# Cryptic diversity of Eviota (Teleostei: Gobiidae) and their habitat use in the shallow waters of Okinawa Island

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**Research Article**

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# Abstract

*Eviota*, known as dwarf gobies, is a diverse genus of marine fishes comprising more than 120 species found throughout the Indo-Pacific Ocean. A previous study has suggested that morphologically similar cryptic species of *Eviota*may exist and that the species diversity may be significantly underestimated. This study evaluated the diversity of *Eviota*species in three habitat types (tidal flats, reef flats, and moats) in the shallow waters (0–1 m in depth at low tide) of Okinawa Islandusing morphological characteristics and molecular phylogenetic analysis. Eleven morphospecies were classified into 17 molecular optical units(MOTUs), and cryptic species for which speciation may have occurred relatively recently were detected in the morphospecies *Eviota abax*, *Eviota japonica*, *Eviota queenslandica*, and *Eviota prasina*. Most MOTUs exhibited only one habitat use, whereas three MOTUs were found in multiple habitats. Among each cryptic *Eviota* species, both habitat segregation and habitat overlaps were found. Therefore, speciation in *Eviota* may occur without habitat segregation, and multiple other factors should be considered regarding speciation in *Eviota*.

# Introduction

*Eviota* (Teleostei: Gobiidae), known as dwarf gobies, is a diverse genus of marine fish (Greenfield 2017). *Eviota* was originally proposed by Jenkins (1903), and Lachner and Karnella (1980) reviewed the diagnostic morphological characteristics of the genus (e.g., cephalic sensory-canal pore patterns, the shape of the male genital papilla, and body coloration) and summarized the geographical distribution. Subsequently, the description of new species continues to progress mainly based on morphology (e.g. Shibukawa and Suzuki 2005; Fujiwara et al. 2020), and 127 valid species are currently recognized throughout the Indo-Pacific Ocean except in the eastern Pacific region (Tornabene et al. 2021). In addition to its small body size and a large number of species, *Eviota* is characterized by a short life span (< 100 days) and, consequently, rapid generational turnover (Depczynski and Bellwood, 2006).

Molecular phylogenetic analysis has been used to delimit species, leading to the discovery of many cryptic species, which are morphologically very similar but distinguishable via molecular analysis (Bickford et al. 2007). This analysis has greatly advanced the understanding of biodiversity and provides a standard method of species delimitation for many organisms (Fišer et al. 2018). Notably, Tornabene et al. (2015) identified the presence of multiple cryptic species of *Eviota* via molecular phylogenetic analysis, suggesting that the number of *Eviota* species may be significantly underestimated. However, studies performing molecular phylogenetic analysis of *Eviota* diversity have been limited; therefore, further research across extensive geographic areas and habitats is required.

Three speciation modes (allopatric, sympatric, and parapatric) have been proposed for coral reef fish (Rocha and Bowen 2008). Tornabene et al. (2015) suggested that local-scale speciation in *Eviota* within the Coral Triangle may be associated with niche partitioning (sympatric speciation) and physical barriers to gene flow (allopatric speciation). *Eviota* species are found in various habitats and substrates, including estuaries, hard corals, coral rocks, tidepools, near-shore reefs with strong currents, mud and silt, sand, rubble, dead coral, and rock (Greenfield 2017). A few *Eviota* species are associated with a single type of microhabitat (Tornabene et al. 2013; Doll et al. 2021). Habitats overlap among some *Eviota* species, but little habitat overlap is observed among pair species, with few exceptions (Doll et al. 2021). However, it is unclear whether habitat segregation and overlap occur among species that would have speciated relatively recently, such as in cryptic species.

This study investigated the diversity of *Eviota* species that were collected from three habitat types (tidal flats, reef flats, and moats) in the shallow waters of Okinawa Island using morphological characteristics and molecular phylogenetic analysis. I have tested the following hypotheses: i) cryptic species are recognized in *Eviota* in the shallow waters of Okinawa Island, and ii) habitat segregation and overlap occur among closely related species.

## Materials And Methods

# Field sampling

*Eviota* species were collected using hand nets during low tide between 2020 and 2022 in the shallow waters (Nakijin, Nago, Onna) of Okinawa Island (Fig. 1; Table 1). These sampling sites were 0–1 m in water depth at low tide. Three habitat types (tidal flats, reef flats, and moats) were identified at these sampling sites. The Nakijin site (Fig. 1a; Table 1) is a coastal lagoon with patch reefs on sand and coral rubble bottoms, and contains habitat-type moats and reef flats. Site Nago (Fig. 1b; Table 1) is a tidal flat adjacent to the mouth of a stream where the bottom is dominated by mud, sand, and coral rubble. Onna (Fig. 1c; Table 1) is a tidal flat adjacent to the mouth of a stream and also a coastal lagoon, and contains habitat-type tidal flats, reef flats, and moats. Data on the habitat type for each *Eviota* specimen were recorded.

Table 1
Substrate and habitat of each sampling site of Okinawa Island.

| Sampling site | Substrate | Habitat |
|---|---|---|
| Nakijin | sand, coral rubbles | moat, reef flat |
| Nago | mud, sand, coral rubbles | tidal flat |
| Onna | sand, coral rubbles | tidal flat, moat, reef flat |

Online Resource 1. Morphological characteristics of each *Eviota* species collected in the shallow waters of Okinawa Island.

## Morphological Observation

The *Eviota* specimens were euthanized in an ice water bath, photographed, fixed in 10% formalin, and preserved in 70% ethanol. Before fixation in formalin, the right pectoral fin of each specimen was taken and stored in 99.5% ethanol until molecular analysis. All specimens were cataloged and deposited at the Okinawa Churashima Foundation.

Morphological species identification was performed based on the studies by Greenfield and Winterbottom (2016a), and subsequent studies describing *Eviota* species (Greenfield and Randall 2016; Greenfield and Winterbottom 2016b; Tornabene and Greenfield 2016; Greenfield and Suzuki 2016; Greenfield and Erdmann 2017; Greenfield and Randall 2017; Greenfield et al. 2017; Greenfield et al. 2018; Greenfield and Gordon 2019; Greenfield et al. 2019; Fujiwara et al. 2020; Greenfield and Erdmann 2020a, b; Winterbottom and Greenfield 2020; Tornabene et al. 2021) were based on fin counts, cephalic sensory-canal pore patterns, urogenital papillae, and pigment patterns. Fin count and morphological measurements were performed using an ocular micrometer under a dissecting microscope (Nikon SMZ645). Cephalic sensory-canal pore patterns were observed as described by Lachner and Karnella (1980). Cyanine Blue staining was used to highlight the sensory pores and urogenital papillae (Akihito et al. 2013).

## Molecular Species Delimitation

Total DNA was extracted using the HotSHOT method (Truett et al. 2000). A segment of the mitochondrial DNA cytochrome oxidase subunit 1 gene (*CO1*) was amplified by polymerase chain reaction (PCR) in a thermal cycler (Gene Atlas 322, Astec, Fukuoka, Japan) using the primer pairs FISHCO1LBC (5 -TCAACYAATCAYAAAGATATYGGCAC) and FISHCO1HBC (5 -ACTTCYGGGTGRCCRAARAATCA) (Ward et al. 2012). For *Eviota japonica*, the newly designed primer pair CO1_EJF (5 -TCAACTAACCATAGGACATTGGCAC) and CO1_EJR (5 -GACCTCAGGGTGTCCGAAGAATCAAAA), based on the FISHCO1LBC primer, FR1d primer (Ivanova et al. 2007), and complete mitochondrial sequence (Accession number AP019334), was used. PCR was performed using the KOD FX Neo DNA polymerase kit (Toyobo, Osaka, Japan) in a total reaction volume of 12.5 µl: 6.25 µl of 2× PCR buffer, 0.5 µl of 10 µM of each primer, 2.5 µl of 2 mM dNTPs, 0.25 µl of KOD FX Neo, 0.25 µl of template DNA, and 2.25 µl $dH_2O$. The PCR cycling conditions were as follows: 94°C for 2 min; 10 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 15 s, and extension at 68°C for 20 s; 25 cycles of denaturation at 98°C for 10 s, annealing at 53°C for 15 s, and extension at 68°C for 20 s; and a final extension at 68°C for 2 min. After purification of amplified PCR samples, sequencing was performed using a BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA), followed by sequencing on a 3730xl DNA Analyzer (Thermo Fisher Scientific). The sequences were deposited in DDBJ/EMBL/GenBank (accession numbers LC753213–LC753261).

Three species delimitation methods were used for the *CO1* dataset to delimit Molecular Operational Taxonomic Units (MOTUs). The first method was the General Mixed Yule Coalescent (GMYC) model (Fujisawa et al. 2013). For the GMYC method, a Bayesian Inference (BI) analysis was performed using BEAST2 (Bouckaert et al. 2019) with the GTR + I + G substitution model, which was inferred as the best fitting model using ModelTest-NG (Darriba et al. 2020), estimated base frequencies with four gamma categories, an optimized relaxed clock, and the Yule model. The length of the MCMC chain was

100 million, with sampling every 5,000 and the first 10% was discarded as burn-in. The effective sample size was confirmed to be greater than 200 using Tracer v1.7.1. Single-threshold GMYC analyses were performed on the webserver (https://species.h-its.org/gmyc/). The second method implemented included the multi-rate Poisson tree process (mPTP) model (Kapli et al. 2017). For the PTP method, the Maximum Likelihood (ML) tree was reconstructed using RAxML v8.2.10 (Stamatakis 2014) based on the GTR + I + G model with 1000 nonparametric bootstrap replicates. PTP analysis was performed using the web server (https://mptp.h-its.org). The last method implemented was Assemble Species by Automatic Partitioning (ASAP) (Puillandre et al., 2021). ASAP analysis was performed on a web server (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html) with default settings.

## Results

# Morphological identification

A total of 49 *Eviota* specimens were collected from the shallow waters of Okinawa Island and 11 morphospecies were identified (Fig. 2; Online Resource 1). Among these, *Eviota hinanoae* and *Eviota bipunctata* were recorded for the first time in Japanese water.

## Motus

Three species delimitation analyses detected the same 17 MOTUs for the 11 identified morphospecies (Fig. 2). Among them, *Eviota afelei*, *Eviota teresae*, *Eviota smargdus*, *Eviota nigramembrana*, *E. hinanoae*, *Eviota rubrimaculata*, and *E. bipunctata* showed correspondence between morphospecies and MOTUs. The morphospecies *Eviota abax*, *Eviota japonica* were classified into two MOTUs (*E. abax* 1, 2 and *E. japonica* 1, 2), and *Eviota prasina* and *Eviota queenslandica* were classified into three MOTUs (*E. prasina* 1, 2, 3 and *E. queenslandica* 1, 2, 3).

## Habitat Use

Most MOTUs exhibited only one habitat use, whereas three MOTUs were found in multiple habitats (Fig. 2; Online Resource 2). *Eviota japonica*1 and 2, *E. abax* 1, *E. queenslandica* 1 and 2, and *E. hinanoae* were found only in the tidal flats. *Eviota afelei*, *E. bipunctata*, *E. teresae*, *E. smargdus*, *E. abax* 2, *E. queenslandica* 1, and *E. nigrimaculata* were found only in the moat. *Eviota prasina* 2 was found only on the reef flat. *Eviota rubrimaculata* and *E. prasina* 3 were found in the tidal flats and moats. *Eviota prasina* 1 was found in tidal flats, moat, and reef flat.

The morphospecies, *E. abax*, *E. japonica*, *E. prasina* and *E. queenslandica* were classified into multiple MOTUs and showed various similarities and differences in habitat use (Fig. 2; Online Resource 2). *Eviota abax* 1 and 2 were found in tidal flats and moats, respectively. *Eviota queenslandica* 1 was found only in moats, whereas *E. queenslandica* 2 and 3 were found only in tidal flats. *Eviota japonica* 1 and 2 were found only in tidal flats, and were found to coexist in the same tidal flats at the Nago and Onna sampling sites. *Eviota prasina* 1 and 2 were found in the reef flat, whereas *E. prasina* 1 and *E. prasina* 3 were found in tidal flats and moats. *Eviota prasina* 1 and 2 coexisted in the same reef flat at the Onna sampling site, and *E. prasina* 1 and 3 coexisted in the same tidal flat at the Nago sampling site.

## Discussion

This study shows how far we are from understanding the diversity of *Eviota* species. Molecular phylogenetic analysis of the mitochondrial DNA of the *CO1* gene revealed that *Eviota* species collected from the shallow waters of Okinawa Island were divided into 17 MOTUs, and the number was more than expected from the morphological identification. Tornabene et al. (2015) also demonstrated the cryptic diversity of *Eviota* species within the Coral Triangle, which is known as the world's richest marine biodiversity hotspot. The fact that the cryptic diversity of *Eviota* species was observed in multiple regions suggests the possibility of finding cryptic diversity in other areas of the Indo-Pacific where *Eviota* occurs.

The life-history traits of *Eviota* species are related to the presence of many cryptic species (Tornabene et al. 2015). The limited adult mobility (< 1 m; Depczynski and Bellwood, 2004) and short pelagic larval duration (24–26 days; Depczynski and Bellwood, 2006) of *Eviota* species produce isolated populations (Tornabene et al. 2015; Brandl et al. 2018). Moreover, the short life span of *Eviota* (< 100 days; Depczynski and Bellwood, 2006), i.e., rapid generational turnover, may lead to subsequent reproductive incompatibility (Brandl et al. 2018). Numerous cryptic species have been identified in the marine gobies *Trimma* and *Schindleria* (Kon et al. 2007; Winterbottom et al. 2014), with many life-history traits consistent with *Eviota* (Kon and Yoshino 2002; Winterbottom and Southcott 2008; Winterbottom et al. 2011).

Niche partitioning, including habitat segregation, is associated with speciation in cryptobenthic reef fish (Brandl et al. 2018). Previous studies have demonstrated that sympatric speciation via niche partitioning occurs within some taxa of these fish (e.g., Rüber et al. 2003; Mundy et al. 2004). In the present study, habitat segregation was observed among multiple cryptic species (between *E. abax* 1 and *E. abax* 2, and between *E. queenslandica* 1 and *E. queenslandica* 2 and 3). Nevertheless, habitat overlap was also observed between multiple cryptic species (between *E. japonica* 1 and 2, *E. queenslandica* 2 and 3, *E. prasina* 1 and 2, and *E. prasina* 1 and 3). Thus, both segregation and habitat overlaps were found among multiple cryptic *Eviota* species, suggesting that speciation may occur in the genus regardless of habitat segregation.

Geographic barriers are another factor in the speciation of cryptobenthic reef fishes, including gobies (Brandl et al. 2018). Tornabene et al. (2015) indicated that speciation in *Eviota* occurred at small scales due to geographic barriers within the Coral Triangle. Moreover, *Trimma* gobies exhibit geographically distinct cryptic lineages in the Indo-Pacific region (Winterbottom et al. 2014). Additionally, Lachner and Karnella (1980) identified morphological differences among geographic populations of *E. prasina*. In the present study, an extensive survey was not performed; therefore, the possibility of geographic variation among the cryptic *Eviota* species identified herein cannot be excluded.

In conclusion, the cryptic diversity of *Eviota* species in the shallow waters of Okinawa Island was identified based on morphological observation and molecular phylogenetic analysis. *Eviota* is widely distributed in the Indo-Pacific Ocean (Greenfield 2017), suggesting that undiscovered cryptic species of *Eviota* may remain. This study, observed both habitat segregation and habitat overlap among cryptic *Eviota* species, thereby highlighting that other multiple factors must be considered in association with speciation in *Eviota*.

# Declarations

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## Conflict of interest

The authors declare no conflict of interest.

## Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the author.

## Sampling and field studies

All necessary permits for sampling and observational field studies have been obtained by the author from the competent authorities and are mentioned in the acknowledgements, if applicable. The study is compliant with CBD and Nagoya protocols.

## Data availability

All specimens obtained were cataloged and deposited at the Okinawa Churashima Foundation. All sequences determined in this study were deposited in the DDBJ/EMBL/GenBank database (accession numbers LC753213–LC753261). All data generated or analyzed during this study are included in this published article and its supplementary information files (Online Resource 1 and 2).

## Author contribution

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# Figures

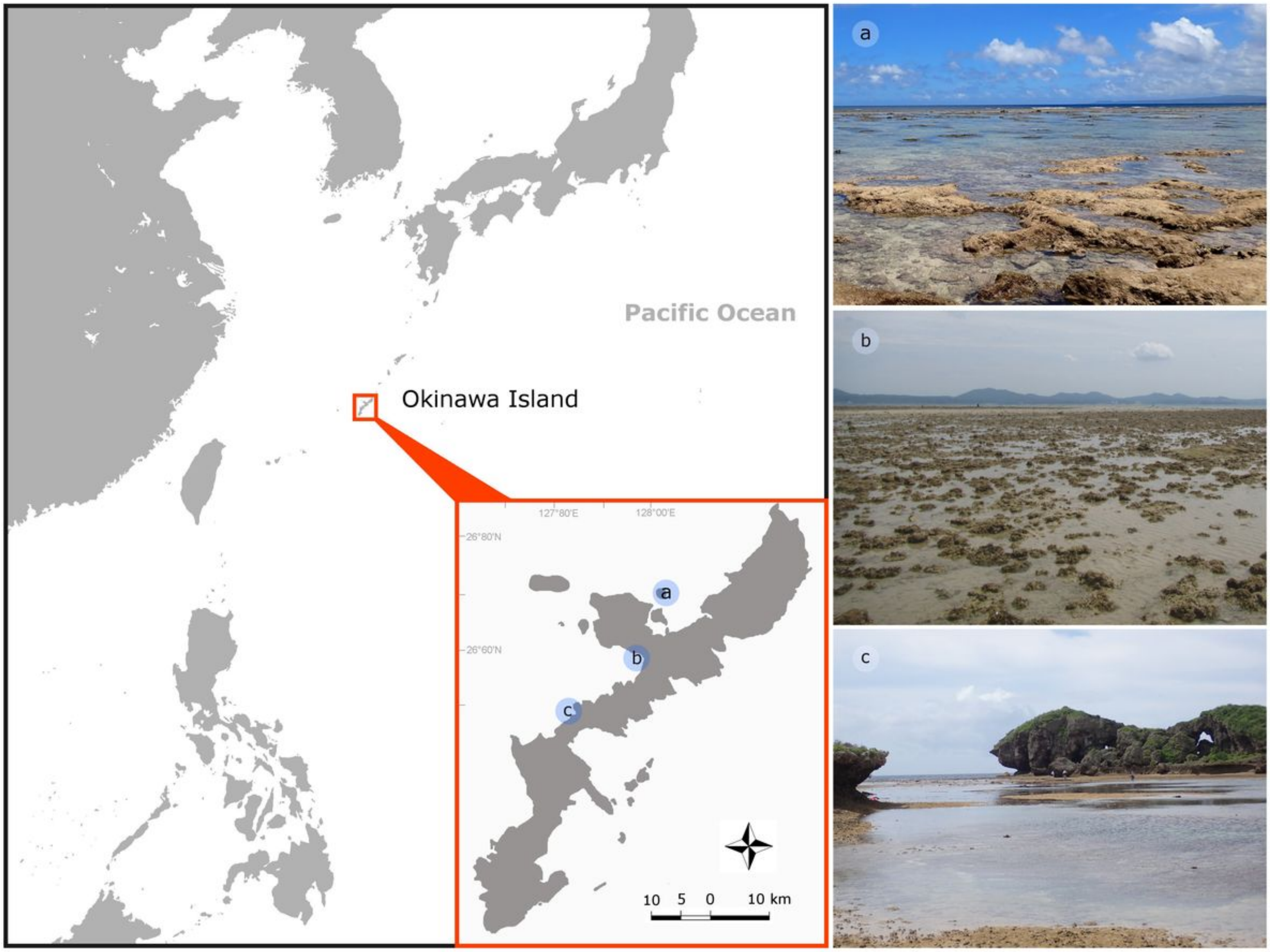


Figure 1

Map showing the locations of the sampling sites near Okinawa Island in the northwesternregion including photographs of the sampling sites (a, Nakijin; b, Nago; c, Onna).

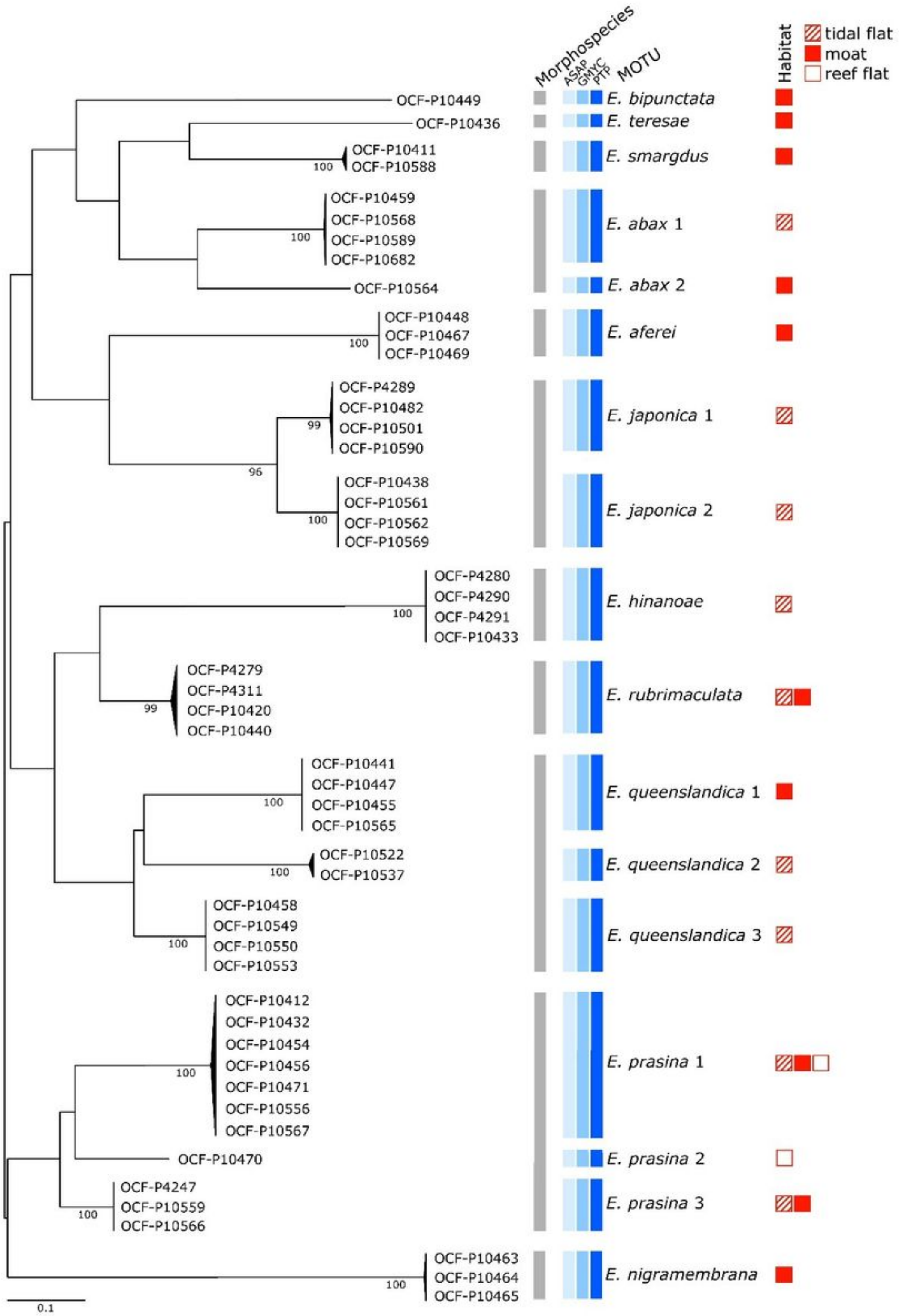
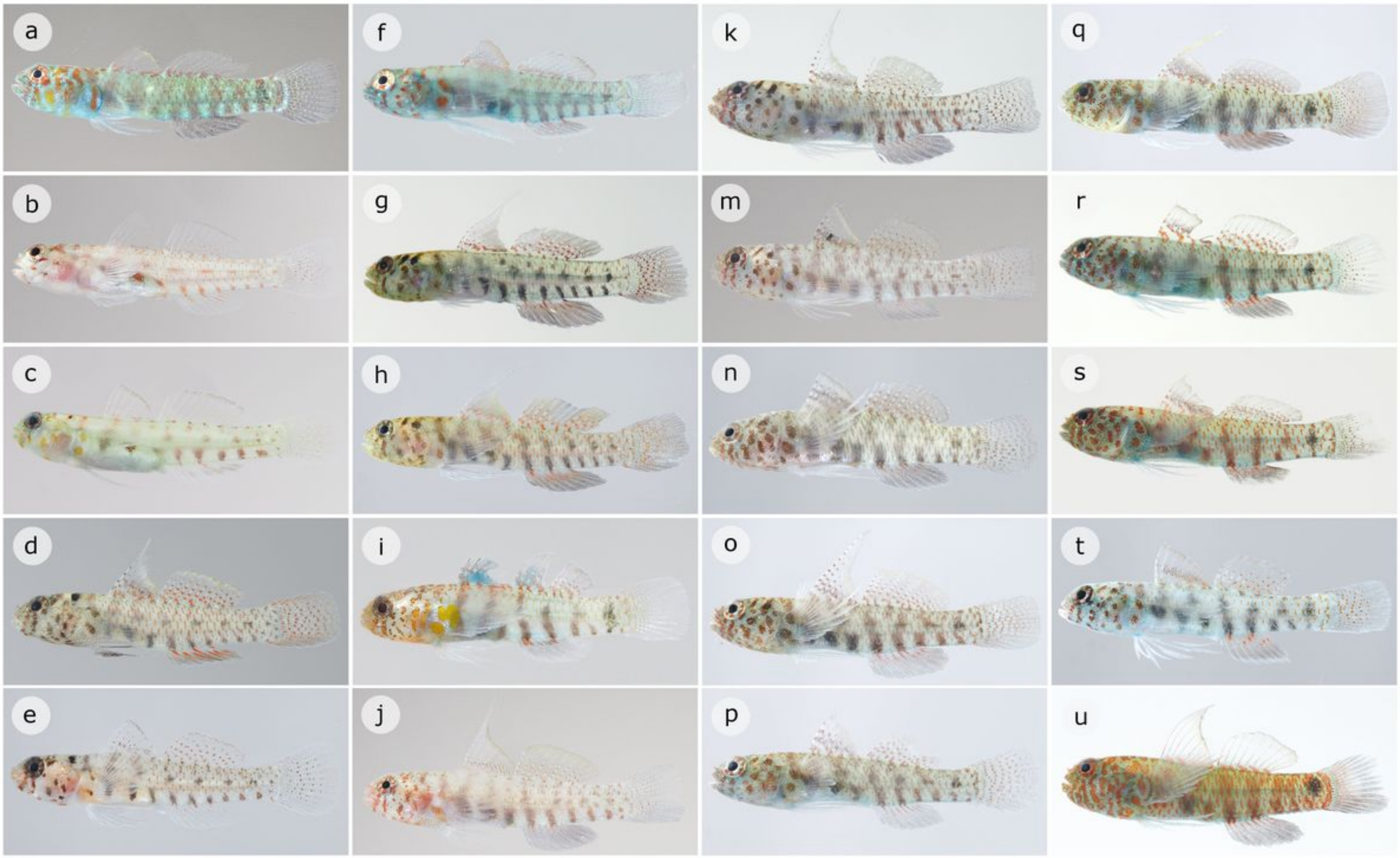


**Figure 2**

Maximum likelihood (ML) tree based on mitochondrial DNA sequences fromthe *CO1*gene with multiple species delimitation and habitat use of *Eviota* species collected in the shallow waters of Okinawa Island.

Numbers on branches indicates the ML bootstrap values; values below 90% are not shown. Color bars indicate the morphospecies (gray) and delineated molecular operational taxonomic unit (MOTU) evaluated by different methods (ASAP, GMYC, and PTP).



## Figure 3

Fresh specimens of *Eviota* species collected in the shallow waters on Okinawa Island, southern Japan. **a**, *Eviota bipunctata*(OCF-P10449, male, 13.3 mm SL); **b**, *Eviota teresae* (OCF-P10436, female, 13.8 mm SL); **c**, *Eviota smaragdus* (OCF-P10411, female, 18.5 mm SL); **d**, *Eviota abax* 1 (OCF-P10459, sex unknown, 15.6 mm SL); **e**, *Eviota abax* 2 (OCF-P10564, female, 14.7 mm SL); **f**, *Eviota afelei* (OCF-P10467, female, 11.9 mm SL); **g**, *Eviota japonica* 1 (OCF-P10501, male, 17.5 mm SL); **h**, *Eviota japonicus* 2 (OCF-P10569, male, 16.8 mm SL); **i**, *Eviota hinanoae*(OCF-P4280, male, 12.0 mm SL); **j**, *Eviota rubrimaculata* (OCF-P10440, male, 15.1 mm SL); **k**, *Eviota queenslandica* 1 (OCF-P10455, male, 16.6 mm SL); **m**, *Eviota queenslandica* 1 (OCF-P10441, sex unknown, 13.5 mm SL); **n**, *Eviota queenslandica* 2 (OCF-P10537, female, 15.3 mm SL); **o**, *Eviota queenslandica* 3 (OCF-P10549, male, 15.4 mm SL); **p**, *Eviota queenslandica* 3 (OCF-P10550, female, 14.9 mm SL); **q**, *Eviota prasina* 1 (OCF-P10567, male, 17.3 mm SL); **r**, *Eviota prasina* 1 (OCF-P10456, female, 15.2 mm SL); **s**, *Eviota prasina* 2 (OCF-P10470, male, 17.1 mm SL); **t**, *Eviota prasina* 3 (OCF-P10559, female, 14.3 mm SL); **u**, *Eviota nigramembrana* (OCF-P10463, male, 15.6 mm SL).

# Supplementary Files

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