragments were purified with Exonuclease I and Shrimp alkaline phosphatase (Fermentas) following the protocol of the manufacturer to remove unincorporated nucleotides and excess primers prior to sequencing. Dideoxy sequencing was performed using the purified PCR fragments following the DYEnamicET cycle sequencing protocol (General Healthcare, USA). Both strands were sequenced using the same primers as for amplification. Separation of fragments and base calling was performed on a MegaBace 500 automated sequencer (General Healthcare, USA). On average, less than 1% of data matrix cells were scored as missing data.

All sequences have been deposited in the EMBL database (for accession numbers, see electronic appendix). Clustal X (Jeanmougin et al. 1998) was used for automatic DNA sequence alignment. Final alignment was done by eye in order to homogenize the positions of insertion/deletion mutations (indels). Indels in the data matrix were coded as additional characters using the simple indel coding approach (Simmons & Ochoterena 2000) implemented in the computer program GapCoder (Young & Healy 2003). The aligned data matrices were 1166, 1108, and 1152 nucleotide positions long for Urgineoideae, Ornithogaloideae and Hyacinthoideae, respectively. Ambiguous and incomplete regions at the beginning and end of sequences were excluded from the analysis: positions 1–48 and 1112–1166 for Urgineoideae, 1–51 and 1044–1108 for Ornithogaloideae, and 1–49 and 1051–1152 for Hyacinthoideae. An AT-rich region resulting in ambiguous alignments at positions 490–540 in Urgineoideae was excluded from the analysis alike. The aligned indel blocks consisted of 102, 113, and 95 positions for Urgineoideae, Ornithogaloideae and Hyacinthoideae, respectively.

Tree searches were performed using the nucleotide data together or without the indel data. Phylogenetic analysis using the maximum parsimony (MP) method were performed with the computer program PAUP* version 4.0b10 (Swofford 2000). Most parsimonious trees were obtained by 1000 replicates of random sequence addition using tree bisection-reconnection (TBR) branch swapping under the Fitch criterion (Fitch 1971). Ten thousand fast bootstrap replicates (Felsenstein 1985) were used to assess confidence limits for the resulting tree topologies.

RESULTS AND DISCUSSION

The phylogenetic trees (figs 1–3) resulting from *trnC*^{GCA}-*ycf6* data match topologies obtained from earlier analyses of different cpDNA regions (Pfosser & Speta 1999, 2001, 2004, Pfosser et al. 2003, 2006, Manning et al. 2004, 2009, Wetschnig et al. 2002, 2007). Also extended taxa sets matching taxon sampling from previous analyses yielded results consistent with previous analyses. As the main focus of this study was to establish a phylogenetic framework for genera distributed in an area spanning from Africa, via Madagascar and Arabian Peninsula to India (*Rhadamanthus*/*Rhodocodon/Drimia cryptopoda*, *Ledebouria*, *Dipcadi*), most clades not related to our study groups were excluded from the analyses. However, a much broader sampling of taxa from Madagascar and India compared to previous analyses, as well as the high amount of sequence divergence of the *trnC*^{GCA}-*ycf6* region in Hyacinthaceae, helped to improve the resolution in our study groups.

In agreement with previous studies (Pfosser & Speta 1999, 2001, 2004, Pfosser et al. 2003, 2006, Manning et al. 2004, 2009, Wetschnig & Pfosser 2003, Wetschnig et al. 2007), analysis of the *trnC*^{GCA}-*ycf6* region yielded evidence for *Bowiea* being sister to the remaining taxa in Urgineoideae and *Pseudoprospero* being sister to all other members of Hyacinthoideae. Therefore, *Bowiea* and *Pseudoprospero* have been used as outgroups for the remaining Urgineoideae

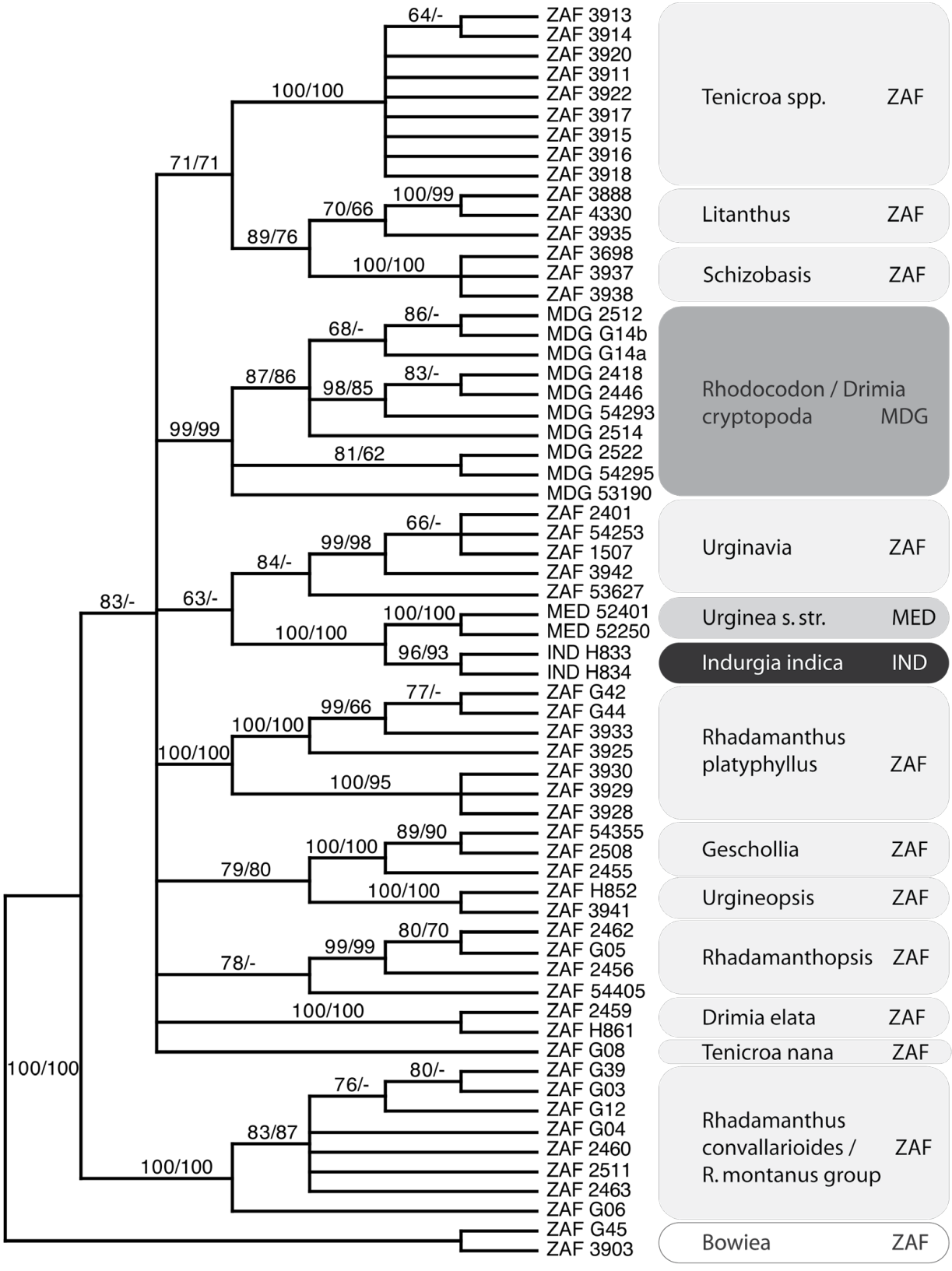

Figure 1 – Phylogenetic reconstruction based on maximum parsimony of members of subfamily Urgineoideae. A strict consensus tree is shown with bootstrap support values (including/excluding indel data) indicated above branches. Major clades are colour-coded by geographic affiliations (light grey shading – South Africa [ZAF], dark grey shading – Madagascar [MDG], black – India [IND], medium grey shading – Mediterranean [MED]; outgroup: *Bowiea* from South Africa [ZAF] – no shading, black frame)

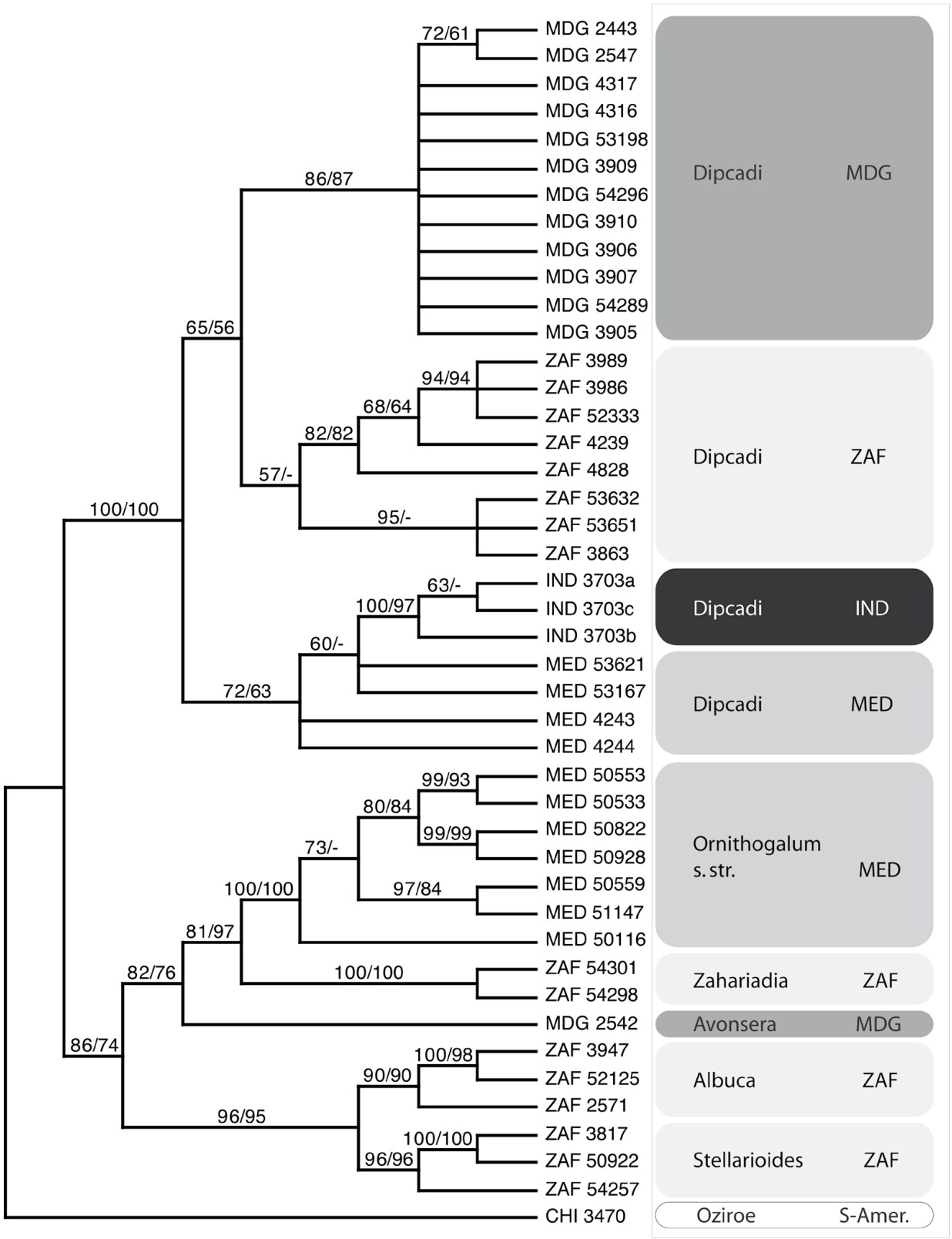

Figure 2 – Phylogenetic reconstruction based on maximum parsimony of members of subfamily Ornithogaloideae using *Oziroe* from South American subfamily Oziroeoideae as outgroup. A strict consensus tree is shown with bootstrap support values (including/excluding indel data) indicated above branches. Major clades are colour-coded by geographic affiliations (light grey shading – South Africa [ZAF], dark grey shading – Madagascar [MDG], black – India [IND], medium grey shading – Mediterranean [MED]; outgroup: *Oziroe* from South America [S-Amer.] – no shading, black frame).

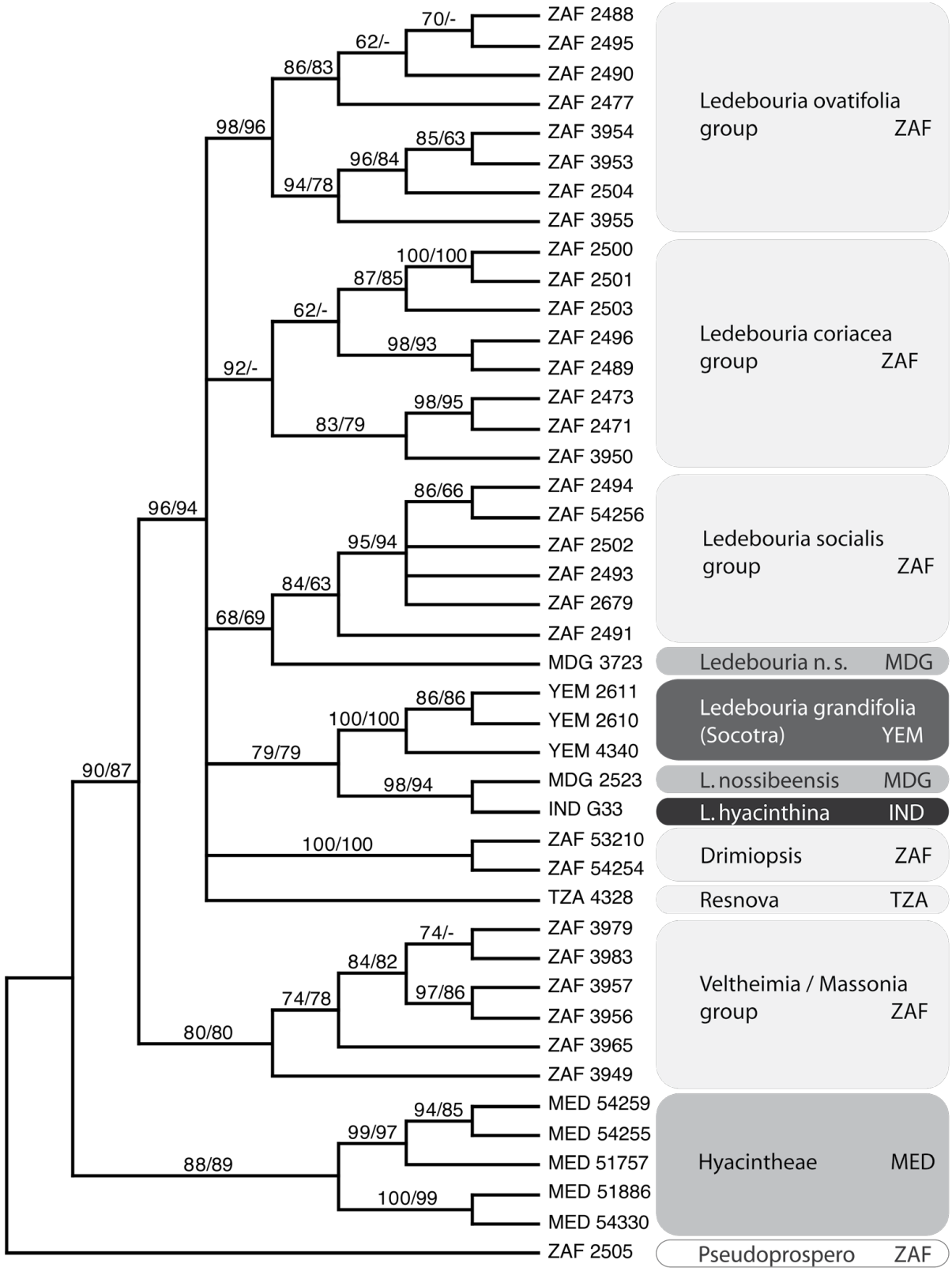

Figure 3 – Phylogenetic reconstruction based on maximum parsimony of members of subfamily Hyacinthoideae. A strict consensus tree is shown with bootstrap support values (including/excluding indel data) indicated above branches. Major clades are colour-coded by geographic affiliations (light grey shading – South Africa [ZAF] and Tanzania [TZA], dark grey shading/black text – Madagascar [MDG], dark grey shading/white text – Arabian Peninsula [Yemen - YEM], black – India [IND], medium grey shading – Mediterranean [MED]; outgroup: *Pseudoprospero* from South Africa [ZAF] – no shading, black frame).

and Hyacinthoideae, respectively. Subfamily Ornithogaloideae was rooted with Oziroeoideae, the most basal subfamily in Hyacinthaceae. Topological findings in subfamilies Urgineoideae, Ornithogaloideae, and Hyacinthoideae, directly address the origin and diversification of Hyacinthaceae in Madagascar and India.

Subfamily Urgineoideae

All species from Madagascar form a clade (fig. 1). This clade (99/99% +indels/-indels bootstrap support) includes all species of *Rhodocodon* as well as the frequently misclassified and morphologically deviant *Drimia cryptopoda*. Originally, the latter species was included in *Hyacinthus* (subfamily Hyacinthoideae), yet it was shown to belong to Urgineoideae instead (Pfosser et al. 2006, Wetschnig et al. 2007). Our results suggest that *D. cryptopoda* is closely related to other Malagasy members of Urgineoideae. The entire clade is nested within a group of predominantly South African species. The monophyly of this well-supported clade suggests a single colonization for the Malagasy alliance from continental Africa. Inclusion of the Malagasy taxa in *Rhadamanthus* as suggested by Pfosser & Speta (2004) is clearly refuted by this topology. The Indian taxa form a well-supported clade (100/100%) with the Mediterranean *Urginea* Steinh. s. str, indicating a Northern Hemisphere diversification after migration of progenitors of this group from South Africa.

Subfamily Ornithogaloideae

Within Ornithogaloideae only *Dipcadi* is found in all of South Africa, Madagascar, India and the Mediterranean. The genus is well supported (100/100%). As in *Rhodocodon*, the Malagasy members of *Dipcadi* show a pattern suggesting a single colonization from mainland Africa followed by rapid radiation and speciation in Madagascar. The Malagasy species form a well-supported clade (86/87% bootstrap support). Although a direct relationship to South African *Dipcadi* is only moderately supported (65/56% bootstrap support), a direct relationship to Indian *Dipcadi* has to be dismissed. Again, an affinity of the Indian taxa to the Mediterranean species is suggested (72/63% bootstrap support). Possible migration routes linking the Mediterranean and India (perhaps via the Arabian Peninsula where this genus is also present) are pending further investigation.

Subfamily Hyacinthoideae

Two species of *Ledebouria* are known from Madagascar. *L. nossibeensis* shows strong relationships to the type species of *Ledebouria*, *L. hyacinthina* from India (98/94% bootstrap support). Sister group relationship of this clade with *Ledebouria grandifolia* from Socotra receives moderate support (79/78% bootstrap support). A second unrelated *Ledebouria* species is found in the south-western parts of Madagascar and remains to be described. This species shows moderate affinity to the *L. socialis*-group of continental Africa (68/69% bootstrap support). In *Ledebouria* therefore, two colonization events must be envisaged to explain the present distribution of the genus in Madagascar. No direct relatives to either the Malagasy, Arabian or Indian members of the genus are found in the Mediterranean, hence excluding the possibility of Northern Hemisphere migration routes having played a role in the diversification of this group. In contrast, the tree topology reflects the most recent tribal classification into strictly Northern Hemisphere (excluding India) Hyacintheae (88/89%), the monotypic South African tribe Pseudoprospereae (genus *Pseudoprospero*) and tribe Massonieae (87/82%) including all remaining African and all Indian and Malagasy species in this subfamily (Manning et al. 2004). Moreover, our data again show the need to revise generic classification within a broadly defined *Ledebouria*. Obviously, in this group phylogenetically unrelated lineages are present, which also show distinct geographic radiation patterns that could warrant further splitting into separate genera as already suggested by Lebatha et al. (2006).

Both *Dipcadi* and *Rhodocodon* (including *Drimia cryptopoda*) are genera with numerous species on Madagascar and show well-supported monophyletic groups for the Malagasy alliances in the phylogenetic reconstructions. This points to single colonization events followed by subsequent radiation on the island. In both cases the island taxa appear to be descendants of dispersers of African origin. Similar scenarios have been evoked for most of the present-day biota of Madagascar (Yoder & Nowak 2006). As both the Urgineoideae and Ornithogaloideae trees do not suggest a direct relationship of these Malagasy groups to members in India, a Gondwana origin leading to vicariance after continental break-up as previously suggested appears to be less likely (Pfosser & Speta 1999). A Gondwana origin as an explanation for the present day distribution of Hyacinthaceae would require an age of the radiations in Madagascar and India similar to the fragmentation of Gondwana in the Cretaceous.

Ledebouria is the only example in Hyacinthaceae where direct relatives are found in Africa, Madagascar, Arabian Peninsula, and India. *Ledebouria* therefore resembles a distribution pattern found also in other groups, where a Gondwana origin was postulated (Conti et al. 2004, Rutschmann et al. 2004, Ashton & Gunatilleke 1987, Ducousso et al. 2004). However, the age of Hyacinthaceae was determined to be less than 40 Myr (Wikström et al. 2001), much younger than the breakup of Gondwana (120–80 Myr ago). A major drawback in dating radiations in Hyacinthaceae is the lack of fossil data. Recently, the arrival of progenitors of the *Rhodocodon/Drimia cryptopoda*-clade in Madagascar was dated by a molecular clock-approach at 5–15 Myr ago (Ali et al. 2011). Interestingly, prevalence of oceanic currents directed eastward from Mozambique towards Madagascar have recently been found based on palaeogeographic reconstruction during the Paleogene (c. 60–20 Myr ago), exactly as required for the establishment of many of the unique floristic and faunistic elements of Madagascar (Ali & Huber 2010) and much closer to the dated arrival of Urgineoideae in Madagascar. According to their hypothesis, rafting (e.g. on large vegetation mats) might have been a common vehicle for progenitors to arrive on Madagascar during this period.

SUPPLEMENTARY DATA

Supplementary data are available at *Plant Ecology and Evolution*, Supplementary Data Site (http://www.ingentaconnect.

com/content/botbel/plecevo/supp-data), and consist of a list of taxa investigated in this study, with vouchers, and EMBL accession numbers (pdf format.)

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