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Multi-locus sequence data reveal a new species of coral reef goby (Teleostei: Gobiidae: *Eviota*), and evidence of Pliocene vicariance across the Coral Triangle

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Here, multi-locus sequence data are coupled with observations of live colouration to recognize a new species, *Eviota punyit* from the Coral Triangle, Indian Ocean and Red Sea. Relaxed molecular clock divergence time estimation indicates a Pliocene origin for the new species, and the current distribution of the new species and its sister species *Eviota sebreei* supports a scenario of vicariance across the Indo-Pacific Barrier, followed by subsequent range expansion and overlap in the Coral Triangle. These results are consistent with the ‘centre of overlap’ hypothesis, which states that the increased diversity in the Coral Triangle is due in part to the overlapping ranges of Indian Ocean and Pacific Ocean faunas. These findings are discussed in the context of other geminate pairs of coral reef fishes separated by the Indo-Pacific Barrier.

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Key words: centre of overlap; dwarfgoby; Indo Australian Archipelago; Indo Malay Archipelago; systematics.

INTRODUCTION

The gobiid genus *Eviota*, commonly known as dwarfgobies, is one of the most diverse genera of marine fishes in the world. There are currently 96 valid species of *Eviota* and many more new species await description. With an evolutionary origin estimated between 6.5 and 16.8 million years ago (MYA) (Tornabene *et al.*, 2015), *Eviota* represents one of the most rapidly diversifying lineages of marine vertebrates, perhaps second only to the gobiid genus *Trimma*, which probably comprises close to 200 species (Winterbottom *et al.*, 2014; Winterbottom & Hoese, 2015). Through the recent increase in the quantity and quality of live underwater photographs of species of *Eviota* and the continued use of multi-locus DNA sequence data and molecular phylogenetic analysis, a clearer picture of the true diversity within the genus is being generated (Tornabene *et al.*, 2013b; Greenfield & Tornabene, 2014).

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Much of the recently described diversity within *Eviota* has been from the Coral Triangle (Greenfield & Erdmann, 2013, 2014; Greenfield & Jewett, 2014; Greenfield & Tornabene, 2014), the biodiversity hotspot that straddles the border between the western Pacific and eastern Indian Oceans and is home to the most diverse assemblage of coral reef fishes in the world (Allen & Erdmann, 2012). The Coral Triangle (also referred to as the Indo-Malay Archipelago, the Indo-Australian Archipelago and other names) includes the Philippines, Indonesia, Malaysian Borneo, Timor-Leste, Papua New Guinea and the Solomon Islands (Veron *et al.*, 2009). Marine species richness decreases with increasing distance from the Coral Triangle both in latitude and longitude (Stehli & Wells, 1971; Veron, 1995; Briggs, 1999; Mora *et al.*, 2003; Bellwood *et al.*, 2012). Several hypotheses have been proposed to explain this pattern of diversity (Bellwood *et al.*, 2012), and the three hypotheses that have received the most attention include: (1) the centre of origin hypothesis, which purports that most species in the region originated within the Coral Triangle, and that the exceptional diversity produced within the hotspot provides a source of biodiversity to adjacent regions; (2) the centre of accumulation hypothesis, which states that species arise outside of the Coral Triangle and accumulate within the present-day hotspot *via* dispersal of individual species (Ladd, 1960) or *via* the movement of entire faunal assemblages following tectonic events (Remington, 1968; McKenna, 1973; Santini & Winterbottom, 2002); (3) the centre of overlap hypothesis, which suggests that high species richness within the Coral Triangle is a by-product of the overlapping distributions of Pacific Ocean fauna and Indian Ocean fauna, with species forming *via* vicariance when the basins were largely separated from one another by the Indo-Pacific Barrier, and subsequently expanding their ranges to overlap within the Coral Triangle (Woodland, 1983). In addition, the Coral Triangle has also been hypothesized to be a 'centre of refuge' for species during glacial cycles due to the temporal stability of coral habitats relative to peripheral areas (Heck & McCoy, 1978; Bellwood & Hughes, 2001; Pellissier *et al.*, 2014). Each hypothesis is supported by a growing body of evidence (Carpenter *et al.*, 2011; Briggs & Bowen, 2013; Gaither & Rocha, 2013), and it has become apparent that the above processes are not mutually exclusive and may occur in concert over differing temporal scales (Mironov, 2006; Barber, 2009; Halas & Winterbottom, 2009; Bowen *et al.*, 2013; Cowman & Bellwood, 2013a).

The exceptional diversity of *Eviota* in the Coral Triangle is due in part to several lineages exhibiting bursts of recent speciation occurring at small geographic scales within the region over the last 1.5 million years, resulting in the formation of numerous endemic species with highly restricted ranges (Tornabene *et al.*, 2015). Although their data provide support for the centre of origin hypothesis, the study by Tornabene *et al.* (2015) focused exclusively on species complexes whose ranges were generally contained within the Coral Triangle, thus precluding their ability to empirically evaluate the importance of the other 'centre of' hypotheses in the evolution of *Eviota*. There are indeed many other species within *Eviota* that have wide ranges that extend well beyond the Coral Triangle (Lachner & Karnella, 1980). One such species is *Eviota sebreei* Jordan & Seale 1906. Prior to the current study, the known range of *E. sebreei* (based solely on examination of preserved specimens) was from the Red Sea east to Samoa (Lachner & Karnella, 1980). Many wide-ranging species of cryptobenthic reef gobies that were described without a comprehensive understanding or appreciation of variation in live colouration have subsequently proved to be complexes of morphologically similar but genetically distinct 'cryptic' species, often times with more restricted

geographic ranges, and frequently identifiable only by live colouration (Victor, 2010; Greenfield & Tornabene, 2014; Winterbottom *et al.*, 2014; Tornabene *et al.*, 2015). As noted in Allen & Erdmann (2012) and numerous recent fish identification guides produced by divers, *E. sebreei* has been considered to comprise two distinct colour morphs, one of which possesses a black lateral stripe and the other with a red lateral stripe. Importantly, no intermediate colour morphs have been observed (pers. obs.) or reported in the literature. During a biodiversity survey conducted off Negara Brunei Darussalam in April 2014, both red and black morphs of *E. sebreei* were observed resting on adjacent coral colonies (often <1 m apart), and it was noted that not only were the red morphs consistently larger, but also showed a different behaviour to the black morphs (M. V. Erdmann, pers. obs.). With suspicions aroused that these two colour morphs might actually represent two distinct species, a conscious effort was made to photograph and collect a number of specimens of both colour morphs, with a sub-set of each of these preserved for genetic sampling.

The purpose of this study was to investigate the taxonomic and geographic species boundaries of *E. sebreei* and to confirm or reject the presence of a second closely related species. The study used multi-locus DNA sequence data, traditional morphological analysis and observations of live colouration to describe a new species, *Eviota punyit*. Finally, the geographic ranges and divergence times of these species are discussed in light of the geologic history of the Coral Triangle and processes contributing to the exceptional diversity within the region.

MATERIALS AND METHODS

Four specimens originally identified as the red colour morph of *E. sebreei* were included in the genetic analysis from Brunei (CAS 238168), the Banda Sea (CAS 238169) and West Papua (CAS 238170). Additional formalin-fixed specimens were included for morphological analysis. The molecular analysis also included seven specimens of the black morph of *E. sebreei* from Wasir (Aru Islands, Indonesia), Pohnpei (Micronesia) and Brunei (sympatric with specimens of the red morph). DNA was extracted from tissue samples taken from whole specimens stored in 95% ethanol using the Qiagen DNeasy Blood and Tissue Kit (Qiagen; www.qiagen.com). A segment of the mitochondrial gene *cytochrome c oxidase subunit I (coi)* was amplified and sequenced using the primers GOBYL6468 and GOBYH7696 (Thacker, 2003). The nuclear locus *protease III (Ptr)* was also sequenced using the primers PtrF2 and PtrR2 (Yamada *et al.*, 2009). The PCRs for both genes used Promega GoTaq Hot Start Master Mix (www.promega.com) and a thermal profile that consisted of 95° C, followed by 35 cycles of 40 s at 95° C, 40 s at 54° C and 90 s at 72° C with a single extra extension period of 5 min at 72° C. Purification and sequencing of PCR products were performed by Beckman Coulter Genomics (www.beckmangenomics.com). Sequences were assembled and aligned in Geneious (ver. 6.0.6) (Biomatters; www.geneious.com). Sequences from this study were combined with those from Tornabene *et al.* (2013a, 2015). New sequences from this study are deposited in GenBank (Appendix). The final alignment consisted of 1204 bp of *coi* and 609 bp of *Ptr*.

Simultaneous Bayesian inference of phylogenetic relationships and divergences times were performed using an uncorrelated log-normal relaxed molecular clock model in BEAST (ver. 2.1.2) (Bouckaert *et al.*, 2014). In the absence of appropriate fossil calibration points, a secondary calibration point was used to date the crown age of *Eviota* (median age 10.63 MYA, 95% highest posterior density interval 6.5–16.8 MYA) based on estimated divergence times from Tornabene *et al.* (2015). The calibration prior was set to closely mimic this age distribution via a gamma distribution prior with a median of 10.6 MYA, and a 95% quantile of 6.9–18.8 MYA (offset = 5.7, gamma shape parameter = 2.3, gamma scale parameter = 2.5). The HKY + I + G and GTR + I + G substitution models were used for the *Ptr* and *coi* partitions, respectively, based on AIC model fit scores calculated using jModeltest vers.2.1.1 (Darriba *et al.*, 2012). The BEAST

Markov Chain Monte-Carlo was run for 10 million generations, using Tracer (Rambaut *et al.*, 2014) to confirm stationarity and mixing. A maximum clade credibility tree was produced from the posterior distribution of trees. Four independent runs were generated, and topologies, node heights and posterior probabilities were visually inspected across runs to ensure convergence. A maximum likelihood tree was also generated using RAxML ver. 8 (Stamatakis, 2014) to provide an independent assessment of topology. The maximum likelihood analysis used GTR + G substitution models for each partition and support for nodes was evaluated using 1000 bootstrap replications. Lastly, individual gene trees were inferred using MrBayes ver. 3.2 (Ronquist *et al.*, 2012) using the models from jModeltest, to confirm that the pattern of divergence between colour morphs was present in both genes. MrBayes analyses were run for 10 000 000 generations, sampling trees every 1000 generations, and convergence and mixing were assessed using Tracer.

To evaluate the contemporary distribution of the two colour morphs, the *E. sebreei* distribution map of Lachner & Karnella (1980) was used as a base map. Additional localities were added based on records of museum specimens of *E. sebreei* retrieved from Fishnet2 (www.fishnet2.net), which searches collection data for 73 natural history collections. These points were supplemented with searches of records from museums outside of the Fishnet2 database (*e.g.* American Museum of Natural History and South African Institute of Aquatic Biodiversity fish collections). Multiple collection records from localities in close proximity to one another are represented by a single point on the resulting distribution map. Whenever data were available on the live colouration of the specimens (either through associated photographs or colour descriptions), this information was incorporated into the distribution map.

Methods for measuring meristics, morphometrics and overall morphological descriptions follow Lachner & Karnella (1980) and Jewett & Lachner (1983). Counts of second dorsal and anal rays include only segmented rays and do not include the first fin element. 'The membrane joining the fifth pelvic-fin rays is always short and weakly developed and the fins lack a frenum. The membranes joining the first four [pelvic] fin rays are considered to be well developed when the membranes extend beyond the bases of the first branches; they are considered to be reduced when they are slightly developed, not extending to the bases of the first branches' and 'The lateral scale row count was made from the upper edge of the gill opening posteriorly to the scale overlying the end of the hypural base' (Lachner & Karnella, 1980). Measurements were made using dial callipers to the nearest 0.1 mm and are presented as per cent of standard length (L_S). Specimen lengths are L_S in mm. Measurements of the holotype are given first followed by the range for all measured specimens and the mean in parentheses. Meristic counts of the holotype are given first followed by the counts for the entire type series in parentheses. Abbreviations for institutional acronyms are as follows: CAS, California Academy of Sciences, USA; NMW, Natural History Museum Vienna, Austria.

RESULTS

The phylogeny resulting from the BEAST analysis shows strong support (posterior probability = 1.0) for the reciprocal monophyly of the red- and black-striped colour morphs of *E. sebreei*, confirming that the two morphs are genetically distinct and represent two evolutionarily distinct species (Fig. 1). Posterior probabilities across the concatenated Bayesian tree are high (>0.9) with only a few exceptions, and nearly all clades are in agreement with the topology from Tornabene *et al.* (2015). Support values are more variable on the RAxML tree (see Dryad repository data package; Dryad Repository, <http://dx.doi.org/10.5061/dryad.n0v50>), although the relationships that were strongly supported (bootstrap >0.85) on the RAxML tree are also found in the Bayesian analysis, including the reciprocal monophyly of the red and black colour morphs. The divergence between the red and black colour morphs based on the BEAST analysis was estimated at 3.1 MYA (95% highest posterior density, HPD, interval 1.5–5.8 MYA).

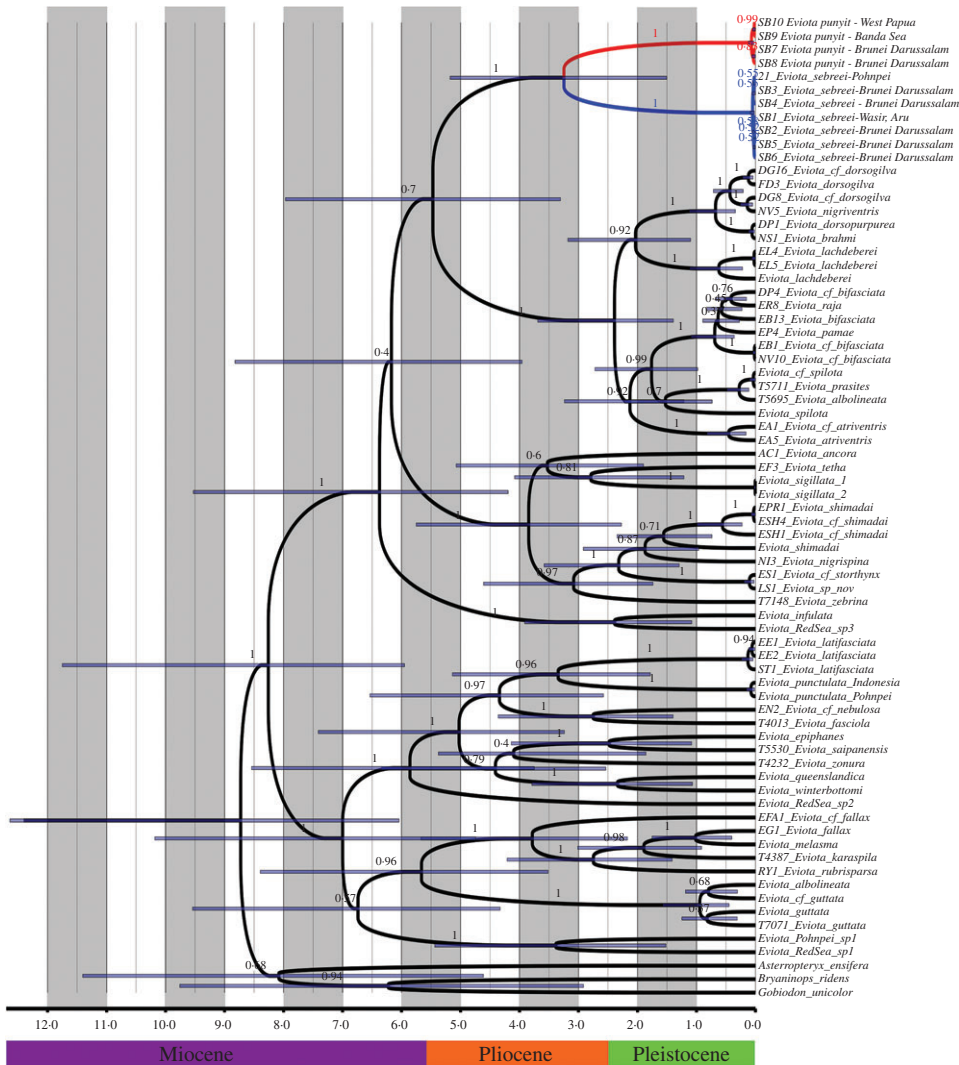


FIG. 1. Time calibrated phylogeny from BEAST. Branch labels are Bayesian posterior probability support values. Node bars represent 95% highest posterior density intervals for divergence times.

Independent phylogenetic analysis of each gene (see Dryad repository data package) show strong support for the monophyly of each colour morph, corroborating the results from the concatenated analysis. The only major difference between genes trees and the concatenated BEAST tree is that a sister relationship between the red and black morph is not recovered in the *coi* gene tree, as many internal nodes across the phylogeny are poorly resolved. This is undoubtedly due, in large part, to homoplasy and possible third codon saturation associated with the hyper-variable nature of this gene in the rapidly evolving genus *Eviota*. This result is similar to past studies, where *coi* alone was not able to resolve many clades within the genus (Tornabene *et al.*, 2013b). The *Ptr* gene tree has a very similar topology to that of the concatenated data set, albeit

with some nodes showing lower support; however, there is still strong support for the reciprocal monophyly of the two colour morphs. The pair-wise genetic distances for *coi* are extremely low within colour morphs (<1%) and very high between colour morphs (20.7–21.8%). Pair-wise genetic distances for *Ptr* are low to non-existent within colour morphs (0–0.3%) and moderate between colour morphs (1.6–1.9%); nine fixed sites in the *Ptr* gene unambiguously diagnose the red morphs from the black morphs.

In order to determine which of the two morphs corresponds to the true *E. sebreei* and which represents the new species, a map of the known distribution of *E. sebreei* was produced and augmented with data on the respective distributions of the black and red colour morphs (Fig. 2). This map reveals two distinct distributions which broadly overlap in the Coral Triangle region, with the black colour morph of *E. sebreei* extending from Fiji and Tonga west to the Maldives, but absent in the western Indian Ocean and Red Sea. By comparison, the red colour morph extends from the Red Sea east to West Papua, Indonesia, but is otherwise absent from the western and central Pacific Ocean. While records of live coloration are unavailable from Samoa, the type locality of *E. sebreei*, this study concludes that the black morph is the true *E. sebreei* based both upon the fact that populations in neighbouring Fiji and Tonga are black and because there is no evidence that the red morph extends into the Pacific. Based on this evidence, it is concluded that the evolutionarily distinct red morph represents a new species, described below as *Eviota punyit*.

EVIOTA PUNYIT N. SP. TORNABENE, VALDEZ & ERDMANN

Punyit dwarfgoby (Figs 3–8)

Holotype. CAS 238167, 18.1 mm female, Porter Patch, Brunei Darussalam, 04° 53.475' N; 114° 24.321', 21 April 2014, M. V. Erdmann.

Paratypes. CAS 237333, two males 17.8–18.8 mm, five females 16.1–18.1 mm, collected with holotype; CAS 238168, two males 17.9–18.9 mm, DNA vouchers SB7 and SB8, collected with holotype; CAS 238170, one female 18.3 mm, DNA voucher SB10, patch reef off Mommon Peninsula, Kaimana, West Papua, Indonesia, 03° 55.703' S; 132° 49.757' E, 8 October 2014, M. V. Erdmann; CAS 238171, two females, 18.2–18.6 mm, patch reef off Mommon Peninsula, Kaimana, West Papua, Indonesia, 03° 55.703' S; 132° 49.757' E, 8 October 2014, M. V. Erdmann; CAS 238169, one female 12.9 mm, DNA voucher SB9, Banda Harbour, Pulau Banda, 04° 30.794' S; 129° 53.589' E, 7 October 2014, M. V. Erdmann; NMW 94957, 14.7 mm, 'Lighthouse', Dahab, Gulf of Aqaba, Northern Red Sea, May 2004, J. Herler; NMW 94956, 14.3 mm, 'Islands', Dahab, Gulf of Aqaba, Northern Red Sea, November 2003, J. Herler.

Diagnosis. Body long and slender with distinctly pointed head; cephalic sensory pore pattern 6 (lacking NA, PITO and IT pores); in life, prominent red lateral stripe beginning on snout and extending to caudal peduncle.

Description: dorsal-fin rays VI+I,9 [9(9), 8(3)]; anal-fin rays I,8 [8(11), 7 (1)]; pectoral-fin rays 17 [15(1), 16(7), 17(3)], all unbranched; the length of fifth pelvic

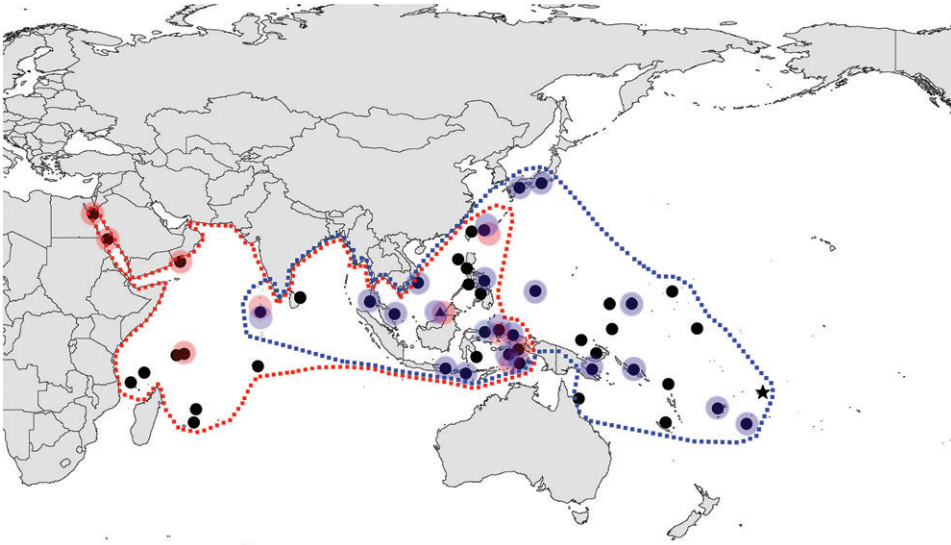


FIG. 2. Distribution of the *Eviota sebreei* species complex. ●, locality of either *E. sebreei* or *E. punyit*, based on museum collections. Localities where species are confirmed based on photographs of fresh and live specimens or colour descriptions are labelled in red (*E. punyit*) and blue (*E. sebreei*). ▲, type locality of *E. punyit*; ★, type locality of *E. sebreei*. Hypothesized ranges are drawn in dotted lines (....., *E. punyit*;, *E. sebreei*).

ray 50% (40–80%) that of fourth pelvic ray; fourth pelvic ray with 12 [11(1), 12(2), 13(3), 14(4), 15 (1)] primary branches; fourth pelvic ray with 0–1 segments between consecutive branches; pelvic-fin membranes well developed; 17 branched and 12 segmented caudal-fin rays [17/12 (6), 16/12 (1), 16/11 (2)]; lateral scale rows 24 [22(2), 23(5), 24(5)]; transverse scale rows 6 [6(7), 7(5)]; cycloid or reduced ctenoid scales on ventral surface of abdomen; first dorsal fin triangular in shape, no spines notably elongate or filamentous; genital papilla in male smooth, long and not fimbriate, extending to the base of the first or second element of the anal fin (Fig. 6); female papilla smooth and bulbous with elongated projections on lateral edges of tip, with several shorter projections medially (Fig. 6); gill opening extending to just below pectoral-fin base; cephalic sensory-pore system pattern 6 with the NA, PITO and IT pores missing, and papillae pattern C (as illustrated for *E. sebreei* in Lachner & Karnella, 1980)

Measurements and sexual dimorphism (based on holotype and 11 other paratypes). Head length 25.4 (25.4–30.6, 27.2); origin of first dorsal fin 34.3 (32.4–36.3, 34.1); origin of second dorsal fin 59.2(54.7–59.1, 57.0); origin of anal fin 64.1 (58.0–65.4, 62.4); caudal-peduncle length 21.5 (19.2–24.8, 22.3); caudal-peduncle depth 10.4 (9.9–12.4, 11.3); body depth 17.4 (17.0–22.4, 19.0); eye diameter 8.3 (7.4–9.3, 8.1); snout length 5.4 (4.9–7.0, 5.9); pectoral-fin length 21.0 (21.0–28.7, 24.9); pelvic-fin length 25.6 (20.9–31.0, 26.0); shape of head and size of jaw sexually dimorphic (Fig. 7); females with front of head steeply sloped, short jaws that never

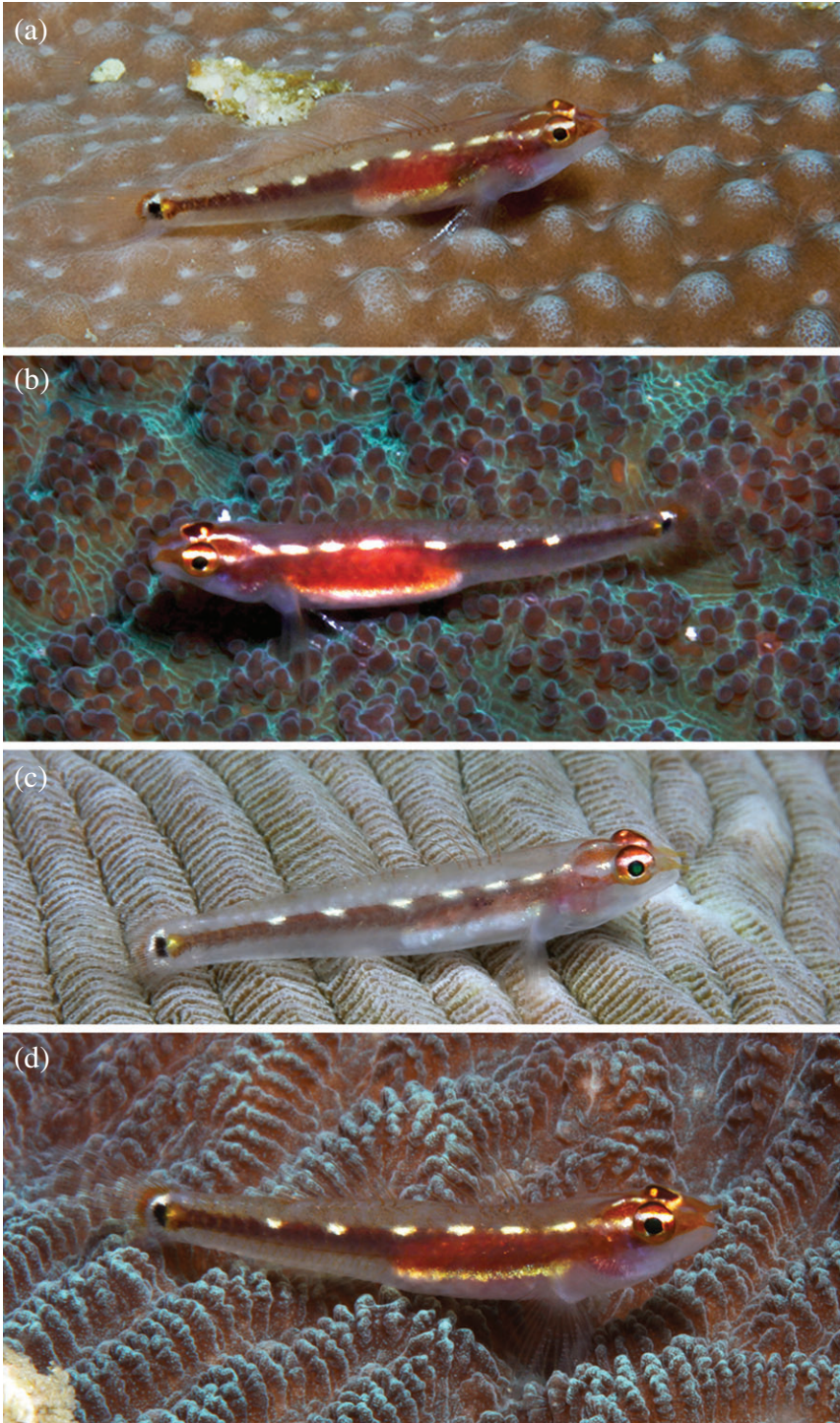


FIG. 3. *Eviota punyit* from localities with genetic samples: (a) West Papua, (b, d), Brunei Darussalam and (c) Banda Sea. Photos by M. V. Erdmann. See online publication for color version of figure.



FIG. 4. *Eviota punyit* and *Eviota sebreei* from Brunei Darussalam, co-occurring on the same reef. Photos by M. V. Erdmann. See online publication for color version of figure.

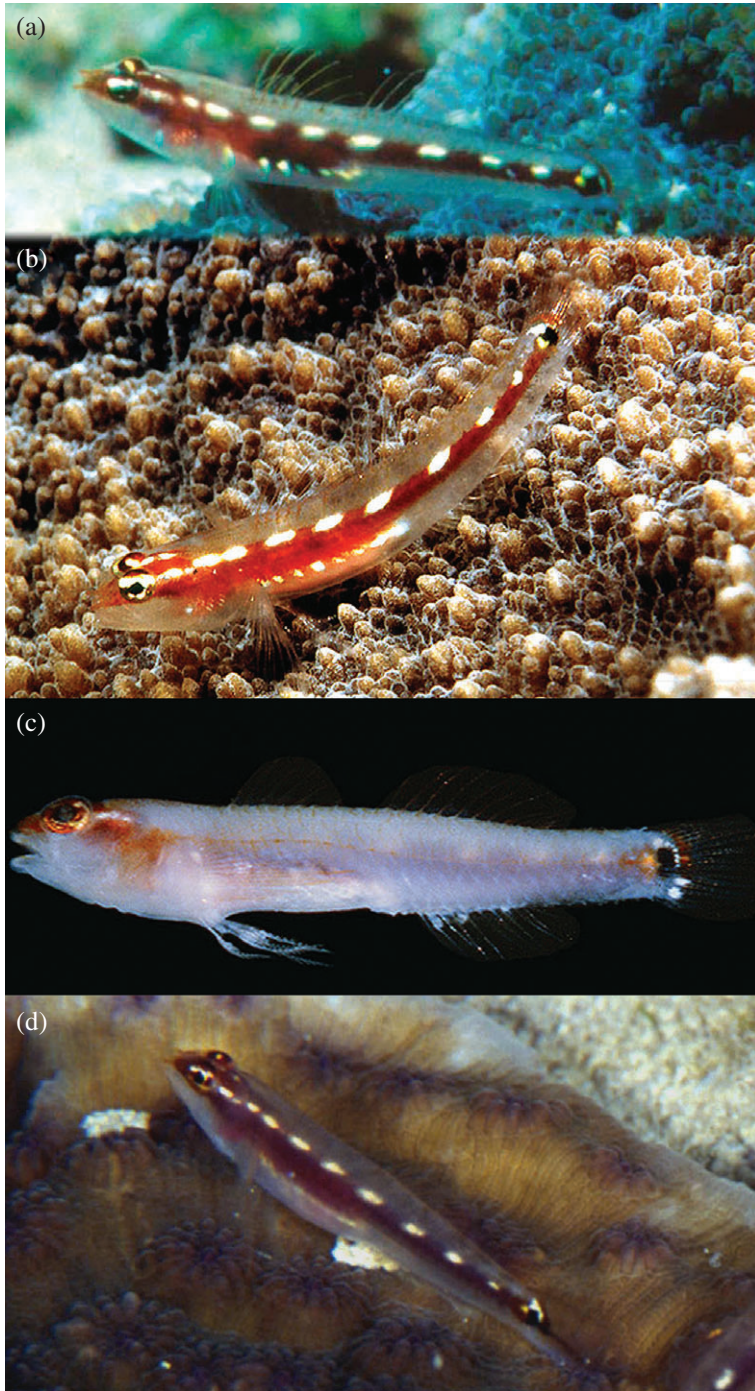


FIG. 5. *Eviota punyit* from localities without genetic samples outside the Coral Triangle: (a) Oman, photo by Richard Field (b) Seychelles, photo by John E. Randall (c) Red Sea, shortly after capture (lateral stripe faded), photo by John E. Randall (d) Okinawa, KPM-NR0067690AF ©Kanagawa Prefectural Museum of Natural History, photo by Yoko Kobayashi. See online publication for color version of figure.

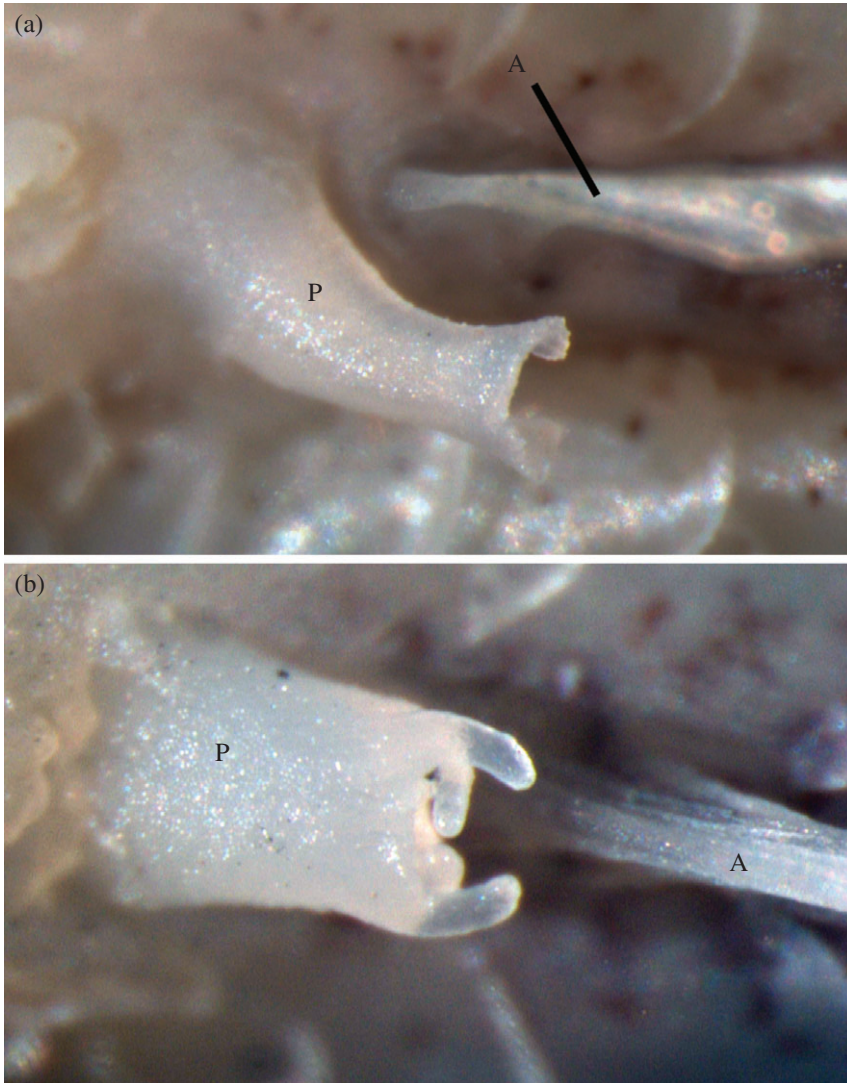


FIG. 6. Urogenital papilla of *Eviota punyit* (a) male and (b) female. A, anal fin; P, urogenital papilla.

extend posteriorly to a point beyond the posterior margin of pupil, often falling well short of this and reaching point below middle of pupil, jaw length 9.3 (8.2–9.9, 9.2); males with front of head more pointed, jaws angled slightly more upwards than in females, at an angle of *c.* 20–30°, jaws large and extending posteriorly to a point below posterior margin of pupil or beyond, mean jaw length of male paratypes 12.9 (12.4–14.1); anterior tubular nares long and unpigmented, reaching to anterior edge of upper lip or nearly so.

Colour in life (Figs 3–5). Head and body with prominent red lateral stripe extending from tip of snout posteriorly to end of caudal peduncle; lateral stripe widest and

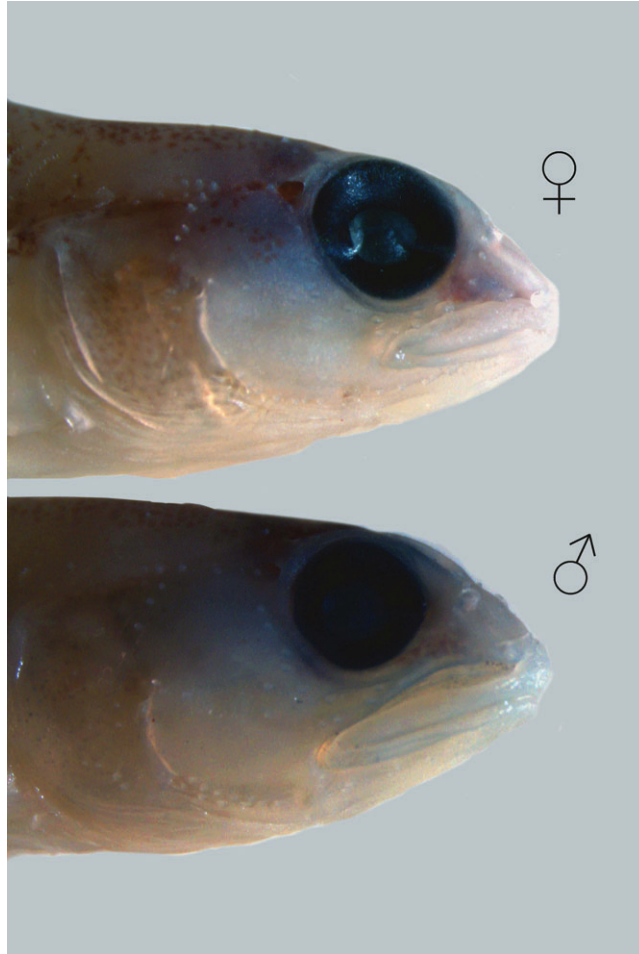


FIG. 7. Sexual dimorphism in head shape and jaw of *Eviota punyit*. Note the larger jaw in males.

brightest on trunk behind pectoral fin and below first dorsal fin, becoming more narrow and dark (sometimes maroon or brownish) posteriorly; anterior half of nape reddish with narrow bright white medial stripe along dorsal midline, posterior half of nape pale; ventral half of head entirely pale, upper half of head reddish brown, including nares tubes; eye reddish with two lateral stripes, upper stripe bright white and passing through upper margin of pupil, lower stripe yellowish and passing through lower margin of pupil; lower posterior corner of preopercle sometimes with small round iridescent white spot; prominent short white stripe posterior to eye, extending to above preopercle; a series of seven or eight bright iridescent white spots or dashes alongside of body at upper margin of red stripe, white spots more elongated anteriorly and shorter posteriorly, anteriormost spot located immediately behind post-orbital stripe, posteriormost white spot minute (sometimes absent), at posterodorsal corner of caudal peduncle; a narrow iridescent white stripe on abdomen below red stripe, extending posteriorly to below second dorsal fin; sides of body above and below red stripe pale



FIG. 8. Preserved holotype of *Eviota punyit*.

or translucent; pectoral-fin base uniformly pale, occasionally with an iridescent white patch on posterodorsal corner; pectoral-fin rays pale and lacking pigment; dorsal-fin elements red with transparent unpigmented membranes; caudal-fin base with prominent black spot bordered anteriorly by a bright yellow spot, and bordered on all other sides by an iridescent white margin, which itself is bordered posteriorly by a narrow reddish brown semicircular band, remainder of caudal fin unpigmented; anal fin and pelvic fins unpigmented.

Colour in preservative (based on holotype, Fig. 8). Background colour of head and body pale; upper half of head and nape uniformly covered with chromatophores, lower half of head below eye pale except for a light scattering of pigment on the posterior margin of operculum; dorsal surface of body with scattered chromatophores that are concentrated around scale margins; scale margins becoming less pigmented ventrally towards the lateral midline, becoming completely pale below midline; lateral midline with a very thin distinct vertical stripe of chromatophores; a very faint indication of a brown subdermal broad lateral stripe (red in life) on body below first dorsal fin; first dorsal fin with narrow horizontal stripe of chromatophores across base of interspinal membranes, remainder of fin without pigment except for a few chromatophores on distal tips of third and fourth spines; second dorsal fin with interradiated membranes uniformly washed with dark chromatophores; caudal-fin base with distinct black, slightly vertically elongated blotch (height slightly shorter than diameter of eye), bordered posteriorly by a white margin followed by a narrow brown margin (across upper half of fin only), middle rays of caudal fin with dusting of chromatophores over their entire length, rays of dorsal and ventral one third of fin without pigment; anal fin with interradiated membranes covered with dark chromatophores except at distal tips, which are pale; pelvic fins unpigmented; pectoral-fin base sometimes with narrow vertical band of pigment near base of rays, remainder of pectoral rays unpigmented.

Distribution. *Eviota punyit* is definitively known from Brunei Darussalam, the Banda Sea and West Papua based on photographic and genetic data presented here. Based on photographs or observations of live or freshly collected specimens, *E. punyit* also

occurs in the Red Sea (Fig. 5; Herler & Hilgers, 2005), the Seychelles (Fig. 5; Randall & van Egmond, 1994), the Maldives (Randall & Goren, 1993), Oman (Fig. 5; Randall, 1995), Halmahera (Indonesia; G. R. Allen, pers. comm.) and the Ryukyu Islands in Japan (Fig. 5; Senou, 2004).

Ecology. Although *E. punyit* is broadly sympatric with *E. sebreei* in the Coral Triangle region and occasionally the two species co-occur on the same reefs (most notably on the shallow coastal patch reefs of Brunei Darussalam), extensive *in situ* observations indicate clear differences in habitat preference and behaviour between the two species. While *E. sebreei* is found in a variety of habitats from coastal to offshore reefs and is generally in shallow water of 1–20 m, *E. punyit* is found exclusively on coastal reefs with significant freshwater influx and terrigenous sedimentary input, and also seems to prefer deeper habitats (12–45 m depth). Moreover, while *E. sebreei* is commonly found in groups of up to 10–15 individuals, *E. punyit* is typically solitary or in small groups of maximally two or three individuals. Finally, although *E. sebreei* can be found resting rather indiscriminately on massive, submassive or foliose corals, *E. punyit* shows a distinct preference for large foliose coral colonies.

Etymology. This species is named *punyit* in honour of Pulau Punyit, Negara Brunei Darussalam, where this beautiful species was first recognized as being distinct from *E. sebreei*. The name is treated as a noun in apposition.

Comparisons. *Eviota punyit* can be distinguished from all other *Eviota* species except *E. sebreei* by having sensory pore pattern 6 of Lachner & Karnella (1980), *i.e.* lacking the nasal pores, posterior interorbital pore and the intertemporal pores. In life, it can be readily distinguished from *E. sebreei* by possessing a red lateral stripe *v.* a black lateral stripe (Fig. 9). There are no apparent morphometric or meristic differences between the two species and thus they may be indistinguishable in preservation (although *in situ* observations in both Brunei Darussalam and West Papua strongly suggest that *E. punyit* reaches a significantly larger maximum size than *E. sebreei*). The presence of a dark spot on the caudal peduncle bordered by white posterodorsally and a yellow dot anteriorly is also a feature of diagnostic importance. With the exceptions of *E. sebreei* and *E. punyit*, the only species to share a similar caudal peduncle pattern is *Eviota cometa* Jewett & Lachner 1983; however, the position of the yellow and white colouration relative to the black spot is different in *E. cometa* (Fig. 10). *Eviota cometa* also possesses a different pore pattern (lacking only the intertemporal pore), but is superficially similar to *E. punyit* in life. Both species have a prominent red lateral stripe with white spots above. The number and position of the spots, however, differ between the two, with *E. punyit* having seven or eight spots along the upper margin of the red stripe, and *E. cometa* having two spots in the centre of the red stripe behind the pectoral fin, a solid white line at the upper margin of the red stripe extending to below the base of the second dorsal fin, and two or three white spots posteriorly. Lastly, *E. cometa* differs from *E. sebreei* and *E. punyit* in eye colouration; the upper half of the eye of *E. cometa* has yellow mottling on a red background, whereas *E. punyit* and *E. sebreei* possess a solid yellow stripe across the upper eye.

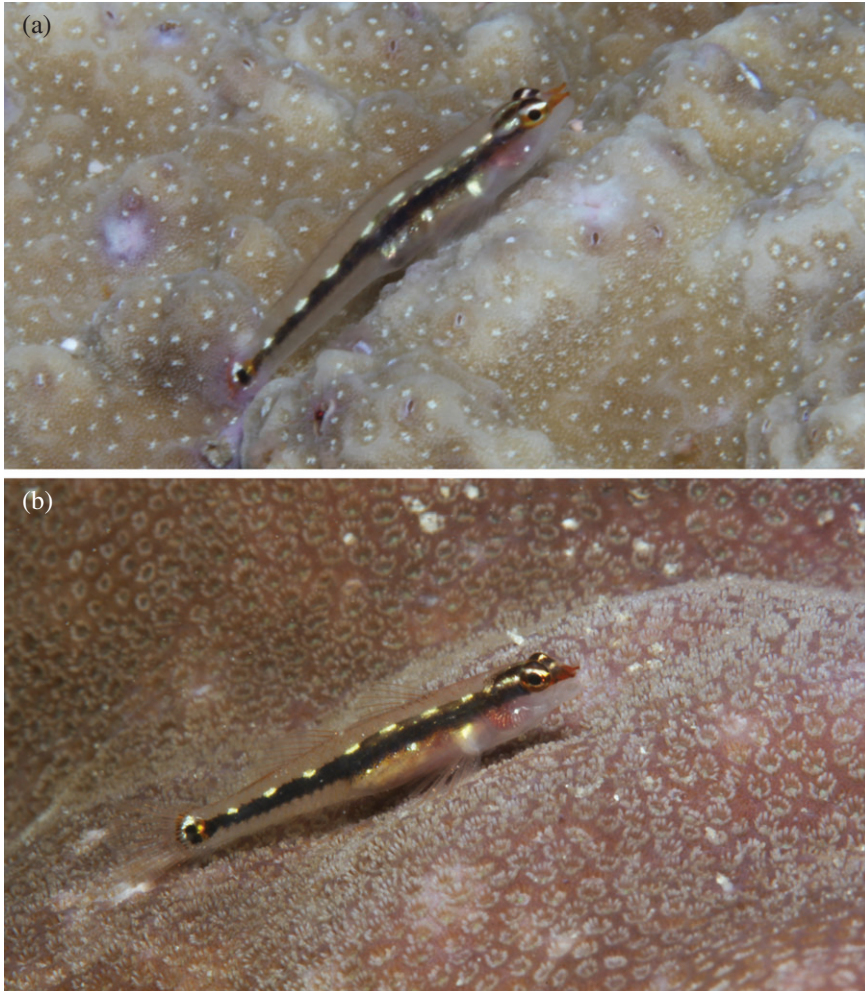


FIG. 9. Live photographs of *Eviota sebreei*: (a) from Brunei Darussalam and (b) from Wasir, Aru. Photographs by M. V. Erdmann. See online publication for color version of figure.

DISCUSSION

The current molecular phylogenetic hypothesis shows *E. punyit* and the morphologically similar *E. sebreei* as being reciprocally monophyletic sister species (Fig. 1). The genetic divergence in both genes between these two species is comparable to that of most species in the genus, and higher than the divergences between the species belonging to the *Eviota nigriventris* Giltay 1933 and *Eviota bifasciata* Lachner & Karnella 1980 complexes, both of which diversified much more recently (Tornabene *et al.*, 2015). The range of *E. punyit* extends from the Red Sea to West Papua, north to the Ryukyu Islands, and is absent from points east of the Sahul Shelf (Fig. 2). Lachner & Karnella (1980) describe the range of *E. sebreei* as extending from Samoa to the Red Sea; however, this undoubtedly included the red-striped form and, based on the current observations of live photographs, the true *E. sebreei* (with a black lateral stripe) appears



FIG. 10. *Eviota cometa* from (a) Indonesia and (b) Fiji. Photos by Dray Van Beek (a) and John E. Randall (b). See online publication for color version of figure.

to extend west only to the Maldives, with most of the distribution being in the Coral Triangle and western Oceania. The overall range of *E. sebreei* including the westward extent of its distribution corresponds well with the Indo-Polynesian Province defined by Briggs & Bowen (2012; Fig. 2), who included the eastern Indian Ocean (including the Maldives and Chagos Archipelago) as part of their Indo-Polynesian Province. Winterbottom & Emery (1986) report *E. sebreei* from the Chagos Archipelago; however, colour photographs from this collection have a faded lateral stripe, and thus it is unclear whether *E. sebreei*, *E. punyit* or both (as in the Maldives) occur there. The fish faunas of the Chagos Archipelago and Maldives are predominantly comprised of species with wide Indo-Pacific distributions (*i.e.* from the Indian Ocean extending east beyond the Andesite line and well onto the Pacific plate), or slightly more restricted Indo-West Pacific distributions (from the Indian Ocean to the Andesite line but not well onto the Pacific Plate), and relatively few species from these atolls are restricted to the

Indian Ocean (Winterbottom & Emery, 1986; Randall & Anderson, 1993; Winterbottom & Anderson, 1997). *Eviota sebreei* more closely resembles an Indo-West Pacific distribution, as it extends only marginally onto the Pacific Plate in the Marshall Islands and Samoa. In the western Indian Ocean and the Red Sea, photographs of live colouration suggest that only *E. punyit* is present. Records of *E. sebreei* from the Comoros Islands, Aldabras Islands, Mauritius and Reunion have not been confirmed to be *E. sebreei* or *E. punyit*, but the presence of *E. punyit* (and absence of *E. sebreei*) in the Seychelles suggests that the same may be the case for the other western Indian Ocean localities.

Eviota sebreei and *E. punyit* broadly overlap from the Maldives to West Papua, as well as the Ryukyu Islands. This pattern is consistent with the centre of overlap hypothesis, which states that the elevated number of species in the Coral Triangle is due to the region being an area where the ranges of distinct Pacific Ocean and Indian Ocean faunas overlap (Woodland, 1983). Under this hypothesis, the mechanism of isolation is the Indo-Pacific Barrier, *i.e.* the shallow Sunda and Sahul shelves of Indonesia, Malaysia, northern Australia and Papua New Guinea that constrict the opening between ocean basins (Woodland, 1983; Gaither & Rocha, 2013). Once speciation has occurred after a period of isolation, species that undergo population expansion may begin to extend their ranges into the Coral Triangle, thus creating the area of overlap. There is considerable support for allopatric speciation across the Indo-Pacific Barrier in the form of Indian and Pacific Ocean geminate pairs (or in more recent cases, genetically structured intraspecific populations) that straddle the Coral Triangle, but the geographic extent to which species expand and re-populate the Coral Triangle after speciation and the timing of the Indian and Pacific Ocean divergence varies somewhat across taxa (Gaither & Rocha, 2013). One commonality regarding all the hypotheses of vicariant speciation across the Indo-Pacific Barrier is that the strength of the isolating mechanism would theoretically be greatest at low sea-level stands, especially those during Pleistocene glacial periods where sea levels dropped as much as 120 m and the Sunda and Sahul shelves were entirely exposed (Voris, 2000). If this is true, then distinctly more speciation events dating to the Pleistocene *v.* the Pliocene or Miocene would be expected to be seen, when sea level was comparatively higher and more temporally stable; however, this is not the case for most marine taxa (Renema *et al.*, 2008; Cowman & Bellwood, 2013*b*). Gaither & Rocha (2013) report a broad range of divergence dates ranging from the Pleistocene (0.3 MYA) to the late Miocene (6.6 MYA) for species pairs straddling the Indo-Pacific Barrier, and Renema *et al.* (2008) demonstrate that far more species of fishes in the Coral Triangle have origins in the Pliocene and Miocene *v.* the Pleistocene. Cowman & Bellwood (2013*b*) compared divergence times for reef fish species pairs distributed between the Indian Ocean and Indo-Australian Archipelago (IAA, broadly similar to the Coral Triangle), and species pairs across the Central Pacific and IAA to other groups of species separated by 'hard barriers' such as the Isthmus of Panama. They found that while the timing of speciation events across hard barriers like the terminal Tethyan Event and the closure of the Isthmus of Panama varied considerably, there was surprising continuity in the timing of speciation events across the soft barriers separating the Coral Triangle from the Indian Ocean and from the Central Pacific Ocean. Divergence times for species pairs between the Coral Triangle and Indian Ocean were largely restricted to within the last 10 MYA, with the average age being 4.1 MYA for the Labridae and 2.2 MYA for the Chaetodontidae (Cowman & Bellwood, 2013*b*).

The estimated divergence time for *E. sebreei* and *E. punyit* is 3.1 MYA (95% HPD interval 1.5–5.8 MYA), which agrees well with the ranges reported by Cowman & Bellwood (2013b) and Gaither & Rocha (2013), and suggests a Pliocene origin. Collectively, the large number of species forming in the Miocene and Pliocene could suggest that the initial formation of the Indo-Pacific Barrier itself *via* the gradual tectonic collision of the Australian and Eurasian Plates beginning in the Miocene may have been the primary driver of speciation (more so than the more recent late Pleistocene sea-level fluctuations). Indeed, by the Late Miocene (*c.* 10 MYA), the connection between the Indian and Pacific Oceans was probably restricted across its entirety by shallow sea or the intermittent landmasses of the Sunda Chain (Hall, 1998). Throughout most of the early to middle Miocene, the South Equatorial Current (SEC) flowed freely through the Indonesia Seaway, but by the late Miocene through mid-Pliocene, the SEC largely reflected northward as the Indonesian Seaway became more restricted, resulting in increased isolation between the basins (Hall, 1998). During this time, the connection between basins may not have been disrupted as completely as in the Pleistocene, but this initial reduction of water exchange (and genetic exchange) between basins may have been sufficient to initiate the speciation process. Alternatively, the late Pleistocene sea-level fluctuations may have indeed been equally as important or even more important drivers of vicariance than the initial formation of the barrier (especially considering the dramatic loss of shallow habitat during glaciations; Ludt & Rocha, 2015), but due to these events being so recent in the evolutionary time scale, the divergent populations may not have had time to complete speciation. Moreover, the detection of the subtle signatures of recent diversification and speciation might not be done as efficiently as the detection of species that have diverged earlier (*e.g.* a taxonomic bias recognizing only well-differentiated species with obvious diagnostic features); with more comprehensive geographic and genetic sampling across putatively wide-ranging species it might be possible to find evidence of recent fine-scale Pleistocene speciation events and cryptic species that have otherwise gone undetected (Winterbottom *et al.*, 2014; Tornabene *et al.*, 2015).

The broad extent to which *E. sebreei* and *E. punyit* overlap is greater than several other species that probably formed *via* vicariance across the Indo-Pacific Barrier. In some cases of putative Indo-Pacific Barrier speciation events, species pairs are present in the Pacific and Indian Oceans but absent entirely from the Coral Triangle, indicating possible local extinctions due to unfavourable ecological conditions during glacial maxima (Springer & Williams, 1990). In other cases, overlap in the Coral Triangle is present but minimal. For example, in two pairs of labrids, *Scarus viridifucatus* (Smith 1956) and *Scarus spinus* (Kner 1868), and *Halichoeres scapularis* (Bennett 1832) and *Halichoeres trimaculatus* (Quoy & Gaimard 1834), the areas of overlap are limited to small regions on the western margin of the Sunda Shelf (Barber & Bellwood, 2005; Choat *et al.*, 2012; Gaither & Rocha, 2013). The overlap is more substantial in the butterflyfishes *Chaetodon trifasciatus* Park 1797 and *Chaetodon lunulatus* Quoy & Gaimard 1825, which overlap over the entirety of the Sunda Chain and into West Papua (Hsu *et al.*, 2007; Bellwood *et al.*, 2010; DiBattista *et al.*, 2012). Other potential examples of Indo-Pacific Barrier vicariance from *Chaetodon* show dramatically asymmetrical distributions where one species (in most cases, the species that also occurs in the Indian Ocean) has a range that extends well into the Pacific Ocean and the other member of the species group is more restricted (Hsu *et al.*, 2007). *Eviota sebreei* and *E. punyit* show intermediate levels of range expansion, but are similar to some examples

from *Chaetodon* in that the expansion is asymmetrical, with *E. sebreei* expanding well outside the Coral Triangle westwards, and *E. punyit* not extending eastwards beyond West Papua. Many factors may contribute to the extent to which sister species overlap in range, including the initial mode of speciation (*e.g.* allopatric, sympatric and parapatric), species dispersal potential, species niche preferences and microhabitat associations, geological and oceanographic factors and the amount of time since the speciation event. Teasing out the relative importance of these factors is complicated. For example, in the genus *Pomacanthus*, Hodge *et al.* (2013) found no evidence of a relationship between the extent of sympatry of sister species and node age, a finding which they attributed to presence of multiple modes of speciation operating across the genus rather than solely an allopatric process followed by range expansion. On the contrary, Hodge & Bellwood (2015) were able to discern a positive relationship between species age and range size by focusing exclusively on younger sister-species pairs (and thus minimizing the effect of missing extinct lineages on estimation of divergence times), but they acknowledge that their methods have low predictive power.

One relevant question regarding sister species with overlapping ranges is whether these species are truly syntopic throughout their overlap. Where they overlap in the Coral Triangle, *E. punyit* is much less common than *E. sebreei*. In Brunei, both species were observed on the same reef (M. V. Erdmann, pers. obs.). The two species were strictly segregated into distinct groups of conspecifics, and both species were never observed on the same coral head, although they did occur in close enough proximity that individuals of one species could easily be chased or 'herded' onto a coral head occupied by the other species, affording excellent opportunities for side by side photographs (Fig. 4). The ability of these sister species to readily differentiate each other, coupled with the apparent role of microhabitat partitioning, and possible species-specific pre-mating rituals (as observed in other species of *Eviota*; Sunobe, 1998; Sunobe & Nakazono, 1999) probably serves to maintain reproductive isolation and species boundaries. A similar scenario where sympatric sister species segregate in groups and partition microhabitats is seen in *Eviota dorsopurpurea* Greenfield & Randall 2011 and *Eviota brahmi* Greenfield & Tornabene 2014. Given the restricted ranges of these latter two species, the extent of overlap between their ranges, and their extremely recent divergence time (60–230 Ka), *E. brahmi* and *E. dorsopurpurea* were interpreted as being a possible example of speciation in sympatry (Tornabene *et al.*, 2015). Speciation in sympatry is also a possibility for *E. punyit* and *E. sebreei*; however, based on the age of the group, the extent of overlap (and non-overlap) between the two species ranges, and the presence of a well-known isolating mechanism positioned in the centre of their region of overlap (the Indo-Pacific Barrier), Pliocene vicariance with subsequent range expansion is here considered the most parsimonious scenario.

Eviota sebreei and *E. punyit* represent a strong case for divergence across the Indo-Pacific barrier, demonstrating the importance of this geographic feature in driving speciation of coral reef fishes. This pattern differs from the patterns observed in the *E. nigriventris* and *E. bifasciata* complexes, where speciation has occurred in many instances without major geographic barriers to gene flow (Tornabene *et al.*, 2015). These groups demonstrate that a variety of abiotic and ecological factors act in concert to produce the high diversity observed in *Eviota*, particularly within the Coral Triangle. Ultimately, the main drivers of speciation within a group depend on the dispersal potential of the species (ability to overcome geographic barriers to gene flow), as well as their propensity to develop ecological or behavioural barriers to gene flow (*e.g.*

habitat partitioning, assortative mating and sexual selection). The pelagic larval duration of 24–26 days in *Eviota* (Depczynski & Bellwood, 2006) suggests that most species are capable of dispersing long distances. Larval behaviour, however, may restrict dispersal by keeping larvae close to reefs in order to avoid starvation (Sunobe & Nakazono, 1987; Woodson & McManus, 2007). Even short periods of genetic isolation, whether geographic or ecological in nature, may be sufficient to enable reproductive isolation in *Eviota* and initiate speciation. Generation time is extremely short in *Eviota*, and a fast evolutionary clock could allow populations to quickly develop species-specific courtship rituals, prominent colour differences, strong secondary sexual characteristics (promoting assortative mating) and specific microhabitat preferences, all of which have been reported in *Eviota* (Sunobe, 1998; Sunobe & Nakazono, 1999; Sekiya & Karino, 2004; Depczynski & Bellwood, 2005, 2006; Tornabene *et al.*, 2013a, 2015; Greenfield & Tornabene, 2014). Collectively, the combination of complex geological features of the Coral Triangle and the diverse ecology of species within *Eviota* help explain the remarkable species richness observed within this group.

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Supporting Information

Supporting Information may be found in the online version of this paper:

FIG. S1. Maximum likelihood tree from concatenated data set. Support values are bootstrap values.

FIG. S2. Bayesian phylogeny of *coi* data set. Support values are posterior probabilities.

FIG. S3. Bayesian phylogeny of *Ptr* data set. Support values are posterior probabilities.

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APPENDIX

New sequences generated from this study

Species	Tissue	Voucher	Locality	GenBank COI	GenBank PTR
<i>Eviota sultani</i>	SB7	CAS 238168	Brunei Darussalam	KT327063	KT327051
<i>Eviota sultani</i>	SB8	CAS 238168	Brunei Darussalam	KT327062	KT327052
<i>Eviota sultani</i>	SB9	CAS 238169	Banda Sea, Indonesia	KT327061	KT327050
<i>Eviota sultani</i>	SB10	CAS 238170	Kaimana, Indonesia	KT327060	KT327049
<i>Eviota sebreei</i>	SB1	CAS 238172	Aru, Indonesia	KT327058	KT327043
<i>Eviota sebreei</i>	SB2	CAS 238172	Brunei Darussalam	KT327054	KT327044
<i>Eviota sebreei</i>	SB3	CAS 238172	Brunei Darussalam	KT327055	KT327045
<i>Eviota sebreei</i>	SB4	CAS 238172	Brunei Darussalam	KT327056	KT327046
<i>Eviota sebreei</i>	SB5	CAS 238172	Brunei Darussalam	KT327057	KT327047
<i>Eviota sebreei</i>	SB6	CAS 238172	Brunei Darussalam	KT327059	KT327048
<i>Eviota sebreei</i>	21	CAS 238173	Pohnpei, Micronesia	KT327053	KT327042