the late diagenetic history. Authigenic growth of kaolinites reduces the permeability/porosity ratio. Flaky and fibrous illites are found to grow in pore spaces offering resistance to fluid flow through the sandstone and thereby reduce permeability. Presence of hairy illite in sandstone pores increases the micro porosity and pore tortuosity, and thereby decreases permeability.

The sandstones of the present study are mainly quartz arenite to sublitharenite and less commonly study. Petrographic associated with SEM study investigation that certain diagenetic changes like precipitation of secondary minerals, quartz overgrowth and precipitation of various cementing materials are some of the important factors responsible for porosity reduction in certain oil-producing horizons of the Upper Palaeocene–Lower Eocene rocks of Nahorkatiya oilfield. Moreover, presence of kaolinites and fibrous nature of illite in the pore throats also reduces porosity and permeability. Conversely, development of intra-particle microfractures, dissolution and partial replacement of the framework grains by cementing materials are some of the important diagenetic changes which contribute towards development and preservation of secondary porosity. So, these diagenetic changes may be responsible for making certain oil-bearing horizons highly productive, whereas others are less productive in spite having good reserves. Also, the sandstones of the present study are found to be the products of craton interior, recycled orogen and quartzose recycled sources, and are mainly derived from plutonic to middle rank metamorphic sources.

1. Mallick, R. K., Characterization and hydrocarbon resource evolution of Lower Eocene source rocks in Upper Assam Basin, using well logs. Reprints of published papers, Department of Petroleum Technology, Dibrugarh University, July 2001.
2. Dott Jr, R. H., Wacke, greywacke and matrix – what approach to immature sandstone classification? *J. Sediment. Petrol*., 1964, **34**, 625–632.
3. Dickinson, W. R. *et al*., Provenance of North American Phanerozoic sandstones in relation to tectonic setting. *Geol. Soc. Am. Bull*., 1983, **94**, 222–235.
4. Basu, A., Young, S. W., Suttner, L. J., James, W. C. and Mack, G. H., Re-evaluation of the use of undulatory extinction and polycrystallinity in detrital quartz for provenance interpretation. *J. Sediment. Petrol*., 1975, **45**, 873–882.
5. Burley, S. D. and Kantorowicz, J. D., Thin section and S.E.M. textural criteria for the recognition of cement-dissolution porosity in sandstones. *Sedimentology*, 1986, **33**, 587–604.
6. Ehenberg, S. N., Relationship between diagenesis and reservoir quality in sandstone of Garn Formation, Haltenbanken, Mid-Norwegian Continental Shelf. *AAPG Bull*., 1990, **74**, 1538–1558.
7. Folk, R. L., *Petrology of Sedimentary Rocks*, Hemphills, Austin, 1980.
8. Grim, R. E., *Clay Mineralogy*, International Series in Earth and Planetary Science, McGraw-Hill Book Company, New York, 1968, 2nd edn.
9. Guven, N., Smectites. In *Review in Mineralogy, Hydrous Phyllosilicates* (ed. Baily J. W.), 1988, pp. 497–558.
10. Handique, G. K., Sethi, A. K. and Sarma, S. C., Review of Tertiary stratigraphy of parts of Upper Assam Valley. *Geol. Surv. India Spec. Publ*., 1989, **23**, 23–36.
11. Handique, G. K. and Mallick, R. K., Hydrocarbon exploration in Upper Assam basin; problems and challenges. *Pet. Asia J*., 1990, **2**, 141–147.
12. Houseknecht, D. W., Assessing the relative importance of compaction processes and cementation to reduction of porosity in sandstones. *AAPG Bull*., 1987, **71**, 633–642.
13. Joshi, G. K., Shyammohan, V., Reddy, A. S., Singh, B. and Chandra, M., Reservoir characterization through log property mapping in Geleki Field of Upper Assam, India. In Proceedings of the 5th Conference and Exposition on Petroleum Geophysics, Hyderabad, 2004, pp. 685–687.
14. Norman, K. W., Hickman, R. G. and Dasgupta, U., Application of a ramp/flat fault model to interpretation of the Naga thrust and possible implications for petroleum exploration along the Naga thrust front. *AAPG Bull*., 2002, **86**, 2023–2045.
15. Pettijohn, F. J., *Sedimentary Rocks*, CBS Publishers and Distributors, New Delhi, 1984.
16. Rittenhouse, G., Pore space reduction by solution and cementation. *AAPG Bull*., 1971, **55**, 80–91.
17. Willson, M. D. and Pittman, E. D., Authigenic clays in sandstones; Recognition and influence on reservoir properties and paleo-environmental analysis. *J. Sediment. Petrol*., 1977, **47**, 3–31.

ACKNOWLEDGEMENTS. We thank the authorities of Oil India Limited, Duliajan, Assam for providing subsurface samples and laboratory facility for SEM analysis. We also thank the University Grants Commission, New Delhi for funds. Views expressed in this paper are those of the authors only.

Received 11 December 2010; revised accepted 1 August 2011

Phylogenetic studies in Indian scleractinian corals based on mitochondrial cytochrome *b* gene sequences

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Phylogenetic relationships within and among three genera of the family Acroporidae, namely *Acropora*, *Montipora* and *Astreopora* were examined based on mitochondrial cytochrome *b* (690 bp) gene sequences with special emphasis on Indian scleractinian corals. Analyses using mitochondrial DNA sequences highlight the usefulness of a molecular approach for examining the phylogenetics, evolution and diversity of corals. The analysis based on various algorithms, including neighbour joining and maximum parsimony along with network analysis clearly establishes the monophyly of *Acropora* and *Montipora*. The average

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number of pairwise differences within the three genera revealed maximum intragenus variability in *Acropora*, followed by *Montipora* and *Astreopora*.

Keywords: Cytochrome *b*, Indian scleractinian corals, mitochondrial DNA, phylogenetic studies.

INDIA is one among the 12 mega-biodiversity countries, 25 hotspots, and the richest and highly endangered eco-regions of the world[1]. India has a long record of coastal and marine biodiversity. The Gulf of Mannar (GOM) Biosphere Reserve, a chain of 21 coral islands covering an area of about 10,500 sq. km, from Tuticorin to Rameshwaram, is one of the four major coral-reef areas in India. Venkataraman *et al.*[2] have accounted for 82 species of hermatypic corals under 27 genera from this reef region. The Lakshadweep Islands, another major reef area in India, lie scattered in the Arabian Sea at about 225–450 km from the Kerala coast. These islands consist of coral formations built up on the Laccadive-Chagos submarine ridge rising steeply from a depth of about 1500–4000 m off the west coast of India[2]. A total of 91 species of hermatypic corals under 34 genera are reported from here[2]. These two tropical reefs are recognized by the International Union for Conservation of Nature's (IUCN) Commission of National Parks and Protected Areas. Corals are also protected under Schedule I of the Indian Wildlife Protection Act (1972). Despite this, the coral reefs have suffered progressive decline due to climate change, coral bleaching, over-fishing, habitat destruction, and other anthropogenic factors[3–5].

Acropora (Scleractinia, Acroporidae) is an extremely speciose genus with more than 120 extant species living sympatrically, exhibiting diverse morphologies and both restricted and nearly pantropic distribution[6]. The fossil record shows that the genus probably originated during the Paleocene[7] or the Eocene[8], and became widely distributed in the early Miocene[9]. It is considered to be a very old genus and thus a promising evolutionary model. The diversity and taxonomy of *Acropora* has been studied extensively using morphological characters and fossil records[10,11]. However, this approach has proved insufficient because of several complex ecological and life-history traits of the genus.

The use of modern genetic markers has made novel insights into the traditional knowledge of ecology, evolution and diversity of corals. Although few workers[12–14] have used nuclear DNA sequences to study relationships among the closely related species in the family Acroporidae, only few molecular studies have addressed relationships within reef-building corals. The present study was an attempt to explore and resolve the phylogenetic relationships of three genera (*Acropora*, *Montipora* and *Astreopora*) of the family Acroporidae using mitochondrial (mtDNA) cytochrome *b* (cyt *b*) sequences, and samples from all the species of the three genera found in Indian waters and sequences of the species worldwide available in the public domain. We think the sampling is adequate and exhaustive to explore and add a new dimension (molecular) to the already existing phylogeny. Cyt *b* gene is most widely used for resolving the phylogenetic relationships within and among different species[15].

A total of 70 coral samples representing 24 species belonging to three genera of the family Acroporidae (*Acropora aspera*, *A. austera*, *A. cerealis*, *A. corymbosa*, *A. cytheria*, *A. echinata*, *A. gemmifera*, *A. humilis*, *A. hyacinthus*, *A. latistella*, *A. microphthalma*, *A. millepora*, *A. muricata*, *A. nobilis*, *A. palifera*, *A. samoensis*, *A. tenius*, *A. valida*, *Montipora aquituberculata*, *M. digitata*, *M. divaricata*, *M. foliosa*, *M. hispida* and *Astreopora myriopthalma*) were collected from Rameshwaram, Tuticorin group of Islands, Gulf of Mannar, southeast coast of India and Lakshadweep Islands (Figure 1 and Table 1). Fresh polyps were collected from these sites and suspended in storage buffer containing 4 M guanidine thiocyanate, 0.1% sodium *N*-lauroyl sarcosine and 0.1 M 2-mercaptoethanol in 10 mM tris-HCl buffer, pH 8.0 (ref. 11) and transported to the laboratory at room temperature for genetic analysis. The collected samples were identified with the help of identification manuals[2,16].

Total genomic DNA was isolated from the polyps by conventional phenol/chloroform (1 : 1) extraction method of Sambrook *et al.*[17] and subsequently precipitated with ethanol. The cyt *b* region of mtDNA was PCR amplified using primers designed from the consensus sequences of *Acropora formosa* (AF099651), *Acropora tenius* (AF338425) and *Montipora cactus* (AY903296), already published in the NCBI database. The partial cyt *b* gene (690 bp) was amplified with primers cyt B F-5′-TGA CTA TGG CGA CCG CTT TTC TG-3′ and cyt B R-5′-GCC CAA TTA AAA TAA AGG GCT CTT C-3′. PCR was carried out in a 0.2 ml thin-wall PCR tube with 50 ng of genomic DNA, 10 pM of each primer, 100 mM of dNTPs, 1× PCR buffer, 1.5 mM $MgCl_2$, and 1.0 U Ampli *Taq* Gold (Perkin Elmer, USA). Amplification conditions were as follows: initial denaturation at 95°C for 10 min; 39 cycles at 95°C for 1 min, 55°C for 1 min, 72°C for 2 min and final extension at 72°C for 10 min. PCR products were electrophoresed in 2% agarose gel and quantified using NanoDrop spectrophotometer (NanoDrop Technologies, USA). All amplicons were directly sequenced using BigDye® Terminator and ABI 3730 (Applied Biosystems, USA) DNA analyser. To promote accuracy, both strands were sequenced.

The sequences were initially edited and aligned using Autoassembler 1.4.0 software (Applied Biosystems, USA). The edited consensus sequences (615 bp) were further aligned using Clustalx[18], visually checked and submitted in GenBank (FJ391978–FJ392006).

Initial sequence comparison, measures of variability and phylogenetic relationships among the haplotypes of different species were examined using neighbour-joining

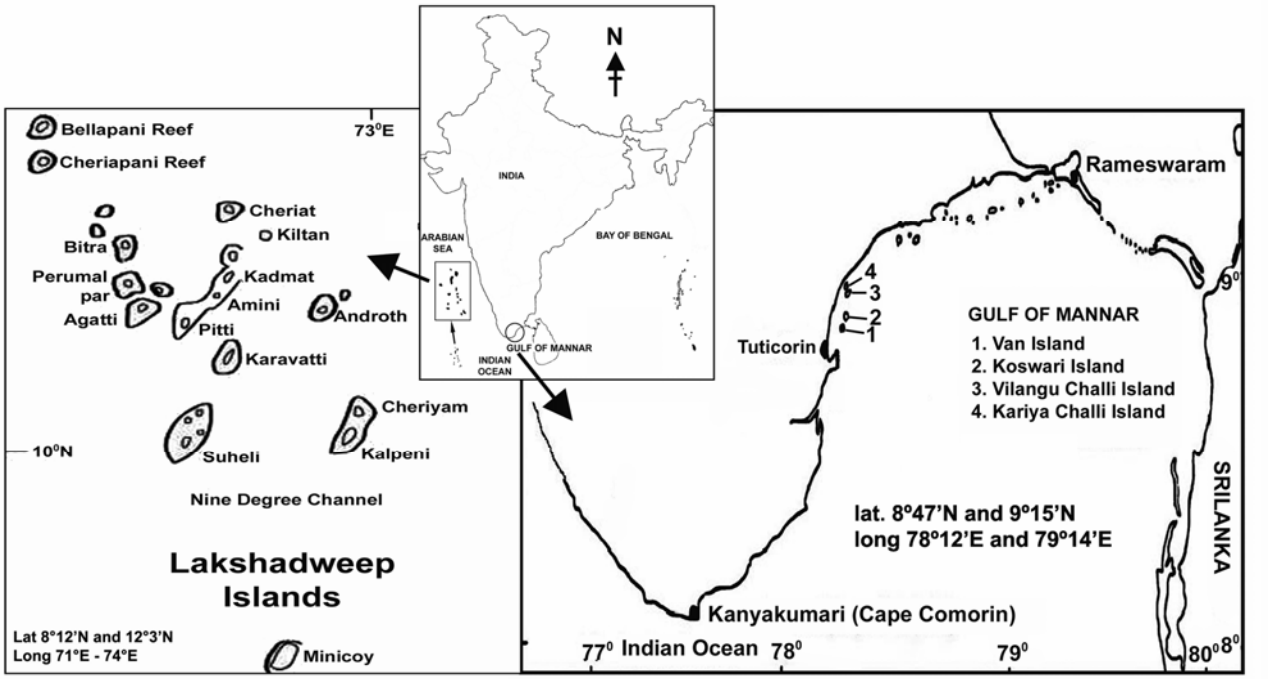

Figure 1. Map showing the sites of sample collection.

Table 1. Samples of Indian scleractinian corals

Species	Location	Number of samples (*N*)
Acropora aspera	LAK (1)	1
Acropora austera	LAK (1)	1
Acropora cerealis	LAK (1)	1
Acropora corymbosa	GOM (2), LAK (1)	3
Acropora cytheria	GOM (2), LAK (1)	3
Acropora echinata	LAK (2)	2
Acropora humilis	GOM (2), LAK (3)	5
Acropora hyacinthus	LAK (2)	2
Acropora gemmifera	GOM (4), LAK (3)	7
Acropora latistella	GOM (2), LAK (3)	5
Acropora muricata	LAK (2)	2
Acropora microphthalma	LAK (2)	2
Acropora millepora	GOM (2), LAK (1)	3
Acropora nobilis	GOM (1), LAK (2)	3
Acropora palifera	GOM (1), LAK (1)	2
Acropora samoensis	LAK (2)	2
Acropora tenius	GOM (1), LAK (2)	3
Acropora valida	GOM (1), LAK (2)	3
Montipora aquituberculata	GOM (2), LAK (1)	3
Montipora digitata	GOM (3), LAK (3)	6
Montipora divaricata	GOM (2), LAK (1)	3
Montipora foliosa	GOM (2), LAK (1)	3
Montipora hispida	GOM (2), LAK (1)	3
Astreopora myriopthalma	GOM (1), LAK (1)	2

LAK, Lakshadweep Islands; GOM, Gulf of Mannar.

(NJ) method[19] based on Kimura two-parameter[20] distance with gamma correction, as implemented in MEGA 4 (ref. 21). Estimation of evolutionary distances of the sequences was computed using Jukes–Cantor method[22]. To find out simplest model of sequence evolution, a maximum parsimony (MP) tree was also constructed using max-mini branch and bound heuristic search in MEGA 4.0. The reliability and robustness of the NJ and MP trees were tested using Felsenstein's bootstrap analysis (based on 500 replicates)[23]. Bootstrap values greater than 50% only were included in the NJ and MP trees. *Poritus poritus* was used as outgroup.

For analysis of genetic divergence within and among groups (species, genera), pairwise F_{ST}, corrected average pairwise differences and percentage variation were computed[24] using the analysis of molecular variance (AMOVA) in Arlequin 3. The transition : transversion (*Ts*/*Tv*) ratio versus genetic distance (Kimura two-parameter[19]) for each pair of haplotype was calculated. Information regarding variable sites, parsimony informative sites, overall *Ts*/*Tv* ratio, and nucleotide base compositions at different codon positions were obtained[21] using MEGA 4. Nucleotide diversity and its variances were calculated according to Nei[25] using DnaSP 3.51 (ref. 26) to analyse the diversification and evolution of various haplotypes in different species of the three genera. A median-joining network of the various haplotypes obtained in the different species of the three genera was drawn using the software Network 4.5.0.0 (ref. 27).

In the present study, a total of 615 sites from the sequence alignment were analysed, after removal of all positions with a gap in any of the mtDNA cyt *b* gene fragment for 70 individuals belonging to the three genera of the family Acroporidae (Table 1). The pairwise comparison of the sequences revealed 21 ($N = 50$), 7 ($N = 18$) and 1 ($N = 2$) haplotypes in *Acropora*, *Montipora* and *Astreopora* respectively (Table 2). These haplotypes were

Table 2. Alignment of cytochrome *b* (cyt *b*) sequences of the different haplotypes of the three genera of Indian scleractinian corals of family Acroporidae. (*Acropora tenuis*, accession no. AF338425, from NCBI database was used as the reference sequence in the alignment shown above. 'L' denotes haplotypes from the Lakshadweep region. Only variable positions are shown.)

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                                    111111 1111111111 1222222222 2222222333 3333333344 4444444444 4444555555 5555555555 556
                             112223577 7889112222 3456677889 9011333445 5677889001 1456679900 0122335567 8889002233 4445566778 890
                            3581479125 8173140369 8432514392 8103147692 8736587035 8240953625 8139586954 0398475817 0365814692 810

Acropora_tenius(AF338425)   CCCCATCGCA ACGATATTTT CCTGCTCTAT TGAAACTGGG TCGATTTACA AAATGTATAA ACTTACATCG ATGTTATTCA ACGAGGGATA GTT
Acropora_tenius             ....G..... .......... .......... .......... .......... .......... .......... .......... .......... ...
Acropora_humilis_I          ...T...... .........C .......... .......... .......... G......... .......... .C........ .......... ...
Acropora_humilis_II         ...T...... .........C .......... .......... .......... G......... .......... .C........ .......C.. ...
Acropora_gemmifera_I        ...T...... .........C .......... .......... .......... G......... .......... .C........ .......... ...
Acropora_gemmifera_II       ...T...... .........C .........A .......... .......... G......... .......... .C........ .......... ...
Acropora_gemmifera_III(L)   ...T...... .........C ..A....... .......... .......... G......... .......... .C........ .......... ...
Acropora_valida             ...T...... .........C .......... .......... .......... G......... .......C.. .C........ .......... ...
Acropora_millepora          ...T...A.. .........C .......... .......... .......... G......... .......... .C........ .......... ...
Acropora_echinata (L)       ...T...... ....G..... .......... .......... .......... .......... .......... .C........ .......... ...
Acropora_microphthalma(L)   ...TG..... .........C .......... .......... .......... G......... .......... .C........ .......... ...
Acropora_aspera (L)         ...TG..... .........C .......... .......... .......... G......... .......... .C........ .......... ...
Acropora_hyacinthus(L)      ...TG..... .........C .......... .......... .......... G......... .......... .C........ .......... ...
Acropora_austera (L)        ...TG..... .......... .......... .......... .......... .......... .......... .C.......T .......... ...
Acropora_cerealis (L)       ...TG..... .........C .......... .......... .......... G......... .......... .C........ .......... ...
Acropora_muricata (L)       .TT.G..... .........C .......... .......... .......... G......... .......... .C........ .....A.... ...
Acropora_latistella         ...T...... .........C .......... .......... .......... G......... .......... .C........ .......... ...
Acropora_samoensis (L)      ...T...... .........C .......... .......... .......... G......... .......... .C...T.... .......... ...
Acropora_cytheria           ...T...... .........C .......... ....G.G... .......... G......... .......... .C........ .......... ...
Acropora_nobilis            ...T...... .........C .......... .......... .......... G......... .......... .C........ .......... ...
Acropora_corymbosa          ...T...... .........C .......... .......... .......... G......... .......... .C........ .......... ...
Acropora_palifera           ...T.C.... .......... ......T... ....GT.... .......... .C......... .......... .C........ T.......C. .C.
Montipora_aquituberculata   ...TGC...T C...CG...C G....CT... C....A...C ...TC..G.. .......C.. .T...TC... .CA..T..T. T...A.C..G .CC
Montipora_digitata_I        ...TGC...T C...CG...C .....CT... C....A...C ...TC..G.. .......C.. .T.A.TC... .CA..T..T. T...A.C..G .CC
Montipora_digitata_II       ...TGC...T C...CG...C .....CT... C....A...C ...TC..G.. ...A...C.. .T.A.TC... .CA..T..T. T...A.C..G .CC
Montipora_digitata_III(L)   ...TGC...T C...CG...C .....CT... C....A...C ...TC..G.. .......C.. .TG..TC..C .CA..T..T. T...A.C..G .CC
Montipora_foliosa           TG.TGC...T C...CG...C .....CT... C....A...C ...TC..G.. .......C.. .T...TC... .CA..T.... T...A.C..G .CC
Montipora_divaricata        TG.TGC...T CTT.CG.AGC .....CT... C....A...C ...TC..G.. .......C.. .T...TC... .CA..T..T. T...A.C..G .CC
Monitpora_hispida           TG.TGC...T C...CG...C .....CT... C....A...C ...TC..G.. .......C.. .T...TC... .CA..T..T. T...A.C..G .CC
Astreopora_myriophthalma    T..T.C...T CT..G..... T....CTC.. C...GA...T C.CT...GTG GT....GC.G GT...T...A GCACCT.CT. T...A..... .CC
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Table 3. Pairwise comparison of genetic distance (F_{ST}) and average pairwise transition–transversion (*Ts*/*Tv*) ratio among the three genera of Indian scleractinian corals of family Acroporidae. Below the diagonal are pairwise genetic distance (F_{ST}) and above it are average pairwise *Ts*/*Tv* ratio between genera

	Acropora	*Montipora*	*Astreopora*	*Porites*	Intragenus
Acropora	–	26.73 : 17	42.61 : 16.28	53.42 : 30.28	11.70
Montipora	0.071	–	33.57 : 14.71	54.57 : 30.42	10.63
Astreopora	0.095	0.078	–	56.00 : 24.00	
Porites	0.136	0.138	0.130	–	

used for further phylogenetic analysis. Of the total 615 sites, 177 were found to be variable, out of which 71 (39.8%) were parsimony informative sites. Most of the variable sites (92) were in the third position of the codon. In addition, substitutions were observed at all 92 sites, out of which 56 were synonymous and the remaining 36 were non-synonymous sites. Transitions (*Ts*) and transversions (*Tv*) at all three codon positions contributed to amino-acid replacement. Transitions (60.45%) and transversions (39.5%) were observed at 107 and 70 sites respectively. The mean *Ts*/*Tv* ratio for overall sequence pair was 1.85 substitutions, which includes all three codons positions. Analysis of average *Ts*/*Tv* ratio in the cyt *b* gene taking pairwise comparison within and among all haplotypes of *Acropora*, *Montipora* and *Astreopora* revealed maximum values for comparison between *Porites* (out group) and *Astreopora*, followed by *Porites* and *Montipora*, and was least between *Montipora* and *Acropora*. These results of *Ts*/*Tv* ratio also indicate that *Acropora*, *Montipora* and *Astreopora* are closely related, but well separated from *Porites* (Table 2).

For testing the genetic structure of scleractinians by AMOVA, the haplotypes of *Acropora*, *Montipora* and *Astreopora* were treated as three groups. AMOVA revealed that 77.68% ($P < 0.01$) of the variation was between groups and 10.63% ($P < 0.01$) of the variation occurred within groups. Within different species, 11.70% ($P < 0.01$) variation was observed. Pairwise comparison of F_{ST} using AMOVA showed that *Acropora*, *Montipora* and *Astreopora* were significantly different ($F_{ST} = 0.0883$, $P < 0.01$). Further, comparisons based on average number of pairwise differences within the three genera, *Acropora*, *Montipora* and *Astreopora*, revealed that the intragenus variability was highest in *Acropora* followed by *Montipora* and *Astreopora* (Table 3). The nucleotide diversity was also found to be highest within *Acropora*, followed by *Montipora* and *Astreopora*.

The pairwise distance in nucleotide transversion substitution among different haplotypes of Indian scleratinian corals showed an overall average of 11.47% (Table 3). Therefore, the genetic distance based on transversion was assumed to be suitable to resolve the genetic relationship

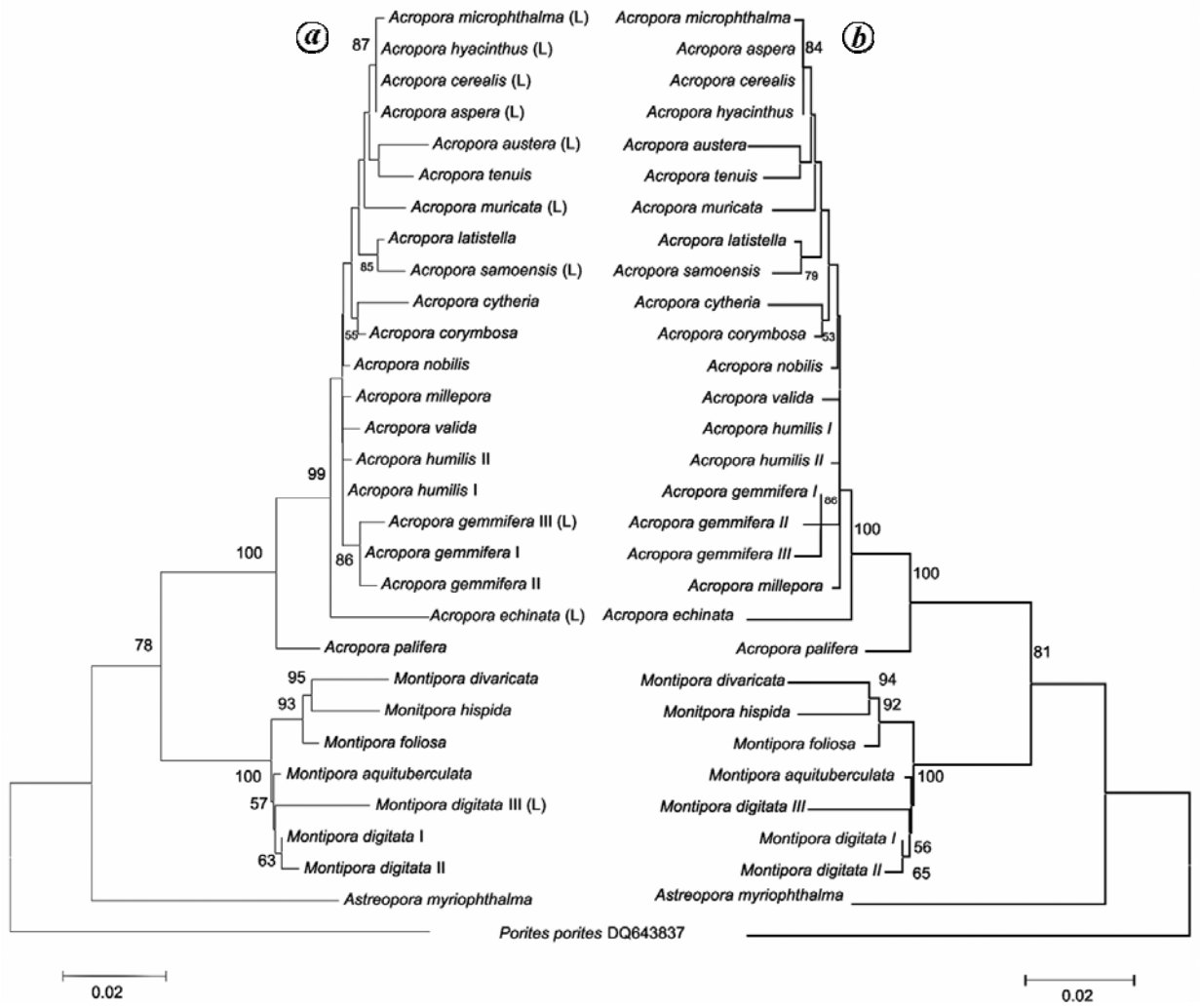

Figure 2. The neighbour-joining (***a***) and maximum parsimony (***b***) tree based on sequences of different haplotypes of cyt *b* in the three genera of Indian scleractinian corals. *Porites porites* was used as the outgroup. Bootstrap values (>50% only) based on 500 replicates are shown. L denotes haplotypes from the Lakshadweep region.

within the genera of Acroporidae. The NJ and MP phylogenetic trees are shown in Figure 2. Both the methods (NJ and MP) used for phylogenetic tree construction resulted in almost similar topologies. Also, both the trees suggest that the family Acroporidae is divided into three major clades composed of 21 haplotypes in the genus *Acropora*, seven haplotypes in *Montipora* and one haplotype in genus *Astreopora*. The species *A. palifera* formed a distinct side branch in the *Acropora* lineage. However, the high bootstrap value suggested that *A. palifera* is phylogenetically close to genus *Acropora*. Similarly, *A. echinata* also formed a side branch in the *Acropora* clade (Figure 2). Phylogenetic analysis based on median-joining network of the different haplotypes also highlights the genetic distinctiveness of the three genera, although there is a sharing of haplotypes within the species of the three genera (Figure 3).

The mitochondrial genomes of scleractinian coral are considered to be uniform in terms of gene content and order[28]. Moreover, the slow rate of evolution of coral mtDNA[29] was generally thought to preclude its use in population genetic studies[30]. The present study shows that the mtDNA cyt *b* gene sequences of 70 corals collected from the Gulf of Mannar and Lakshadweep Islands affiliated to the genera *Acropora*, *Montipora* and *Astreopora* have high levels of intraspecific variation (0–11.70%) in the cyt *b* regions compared to the *Acropora* corals from Okinawa (0–8.36%)[31] and the Caribbean (0–0.6%)[32]. The high intra- and interspecific levels of sequence heterogeneity in *Acropora* have been attributed to introgressive hybridization[33–35].

This phenomenon of introgressive hybridization during speciation has been reported by several group of workers as a characteristic feature in corals. Vollmer and Palumbi[36] reported interspecific hybridization between *A. cervicornis* and *A. palmata*. In the present study also, interspecific hybridization was observed between several species, for e.g. *A. cerealis* and *A. aspera*, as evident from shared haplotypes. Hybridization occurred most readily between species that are morphologically similar.

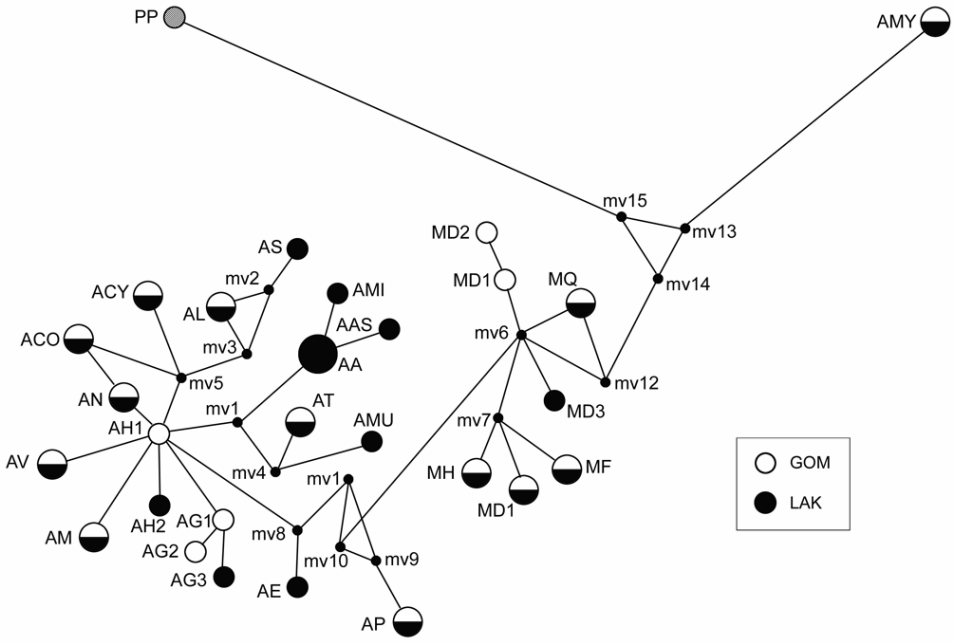

Figure 3. Median-joining network of Indian scleractinian coral haplotypes constructed using the NETWORK programme. The length of the line represents the number of mutational steps; small circles are median vectors. AH1, *Acropora humilis* 1; AH2, *A. humilis* 2; AG1, *A. gemmifera* 1; AG2, *A. gemmifera* 2; AG3, *A. gemmifera* 3; AV, *A. valida*; AM, *A. millepora*; AE, *A. echinata*; AMI, *A. microphthalma*; AA, *A. aspera*; AHY, *A. hyacinthus*; AAS, *A. austere*; AC, *A. cerealis*; AT, *A. tenius*; AMU, *A. muricata*; AL, *A. latistella*; AS, *A. samoensis*; ACY, *A. cytheria*; AN, *A. nobilis*; ACO, *A. corymbosa*; AP, *A. palifera*; MQ, *Montipora aquituberculata*; MD1, *M. digitata* 1; MD2, *M. digitata* 2; MD3, *M. digitata* 3; MF, *M. foliosa*; MDI, *M. divaricata*; MH, *M. hispida*; AMY, *Astreopora myriophthalma* and PP, *Porites porites* (outgroup). MV1–MV15 are mutation sites.

Coral species generally appear to be reproductively isolated from other species in the *Acropora* genus through differences in spawning times. For example, *A. latistella* typically spawns two weeks out of phase with most *Acropora* species[37,38]. Similarly, *A. tenius* is likely to be effectively isolated genetically by a temporal separation in breeding, since it spawns 2–3 h earlier than its congeners[38]. However, in the absence of reproductive barriers and situations like mass spawning, different species of the same genus may hybridize resulting in shared DNA sequences among species[33–35,39–43].

Further, phylogenetic analyses clearly separated *Acropora*, *Montipora* and *Astreopora* into three distinct groups (Figures 2 and 3). It has been suggested that sympatric taxa maintain distinct species boundaries due to accumulation of some fixed differences through evolutionary time as a result of genetic or geographic reproductive barriers[44]. The fixed differences between the different genera in the present study proves the existence of such genetic mechanisms. It has also been suggested that disruptive selection could maintain ecologically differentiated coral morphotypes[45], whereas a certain level of assortative mating can maintain these morphotypes genetically distinct[46].

The important finding in the present study was the significant genetic distance between *A. echinata* from other species of *Acropora*, which suggests their early evolution. The fossil record shows that *A. echinata* has evolved in the Pleistocene era, while many other acroporids were found as fossils in the Eocene to Miocene[47]. *A. palifera* has been already reported to be morphologically distinguished from other *Acropora*[48]. This study provides the genetic evidence for the same.

In conclusion, some of the species in genus *Acropora* shared haplotypes, suggesting that hybridization occurred between these species. However, the three genera were found to be genetically distinct. The existence of fixed differences between sympatric closely related taxa provides evidence for certain intrinsic factors, which help in maintaining them as discrete species *in situ*. Certainly, there is an urgent need to look into the reproductive biology of these taxa to have greater insights into their evolution.

1. Myers, N., Mittermeier, R. A., Mittermeier, C. G., da Fonseca, G. A. B. and Kent, J., Biodiversity hotspots for conservation priorities. *Nature*, 2000, **403**, 853–858.
2. Venkataraman, K., Satyanarayana, Ch., Alfred, J. R. B. and Wolstenholme, J., *Handbook on Hard Corals of India*, Zoological Survey of India, Kolkata, 2003, p. 266.
3. Hughes, T. P. *et al.*, Climate change, human impacts, and the resilience of coral reefs. *Science*, 2003, **301**, 929–933.
4. Pandolfi, J. M. *et al.*, Global trajectories of the long-term decline of coral reef ecosystems. *Science*, 2003, **301**, 955–958.

5. Wilkinson, C. (ed.), New initiatives in coral reef monitoring, research, management and conservation. In *Status of Coral Reef of the World: 2004*, Australian Institute for Marine Science, Townsville, Queensland, 2004, vol. 1, pp. 93–114.
6. Veron, J. E. N., *Corals of the World*, Australian Institute of Marine Science, Townsville, Queensland, 2000, vol. 2, p. 490.
7. Carbone, F., Matteucci, R., Pignatti, J. S. and Russo, A., Facies analyses and biostratigraphy of the Auradu Limestone Formation in the Berbera–Sheikh area, northwestern Somalia. *Geol. Romana*, 1994, **29**, 213–235.
8. Latham, M. H., Jurassic and Kainozoic corals from Somaliland. *Trans. R. Soc. Edinburgh*, 1929, **56**, 273–290.
9. Wells, J. W., Scleractinia. In *Treatise on Invertebrate Paleontology* (ed. Moore, R.), Part F. Coelenterata. Geological Society of America and University of Kansas Press, 1956, pp. 328–478.
10. Veron, J. E. N. and Wallace, C. C., *Scleractinia of Eastern Australia, Part 5, Acroporidae*, Australian Institute of Marine Science Monograph Series 6, 1984, p. 485.
11. Fukami, H., Budd, A. F., Paulay, G., Solé-Cava, A., Chen, C. A., Iwao, K. and Knowlton, N., Conventional taxonomy obscures deep divergence between Pacific and Atlantic corals. *Nature*, 2004, **427**, 832–835.
12. McMillan, J., Mahony, T., Veron, J. E. N. and Miller, D. J., Nucleotide sequencing of highly repetitive DNA from seven species in the coral genus *Acropora* (Cnidaria: Scleractinia) implies a division contrary to morphological criteria. *Mar. Biol.*, 1991, **110**, 323–327.
13. Chen, C. A., Wallace, C. C. and Wolstenholme, J., Analysis of the mitochondrial 12S rRNA gene supports a two-clade hypothesis of the evolutionary history of scleractinian corals. *Mol. Phylogenet. Evol*., 2002, **23**, 137–149.
14. Frank, U. and Mokady, O., Coral biodiversity and evolution: recent molecular contributions. *Can. J. Zool*., 2002, **80**, 1723–1734.
15. Budd, A. F., Romano, S. L., Smith, N. D. and Barbeitos, M. S., Rethinking the phylogeny of scleractinian corals: A review of morphological and molecular data. *Integr. Comp. Biol.*, 2010, **50**, 411–427.
16. Edward, J. K. P., Patterson, J., Venkatesh, M., Mathews, G., Chellaram, C. and Wilhelmsson, D., *A Field Guide to Stony Corals* (*Scleractinia*) *of Tuticorin in Gulf of Mannar*, South East Coast of India, Suganthi Devadason Marine Research Institute Special Research Publication, 2004, vol. 4, pp. 1–80.
17. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, 2nd edn.
18. Thompson, J. D., Gibson, T. J., Plewniack, F. and Jeanmougin, F. D. G., The Clustalx windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*., 1997, **24**, 4876–4882.
19. Saitou, N. and Nei, M., The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, 1987, **4**, 406–425.
20. Kimura, M., A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol*., 1980, **16**, 111–120.
21. Tamura, K., Dudley, J., Nei, M. and Kumar, S., MEGA 4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol*., 2007, **24**(8), 1596–1599.
22. Jukes, T. H. and Cantor, C., Evolution of protein molecules. In *Mammalian Protein Metabolism* (ed. Munro, H. N.), Academic Press, New York, 1969, pp. 21–32.
23. Felsenstein, J., PHYLIP version 3.6 (phylogeny inference package), University of Washington, Seattle, WA, 1994.
24. Excoffier, L., Laval, G. and Schneider, S., Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evol. Bioinf. Online*, 2005, **1**, 47–50.
25. Nei, M., *Molecular Evolutionary Genetics*, Columbia University Press, New York, 1987.
26. Rozas, J., Sanchez-DelBarrio, J. C., Messeguer, X. and Rozas, R., DnaSP. DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, 2003, **19**, 2496–2497.
27. Bendelt, H., Froster, P. and Rohl, A., Median joining network for inferring intraspecific phylogenies. *Mol. Biol. Evol*., 1999, **16**, 37–48.
28. Medina, M., Collins, A. G., Takaoka, T. L., Kuehl, J. V. and Boore, J. L., Naked corals: skeleton loss in Scleractinia. *Proc. Natl. Acad. Sci. USA*, 2006, **103**, 9096–9100.
29. Shearer, T. L., Van Oppen, M. J. H., Romano, S. L. and Worheide, G., Slow mitochondrial DNA sequence evolution in the Anthozoa (Cnidaria). *Mol. Ecol*., 2002, **11**, 2475–2487.
30. Hellberg, M. E., No variation and low synonymous substitution rates in coral mtDNA despite high nuclear variation. *BMC Evol. Biol*., 2006, **6**, 24.
31. Fukami, H., Omori, M. and Hatta, M., Phylogenetic relationships in the coral family Acroporidae, reassessed by inference from mitochondrial genes. *Zool. Sci*., 2000, **17**, 689–696.
32. Van Oppen, M. J. H., Willis, B. L. and Miller, D. J., Atypically low rate of cytochrome *b* evolution in the scleractinian coral genus *Acropora*. *Proc. R. Soc. London, Ser. B*, 1999, **266**, 179–183.
33. Hatta, M. *et al.*, Reproductive and genetic evidence for a reticulate evolutionary history of mass-spawning corals. *Mol. Biol. Evol*., 1999, **16**, 1607–1613.
34. Van Oppen, M. J. H., Willis, B. L., Vugt, H. W. J. A. and Miller, D. J., Examination of species boundaries in the *Acropora cervicornis* group (Scleractinia, Cnidaria) using nuclear DNA sequence analyses. *Mol. Ecol*., 2000, **9**, 1363–1373.
35. Van Oppen, M. J. H., McDonald, B. J., Willis, B. L. and Miller, D. J., The evolutionary history of the coral genus *Acropora* (Scleractinia, Cnidaria) based on a mitochondrial and a nuclear marker: reticulation, incomplete lineage sorting, or morphological convergence? *Mol. Biol. Evol.*, 2001, **18**, 1315–1329.
36. Vollmer, S. V. and Palumbi, S. R., Hybridization and the evolution of reef coral diversity. *Science*, 2002, **296**, 2023–2025.
37. Willis, B. L., Babcock, R. C., Harrison, P. L., Oliver, J. K. and Wallace, C. C., Patterns in the mass spawning of corals on the Great Barrier Reef from 1981 to 1984. In Proceedings of the 5th International Coral Reef Symposium, Tahiti, French Polynesia, 1985, vol. 4, pp. 343–348.
38. Babcock, R. C., Bull, G. D., Harrison, P. L., Heyward, A. J., Oliver, J. K., Wallace, C. C. and Willis, B. L., Synchronous spawning of 105 coral species on the Great Barrier Reef. *Mar. Biol*., 1986, **90**, 379–394.
39. Odorico, D. and Miller, D., Variation in the ribosomal internal transcribed spacers and 5.8 S rDNA among five species of *Acropora* (Cnidaria: Scleractinia): patterns of variation consistent with reticulate evolution. *Mol. Biol. Evol*., 1997, **14**, 465–473.
40. Van Oppen, M. J. H., Catmull, J., McDonald, B. J., Hislop, N. R., Hagerman, P. J. and Miller, D. J., The mitochondrial genome of *Acropora tenius* (Cnidaria; Scleractinia) contains a large group I intron and a candidate control region. *J. Mol. Evol*., 2002, **55**, 1–13.
41. Van Oppen, M. J. H., Willis, B. L., Van Rheede, T. and Miller, D. J., Spawning times, reproductive compatibilities and genetic structuring in *Acropora aspera* group: evidence for natural hybridization and semi-permeable species boundaries in corals. *Mol. Ecol*., 2002, **11**, 1363–1376.
42. Diekmann, O. E., Bak, R. P. M., Stam, W. T. and Olsen, J. L., Molecular genetic evidence for probable reticulate speciation in the coral genus *Madracis* from a Caribbean fringing reef slope. *Mar. Biol.*, 2001, **139**, 221–233.
43. Fukami, H., Omori, M., Shimoike, T., Hayashibara, T. and Hatta, M., Ecological and genetic aspects concerned with reproductive isolation by differential spawning timing in *Acropora* corals. *Mar. Biol*., 2003, **142**, 679–684.

44. Avise, J. C. and Ball, R. M., Principles of genealogical concordance in species concepts and biological taxonomy. *Oxf. Surv. Evol. Biol*., 1990, **7**, 45–67.
45. Willis, B. L., Babcock, R. C., Harrison, P. L. and Wallace, C. C., Experimental hybridization and breeding incompatibilities within the mating systems of mass spawning reef corals. *Coral Reefs* (*Suppl.*), 1997, **16**, S53–S65.
46. Dieckmann, U. and Doebeli, M., On the origin of species by sympatric speciation. *Nature*, 1999, **400**, 354–357.
47. Wallace, C. C. and Rosen, B. R., Diverse staghorn corals (*Acropora*) in high latitude Eocene assemblages: implications for the evolution of modern diversity patterns of reef corals. *Proc. R. Soc. London, Ser. B*, 2006, **273**, 975–982.
48. Randall, R. H., Morphologic diversity in the scleractinian genus *Acropora*. In Proceedings of the 4th International Coral Reef Symposium, Manila, Philippines, 1981, vol. 2, pp. 157–164.

ACKNOWLEDGEMENTS. We thank the Department of Biotechnology and Council of Scientific and Industrial Research, Government of India for financial support. We also thank Dr Ch. Sathyanarayana, Zoological Survey of India, Kolkata for sample collection and identification.

Received 26 April 2010; revised accepted 25 July 2011

Determining the sex of a monomorphic threatened, endemic passerine in the sky islands of southern India using molecular and morphometric methods

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Identifying the sex of an individual is often a basic requirement for many biological studies. This is often critically important for threatened or endangered species that may require different conservation strategies for the two sexes. In many passerine birds, like the threatened, endemic White-bellied Shortwing, *Brachypteryx albiventris*, found only in the Shola forests of the Western Ghats, however, the sexes are often monomorphic and indistinguishable in the field. There has been some conflicting information in the historic incidental records on how the sexes can be identified in this species. We conducted molecular sexing to determine the sexual identity of 99 individuals captured in the field and examined the possibility of using some key morphological and morphometric variables to predict the sex of these individuals. Of all the variables tested, the sexes could be distinguished only by the relatively greater wing and tarsus length of the males. We then examined the sexual identity of 149 individuals that were captured over four years of fieldwork during a long-term study of this species. This study thus provides important baseline data for an ongoing exploration of the ecology and demography of the two sexes of this unique threatened bird.

Keywords: Molecular sexing, monomorphic and threatened species, morphometric variables, Shola forest.

KNOWING the sexual identity of an individual is often a basic requirement for any biological study, whether at the level of a population or an individual. This usually becomes critically important in ecological and behavioural field studies[1]. In population demography, for example, it is essential to know the sex of individuals as there may be sex-specific dispersal or mortality[2]. In some cases, the two sexes may have different methods of foraging[3] and may even experience differential predation pressures[4]. Identifying the sex of an individual is thus clearly necessary for a complete understanding of many natural processes.

Many species of birds are, however, monomorphic and difficult to sex visually, particularly in the field and some even in hand. Some examples are the Hill Mynah, *Gracula religiosa* and the Black-capped Chickadee, *Parus atricapillus*. The White-bellied Shortwing, *Brachypteryx albiventris* in the Western Ghats is one such species. The Shortwing is a small bird (< 25 cm, < 25 g) found only in the dense understorey (within 2 m off the forest floor) of the Shola forests in the high-elevation sky islands of the Western Ghats[5]. Being a highly specialized species adapted to a unique habitat, its biology is likely to have been shaped by various ecological processes, which may have also acted differentially on the two sexes. The Shortwing is also a threatened, endemic species which was thought to be rare, with less than 100 sightings in 150 years, until recently[6]. Like most endemic, understorey bird species, the Shortwing may also be sensitive to habitat disturbance[7]. Hence understanding the impact of various ecological processes, both natural and more recently anthropogenic, is likely to be of critical importance in developing conservation strategies for the species. Such strategies may also need to consider individual life-history traits, which usually differ between the two sexes. Establishing the sexual identity of individuals is thus essential for any field study of this bird. Previous information[8] and our own studies[5,6,9] have, however, clearly established that the sexes of this species cannot be visually distinguished during non-invasive observational studies in the wild.

In some avian species, the sex of captive individuals can be determined by examining the cloacal protrusion[10,11], but this method involves examining the angle of the cloaca. The male cloaca is directed upwards, whereas the female one points backwards. This method is, however, difficult to execute unless adequately trained with sexually dimorphic birds[10]. Cloacal protrusions are also known to enlarge

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