

Mitochondrial Insights into Cryptic Diversity and Taxonomic Complexity of *Ecsenius* (Blenniidae)

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Abstract

The genus *Ecsenius* (Blenniidae) is the most species-rich group of combtooth blennies, yet its evolutionary relationships remain poorly resolved. Here, we aim to examine phylogenetic patterns across Indo-West Pacific (IWP) *Ecsenius* (n=49) using three mitochondrial markers (16S, Control Region, and Cytochrome B). The results of pairwise genetic distance analyses revealed substantial diversity (0.00–0.21), with a species delimitation threshold of >2% based on the distance consistent with the divergence between *E. schroederi* and *E. kurti*. The phylogenetic analyses also confirmed 10 different clades within *Ecsenius* with a clade delimitation cutoff >8%. Neighbor-joining, Maximum Likelihood, and Bayesian Inference phylogenetic reconstructions consistently supported the monophyly of most clades, while uncovering cases of crypticity, particularly within *E. yaeyamensis* and *E. bicolor*. Complex speciation was also observed within the bandanus-randalli clade. This study suggested hidden diversity and potential undescribed taxa by the presence of recognized taxonomic units (indicated by cf.). However, a cautious note should be taken that this thesis was addressed based on mitochondrial perspectives, which are exclusive to maternal evolutionary traits. In this sense, this study generates novel mitochondrial phylogenies for the Indo-West-Pacific *Ecsenius* to resolve cryptic diversity, refine taxonomy, and enhance reef fish biodiversity databases.

Introduction

Habitat complexity, latitude, depth gradients, oceanographic processes, and geological history drive the genetic diversity and evolutionary dynamics by shaping the spatial distribution of global species diversity of teleosts, including reef fishes (Bellwood et al., 2012; Huang et al., 2018; Jablonski et al., 2017; Ma et al., 2016). The Indo-West Pacific (IWP) represents the global centre of coastal fish diversity and has undergone dynamic geological events that created physical and geographic barriers, facilitating allopatric speciation. Its equatorial position further promotes highly concentrated species richness (Lo et al., 2017; Jablonski et al., 2017).

Reef fish diversity is critical to ecosystem stability and is widely used as an indicator in reef health monitoring and conservation. However, conventional survey methods such as “Reef Check” often focus on visible indicator taxa (e.g., Haemulidae and Serranidae) (Hodgson, 2001), while cryptobenthic groups such as Blenniidae and Gobiidae remain underrepresented due to its hidden characteristics (Bessey et al., 2023; Brandl et al., 2018; Willis, 2001). Bickford et al. (2007) emphasized that cryptic species recognition is central to evolutionary theory, biogeography, and conservation, as it can reveal hidden introductions of invasive taxa that threaten native biodiversity (Clavero & García-Berthou, 2005; Willis, 2001). Moreover, the partitioning of diversity and biogeographic distinctiveness often indicates ongoing

evolutionary processes contributing to the high biodiversity observed in these systems (Faria et al., 2021).

Molecular approaches provide Rapid and accurate methods that have successfully revealed cryptic lineages, such as in Rainbowfishes (Melanotaeniidae) in the Indo-Australian region (Kadarusman et al., 2012) and demonstrated endemism in the Blueline Surgeonfish (*Acanthurus nigroris*) in Hawaii (DiBattista et al., 2011). Within this context, *Ecsenius* is a benthic teleost genus and the most species-rich member of Blenniidae, comprising approximately 62 morphologically recognized species according to World Register of Marine Species (WoRMS). While superficially resembling *Salarias*, it differs in dentition (McCulloch, 1923) and forms a distinct clade within Blenniidae (Hundt et al., 2014).

Despite its taxonomic and ecological significance, genetic information on *Ecsenius* remains scarce, particularly in the IWP. Phylogenetic analyses of *Ecsenius* have previously been conducted primarily to support the description of the new species *E. springeri* (Allen et al., 2019). Additionally, the genus has been included in family-level phylogenetic studies of Blenniidae (Sharifiniya et al. 2024; Lin & Hastings, 2013). Nevertheless, comprehensive phylogenetic assessments across *Ecsenius* remain limited, leaving broader evolutionary relationships and patterns of lineage diversification within the genus insufficiently resolved.

This study, therefore, aimed to examine the phylogenetic profile of *Ecsenius*, a genus regarded as diverse, complex, and partly cryptic among the Blennies. Three mitochondrial markers were used to generate a baseline maternal phylogenetic profile of this Genus. In addition, the use of these markers was due to their universality and distinct evolutionary rates of each marker, with a high success rate of amplification that has been widely used in fish phylogenetic studies (Hassler et al., 2022; Ooi et al., 2021; Bronstein et al., 2018; Okumu & Çiftci, 2003). This study allows us to contribute to documenting reef fish biodiversity and refining regional taxonomic databases.

Materials and Methods

Sample Collection

Tissue samples from several species of *Ecsenius* were collected from several regions: Indonesia (SE Misool, Misool, Cendrawasih Bay, Raja Ampat, Batanta, Watubela Island, Fam Island, Aru, Anambas, Mapia Atoll, and Halmahera), Philippines (Palawan), Myanmar (Mergui), and Timor-Leste between 2009 and 2014 (Figure 1). The list of locations and number of specimens for each species can be accessed in Supplementary 2. All specimens were deposited at BIONESIA, Denpasar, Indonesia. Specimen collection was conducted in accordance with animal care and preservation protocols



Figure 1. The map of Indo-West Pacific Region. The red placemarks indicated the locations of *Ecsenius* sample collection.

established by the Indonesian Ministry of Forestry (Government Regulation No. 7/1999). During the collection, clove oil was used to anaesthetize the fish prior to the preservation. The specimens were preserved in 95% EtOH and stored at room temperature until they were extracted.

DNA Extraction, PCR, and Sequencing

DNA template was extracted using a 10% Chelex solution (Walsh et al. 1991). Extracted samples were amplified using a portion of mitochondrial DNA *Control Region* (Lee et al., 1995), 16s (Romano & Palumbi, 1997), and *Cytochrome b* (Liu et al., 2014); HIMB01: 5'-GTGACTTGAAAAACCACCGTTG-3' and HIMB02: 5'-AATAGGAAGTATCATTCGGGTTTGAT-3' via PCR. These markers were selected based on their relative sensitivity to evolutionary variation among species within the same genus. The 16s rRNA gene is the most conserved of the three markers, providing essential information on deeper evolutionary divergence while offering a robust for framework lineage reconstruction (Hassler et al., 2022). In contrast, the *Control Region* (CR) is a non-coding region characterized by a mutation rate, making it particularly suitable for assessing diversity, especially among closely related species or taxa with low evolutionary rates (Bronstein et al., 2018). *Cytochrome b* (Cyt b) was selected for its capacity to discriminate species-level differences with adequate phylogenetic resolution (Tobe et al., 2010).

DNA amplification was performed via PCR using the Hot-Start protocol (Eun, 1996) of 25 µL volumes, using 1 µL of DNA template. Each reaction included 1.5 µL 10x PCR buffer (Applied Biosystems), 2.5 µL 10 mM dNTPs, 1.25 µL of each primer at 10 mM, 2 µL 25 mM MgCl₂ solution, 0.125 µL AmplyTaq Red (Applied Biosystems), and 14.5 µL ddH₂O. PCR conditions included an initial denaturation of 94°C for 15s, 38 cycles of 94°C for 30s, 50°C for 30s, and 72°C for 45s, with a final extension of 72°C for 5 minutes (Pertiwi et al., 2024; Borsa et al., 2014). This protocol was confirmed to support the optimum amplification conditions for three targeted markers. All PCR reactions were checked on 1% agarose gels stained with Ethidium Bromide to confirm the DNA amplification and specificity. The successfully amplified product with a clear single band was then sequenced at the UC Berkeley sequencing facility.

Phylogenetic Analysis

Forward and reverse sequences were edited and aligned using MEGA12 (Kumar et al., 2024). Aligned sequences from three mtDNA markers were concatenated using Geneious R7. Phylogenetic tree reconstruction was done using Neighbor Joining (NJ), Maximum Likelihood (ML), and Bayesian Inference (BI). The NJ analysis was done on MEGA12. Regarding ML analysis, JModelTest (Posada, 2008) was used to obtain the best evolutionary model that fit the data. According

to this, General Time Reversible included Gamma distribution, and Invariable site (GTR+G+I) was suggested as the best model. The ML phylogenetic tree was constructed using RaxML (Stamatakis, 2014). Both the NJ and ML trees were tested using 1000 bootstrap replicates. BI analysis was done Markov Chain Monte Carlo approach with four chains on MrBayes 3.2 (Ronquist et al., 2012). This analysis was run for 1,000,000 generations with an initial burn-in fraction of 25,000 to obtain the posterior probability as a support value on the phylogenetic tree. Pairwise K2P distance was calculated using MEGA12. A sequence of *Cirripectes filamentosus* was used as an outgroup to result in a rooted-phylogenetic tree. The genetic lineage cladistics were later defined as Operational Taxonomic Units (OTUs) based on the similarity value that was clustered using Mothur (Schloss et al., 2009). DnaSP 5 (Librado & Rozas, 2009) was used to calculate nucleotide and haplotype diversities for the generated clades comprising at least two individuals. Since the sample sizes were uneven across species, this diversity estimation was conducted to confirm only the diversity of species with high sample sizes.

Results

A total of 49 individual fish were successfully amplified using three different mitochondrial markers (16s, CR, and Cyt B). Among these, 16 species of *Ecsenius* were examined in this study. Molecular and bioinformatic analyses successfully resulted in 1884 bp of concatenated sequences from approximately 595 bp of 16s (Genbank Acc. No. PV549456- PV549504), 637 bp of CR (Genbank Acc. No. PV599327- PV599375), and 750 bp of Cyt B (Genbank Acc. No. PV390008-PV390056). Successfully amplified sequences were submitted to NCBI with accession number listed in Supplementary 1.

A neighbor-joining (NJ) tree was constructed to represent the phylogenetic relationships of *Ecsenius*. Bootstrap values from NJ and maximum likelihood (ML) analyses, as well as posterior probabilities from Bayesian inference (BI), were added to each node to indicate lineage support. The tree showed that bootstrap values were >60 and posterior probabilities were >90 for most lineages, providing strong monophyletic support for each species clade. Most clades were concordant and consistently supported across the three analyses, although some nodes showed minor incongruences among the methods.

The topological patterns showed that both *E. bicolor* and *E. yaeyamaensis* were divided into three internal clades. These divergences were statistically supported by bootstrap values (NJ and ML) and posterior probability (BI, Figure 2).

The overall pairwise evolutionary distances among species groups ranged from 0 to 0.21 (Table 1). The greatest distances were observed between *E. midas* and *E. pictus*, as well as between *E. midas* and *E. lineatus*, whereas the closest relationship was between *E.*

bandanus and *E. randalli* (0.00). Additionally, intraspecific genetic divergence ranged from 0.00 to 0.03, with *E. yaeyamaensis* showing the highest value (0.03, Table 2).

Based on genetic lineage clustering generated by mothur, sequences divergence threshold of >8% was applied, resulting in 10 clades of genetic lineages, which will be called Otu groups. This grouping system excluded *E. pictus*, which would not be processed for the diversity analyses. Only groups with at least two sequences were processed for this analysis (Table 3). This cladistic threshold showed how each species evolved and the

closest taxon that shared common ancestors, which were further supported by high bootstrap values and posterior probability estimates. Indicating the evolutionary rates within *Ecsenius* may differ according to the genetic lineage (Figure 2 and Table 3).

Among these, the most diverse group in terms of nucleotide diversity was Otu03 ($\pi = 0.04719$), comprising *E. stigmatura*, *E. cf. tricolor*, and *E. ops* (Figure 1). High haplotype diversity ($H_d = 1$) was observed in most Otu groups. Otu01 exhibited a slightly lower H_d (0.891), but it contained the largest number of analyzed sequences ($n = 11$, Table 3).

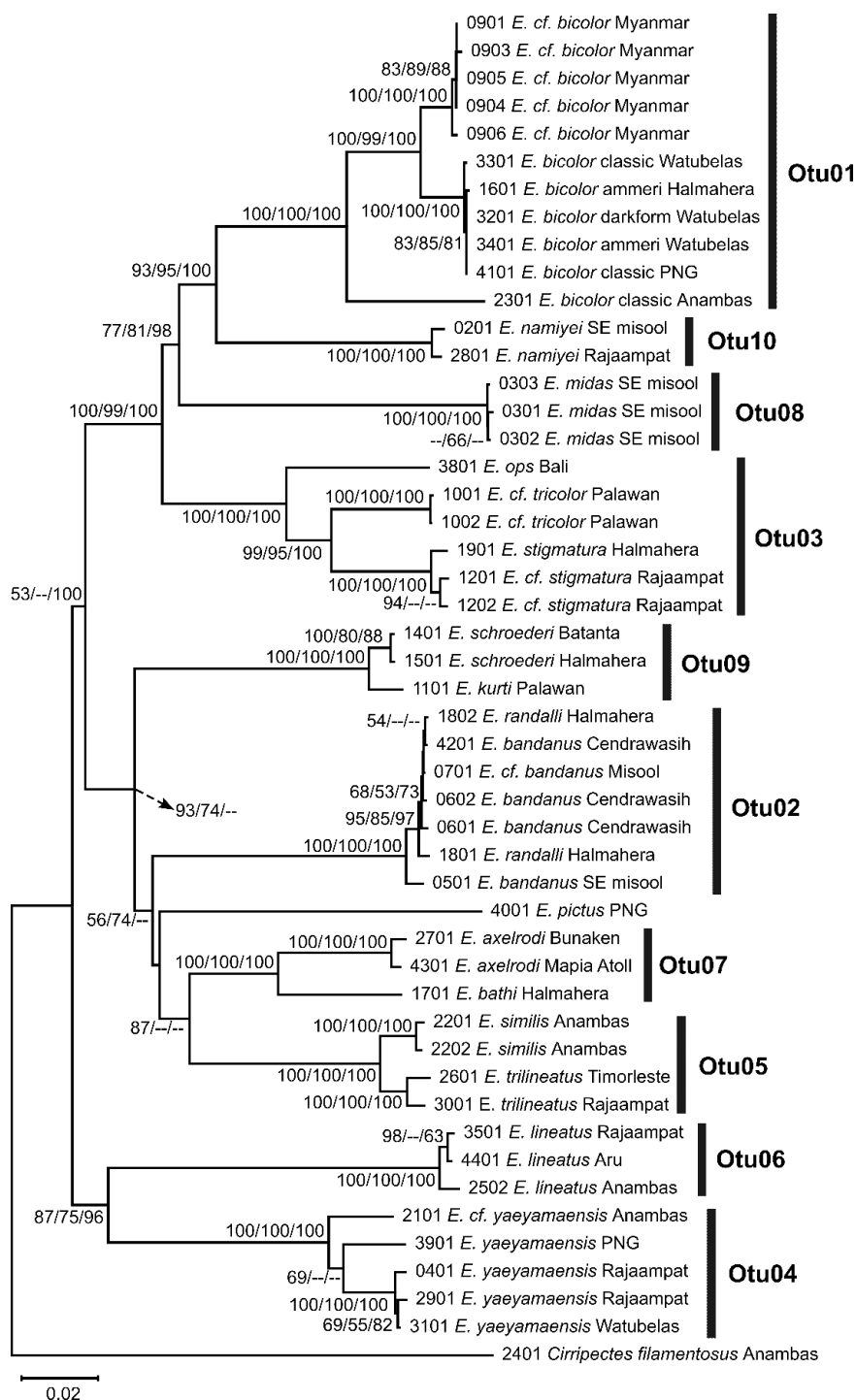


Figure 2. Phylogenetic tree based on concatenated 16S, CR, and Cyt B. Values on internal nodes represent bootstrap support of Neighbor Joining, Maximum Likelihood, and posterior probability of Bayesian Inference Analysis.

Table 1. Pairwise distance between groups of species

No.	Species Group	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1.	<i>E. namiyei</i>		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
2.	<i>E. midas</i>	0.15		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
3.	<i>E. yaeyamaensis</i>	0.18	0.20		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
4.	<i>E. bandanus</i>	0.18	0.18	0.18		0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01
5.	<i>E. bicolor</i>	0.12	0.16	0.19	0.19		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
6.	<i>E. tricolor</i>	0.14	0.16	0.18	0.17	0.15		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
7.	<i>E. kurti</i>	0.18	0.19	0.18	0.15	0.18	0.18		0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
8.	<i>E. stigmatura</i>	0.14	0.16	0.18	0.18	0.16	0.06	0.18		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
9.	<i>E. schroederi</i>	0.17	0.19	0.18	0.15	0.18	0.18	0.02	0.18		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
10.	<i>E. bathi</i>	0.17	0.18	0.19	0.14	0.18	0.17	0.13	0.18	0.13		0.01	0.01	0.01	0.01	0.01	0.01	0.01
11.	<i>E. randalli</i>	0.18	0.19	0.18	0.00	0.19	0.17	0.15	0.18	0.15	0.14		0.01	0.01	0.01	0.01	0.01	0.01
12.	<i>E. similis</i>	0.18	0.18	0.19	0.14	0.19	0.19	0.14	0.18	0.14	0.12	0.14		0.01	0.00	0.01	0.01	0.01
13.	<i>E. lineatus</i>	0.19	0.21	0.17	0.19	0.20	0.18	0.19	0.18	0.19	0.19	0.18	0.19		0.01	0.01	0.01	0.01
14.	<i>E. trilineatus</i>	0.18	0.17	0.19	0.14	0.19	0.19	0.14	0.18	0.14	0.11	0.15	0.02	0.19		0.01	0.01	0.01
15.	<i>E. axelrodi</i>	0.18	0.18	0.19	0.13	0.18	0.17	0.14	0.17	0.13	0.06	0.14	0.12	0.19	0.12		0.01	0.01
16.	<i>E. ops</i>	0.13	0.16	0.17	0.18	0.15	0.07	0.17	0.08	0.17	0.17	0.19	0.18	0.19	0.18	0.16	.	0.01
17.	<i>E. pictus</i>	0.19	0.21	0.20	0.16	0.20	0.20	0.16	0.20	0.16	0.15	0.16	0.15	0.20	0.16	0.14	0.19.	

Note: *The Lower left part of the matrix indicates the distance value between groups. **The Above right part of the matrix indicates the standard deviation of the distance between groups.

Table 2. The average of the genetic distance within a group of species

No.	Species	Distance	Std. Error
1.	<i>E. namiyei</i>	0.01	0.00
2.	<i>E. midas</i>	0.00	0.00
3.	<i>E. yaeyamaensis</i>	0.03	0.00
4.	<i>E. bandanus</i>	0.00	0.00
5.	<i>E. bicolor</i>	0.02	0.00
6.	<i>E. tricolor</i>	0.00	0.00
7.	<i>E. stigmatura</i>	0.01	0.00
8.	<i>E. schroederi</i>	0.00	0.00
9.	<i>E. randalli</i>	0.00	0.00
10.	<i>E. similis</i>	0.00	0.00
11.	<i>E. lineatus</i>	0.01	0.00
12.	<i>E. trilineatus</i>	0.01	0.00
13.	<i>E. axelrodi</i>	0.01	0.00

In *E. bicolor*, three colormorphs (Figure 3; dark form, classic, and ammeri) did not correspond directly to the three internal clades recovered, which were clearly separated with strong bootstrap and posterior probability support (Figure 2). The average genetic distance among *E. bicolor* clades was 0.02. In contrast, most other clades showed values of 0.00 (Table 2). This suggests hidden diversity within the species. Diversity indices showed that *E. bicolor* was grouped into Otu01, with seven haplotypes identified from 11 sequences and no other species included, resulting in relatively high diversity ($\pi = 0.02155$; $Hd = 0.891$, Table 3).

Morphological variation was also observed in several species, including *E. yaeyamaensis* (Figure 4), *E. stigmatura*, and *E. Bandanus* (Figure 5). Phylogenetic analyses showed that *E. stigmatura* and *E. cf. tricolor* were closely related as sister taxa with a low genetic distance (0.06) (Table 1). Despite this low divergence, the two species were resolved into distinct clades with strong support from NJ, ML, and BI analyses (bootstrap values of 99 and 95, and posterior probability of 100, respectively). Both species were visually identified by their bright blue-orange body coloration and black-white horizontal lines beneath the eyes (Figure 5a–c). The individual identified as *E. cf. stigmatura* shared most key traits of *E. stigmatura*, except for reduced blue coloration (Figure 5a–b). Based on phylogenetic clustering and genetic distance, this individual was classified as *E. stigmatura*. In contrast, *E. cf. tricolor* lacked the distinctive black spot with a circular white outline that characterizes its sister taxon (Figure 5c).

In a different pattern, *E. bandanus* and *E. randalli* appeared to be genetically almost identical, with a very low distance value (0.00), despite having distinct body patterns (Figure 5d–f). Similarly, *E. trilineatus* specimens from Timor Leste and Raja Ampat were morphologically identical and clustered together as sister taxa with *E. similis* and also confirmed as one lineage group (Otu05) based on 8% similarities (Table 3). In the meantime, despite their minimal morphological differences, phylogenetic analyses showed a clear bifurcation between *E. similis* and *E. trilineatus*, supported by strong bootstrap values and posterior probabilities across all three phylogenetic methods. The average genetic distance between the *E. trilineatus* and *E. similis* was 0.02 (Table 1).

Discussion

Blenniidae, the second most species-rich reef fish family, comprises more than 400 species listed in the World Register of Marine Species (WoRMS), of which ~60 belong to the genus *Ecsenius*. Previous studies have shown that monophyly, crypticity, and taxonomic complexity are common within this family (Lin & Hastings., 2013; Hundt et al., 2014). Integrative genetic and morphological analyses have further highlighted taxonomic challenges in general, such as *Omobranchus* and *Cirripectes* (Araujo et al., 2020; Cabezas et al., 2022; Delrieu-Trottin et al., 2018; Hoban & Williams, 2020). Despite being the most diverse genus in the family, phylogenetic studies on *Ecsenius* remain limited. This study therefore, aimed to clarify its phylogenetic profile and contribute to understanding the evolutionary patterns of Blenniidae using mitochondrial sequences.

The findings demonstrate considerable genetic diversity within *Ecsenius*, with indices of evolutionary distance, nucleotide diversity, and haplotype diversity consistently supporting this pattern. Although most clades were resolved as monophyletic with clear speciation signals across three mitochondrial markers (16S, Cyt B, and CR), cases of crypticity and lineage complexity were also evident. A divergence threshold of >2% was inferred for species delimitation, based on the genetic distance between *E. schroederi* and *E. kurti* as well as *E. trilineatus* and *E. similis*. However, this threshold was not applicable to other clades, such as *E. bicolor*, which appears to require a higher divergence cutoff for reliable delimitation. This threshold is also inapplicable to *E. bandanus* and *E. randalli* due to potential incomplete lineage sorting. This indicated that no universal mitochondrial threshold applies in *Ecsenius*, and the divergence threshold was more framed for clade-specific indicators rather than strict taxonomic rules. A similar threshold was reported with broader trends observed in another genus of Blenniidae, where *Cirripectes* shows species-level divergence that ranges widely at 3 to 9% using COI (Delrieu-Trottin et al., 2018; Hoban & Williams, 2020), suggesting a comparable evolutionary scale across related taxa.

As indicated by *E. bicolor*, which represents discordant substantial genetic and phenotypic variability, a complex speciation pattern among the

Table 3. Genetic diversity of *ecsenius* by clade (Clustering cutoff > 0.08)

Otu Group	No. of Seq (n)	π	SD of π	H	Hd	SD of Hd
Otu01	11	0.02155	0.00758	7	0.891	0.074
Otu02	7	0.00431	0.00136	7	1.000	0.076
Otu03	6	0.04719	0.01073	6	1.000	0.096
Otu04	5	0.02599	0.00714	5	1.000	0.126
Otu05	4	0.01740	0.00419	4	1.000	0.177
Otu06	3	0.00649	0.00229	3	1.000	0.272
Otu07	3	0.04330	0.01858	3	1.000	0.272
Otu08	3	0.00146	0.00042	3	1.000	0.272
Otu09	3	0.01048	0.00445	3	1.000	0.272
Otu10	2	0.00554	0.00277	2	1.000	0.500

Bleniidae was also attributed in this study. The results showed that these sequences were divided into three separate genetic lineages with no association to the observed color polymorphisms which consist of bicolored-form, black striped-form, pale head form, grey form and brown back form (Springer, 1988; Allen & Erdmann, 2024) (Figure 2). Studies had mentioned that

this polymorphic traits is often linked to various ecological diverging factors including sexual-dimorphism, spatial or geographic isolation, and habitat prevalency (Gaither et al., 2020; Mclean & Stuart-Fox, 2014). However, factors underlying the *Ecsenius* colormorphisms remain unclear due to the absence of supporting environmental and behavioral data. In the



Figure 3. Colormorphisms in *Ecsenius bicolor* a) *E. bicolor* (dark form) from Watubelas, b-c) *E. bicolor* (ammeri or black striped-form) collected from Halmahera and Watubela Island, respectively, d) Recognized taxonomic unit of *E. bicolor* that morphologically resembles the ammeri form, e-g) *E. bicolor* (classic or bicolored-form) from Papua New Guinea, Anambas, and Watubelas, respectively.

wider perspective, mitochondrial markers used in this study revealed *E. bicolor* to be a sister taxa with *E. namiyei*, which has been described as the Bicolor group, confirming the primary clusterization of *Ecsenius* (Springer, 1988). The recently described *E. springeri* was also mentioned to possess similar color features; however, the sequence was not included in the analyses due to the unavailability of a comparable sequence in the NCBI (Allen et al., 2019).

E. yaeyamaensis showed the highest genetic and haplotype diversity, with clear internal clades in phylogenetic analyses, indicating strong genetic structuring and hidden diversity within the species. Morphologically, the species is readily distinguished by two dark dashed lines positioned behind the eyes (Allen & Erdmann, 2024). The taxon was originally described as *Salarias yaeyamaensis* by Aoyagi in 1954 and subsequently revised as *E. yaeyamaensis* by Springer (1972). Notably, a specimen from Anambas displayed slight morphological divergence: in most individuals, white dots were scattered across the body with varying intensity, but in one specimen –identified as *E. cf. yaeyamaensis* from Anambas— these markings were absent, despite overall similarity to other conspecifics. This incongruence between genetic structuring and subtle morphological differentiation suggests the potential presence of cryptic diversity within the species, which is assumed to be a result of geographical isolation. This highlights the need for more

comprehensive sampling and integrative analyses to clarify species boundaries within *E. yaeyamaensis* (Hobbs et al., 2013; Liu et al., 2023; Mclean and Stuart-Fox, 2014; Quadroni et al., 2023; Springer, 1972). Alternatively, the recognized taxonomic units species likely represent a recent speciation event supported by strong phylogenetic evidence and high genetic distance. However, true evolutionary relationships among *Ecsenius* species remain unresolved, given the absence of nuclear data, and detailed morphological analysis is needed for formal species recognition.

In the case of *E. bandanus* and *E. randalli*, both species were placed within a single genetic lineage, showing very low genetic divergence (Table 1). Morphologically, *E. bandanus* is characterized by a brown upper body, a light grey to dusky white lower head, a bluish abdomen, and a yellow-bordered black stripe passing through the eye above and below the pupil (Springer, 1988; Allen & Erdmann, 2024). *E. randalli* shares several of these traits, including the bluish abdomen and eye stripe, but differs in having a narrower stripe and nine distinct dark bars along the body flank (Allen & Erdmann, 2024). The discrepancy between their minimal mitochondrial genetic differentiation and their morphological distinctions raised a thesis that both species underwent an incomplete lineage sorting where the evolutionary process is progressing, thus their true evolutionary relationships are unresolved (Yu et al., 2024). Notably,

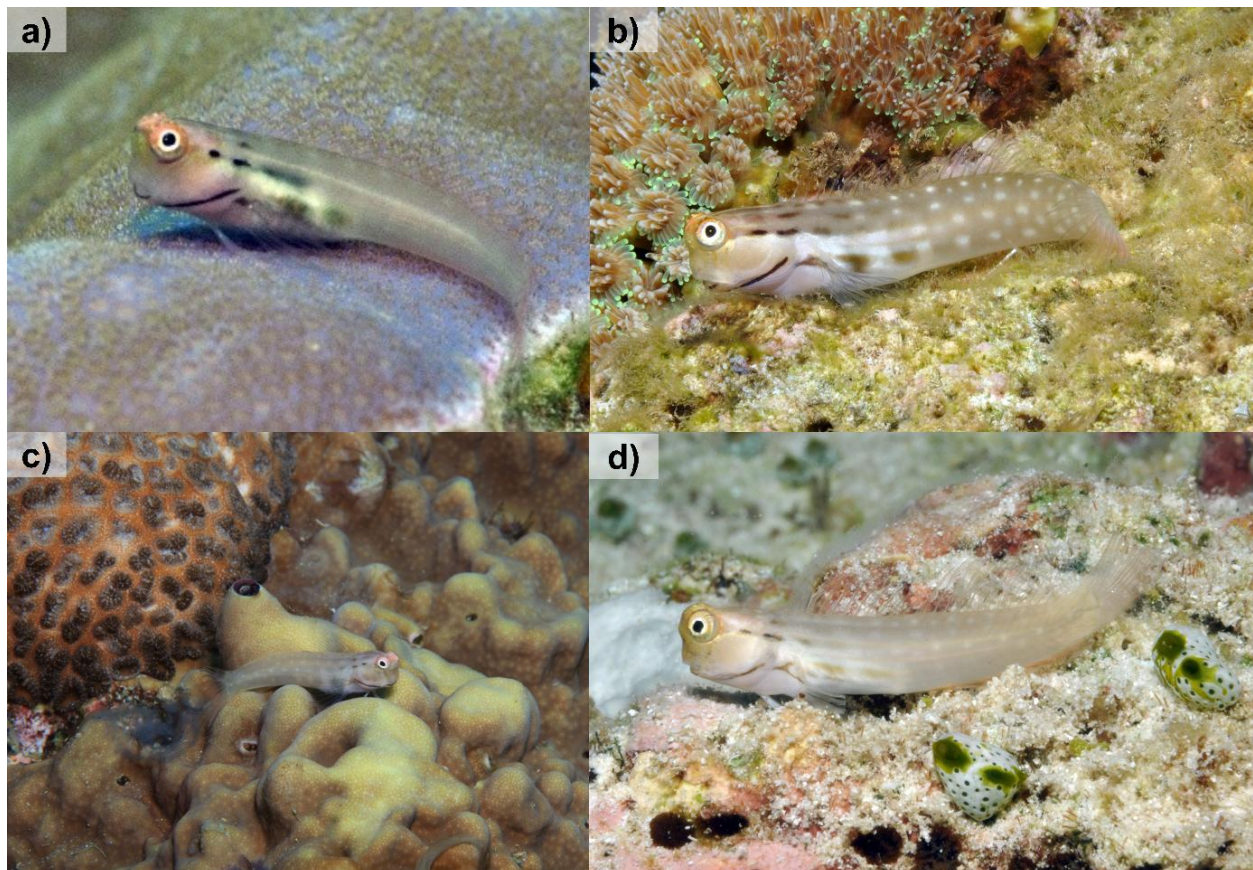


Figure 4. Morphological variations of *Ecsenius yaeyamaensis*. a) *E. cf. yaeyamaensis* from Anambas, b) *E. yaeyamaensis* from Raja Ampat, c) *E. yaeyamaensis* from Papua New Guinea, and d) *E. yaeyamaensis* from Watubela Island.

this finding constitutes a foundational reference for interpreting *Ecsenius* phylogeny based on maternal lineage. In fishes, such cases often result in blurred diversification patterns, where boundaries among species remain indistinct despite morphological or ecological differences. Evidence from the *bandanus-randalli* clade clarified that *Ecsenius* represents a species complex shaped by ongoing evolutionary processes. Moreover, a study of mitochondrial cytochrome oxidase I (COI) failed to provide a clear resolution of the placement of another species of Blennies from the genus *Hypoleurochilus*, indicating complexities are common among Blennidae and the requirements of multi-locus with higher resolution to resolve these discordances (Carter et al., 2023).

E. trilineatus was consistently supported across three phylogenetic methods as a lineage and became a sister taxa to its morphologically look-alike species, *E. similis*, which was just recently confirmed as a new species (Allen & Erdmann, 2024). The species was originally described based on the presence of three horizontal body stripes (Springer, 1972). Although morphological similarities suggest a single taxon, phylogenetic analyses revealed two distinct clades separating the two species (Figure 4g–i). Morphological similarities, combined with the statistically distinct phylogenetic clades, further proved the crypticity of Genus *Ecsenius*. On one hand, this crypticity may indicate the critical role in enhancing genetic diversity within this genus. On the other hand, such phenomena

often raise the potential risk of misidentification during visual census because of phenotypic noise (Ekanayaka et al., 2025). Thus, in addition to documenting biodiversity, comprehensive phenetic assessment may require genetic data, essential for taxonomic validation as well as species delimitation (Mallet, 2007; Oswald et al., 2023)

The occurrence of recognized taxonomic units was also detected in several lineages, namely *E. cf. yaeyamaensis* and *E. cf. bandanus*. Genetic analyses inferred a monophyletic relationship between each recognized taxonomic unit and its respective described counterpart. These findings align with the high incidence of undescribed and cryptic species reported in reef fishes, which contribute to incomplete phylogenetic sampling of reef assemblages (Cowman, 2014; Mora et al., 2008; Zapata & Ross Robertson, 2006). The presence of recognized taxonomic units expands morphological diversity in *Ecsenius*, while genetic evidence reveals its cryptic nature, underscoring the value of molecular tools in uncovering hidden diversity. (Hebert et al., 2004; Lo et al., 2017; Struck & Cerca, 2019).

The phylogenetic profile of *Ecsenius* based on three mitochondrial markers corroborated the earlier cladistic framework proposed by Springer (1972), which grouped species according to phenetic relatedness into the *Yaeyamaensis*, *Mandibularis*, *Opsifrontalis*, *Isos*, *Bicolor*, *Lineatus*, and *Stigmatura* groups. These findings demonstrate that mitochondrial markers remain a reliable tool for genetically-based species identification

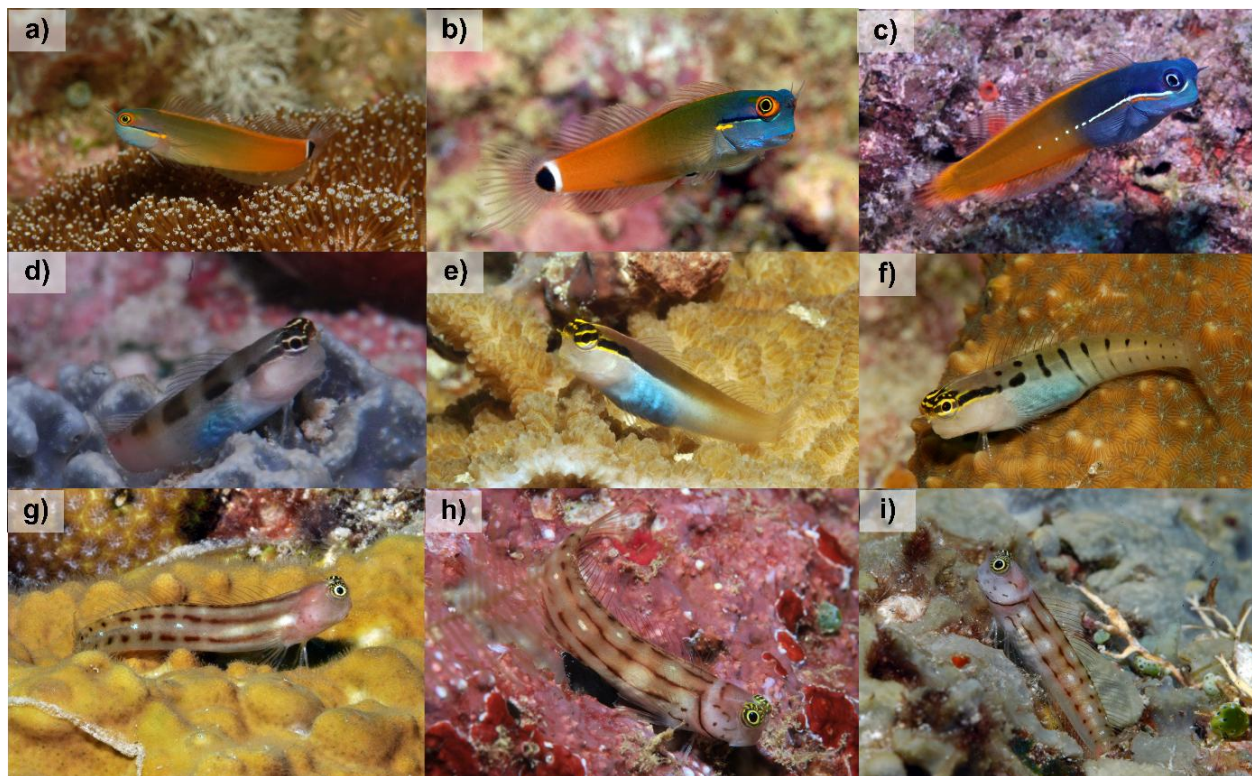


Figure 5. Morphological variations and similarities of *Ecsenius* indicating species crypticity. a) *E. stigmatura* from Halmahera, b) *E. cf. stigmatura* from Raja Ampat, c) *E. cf. tricolor* from Palawan, d) *E. cf. bandanus* from SE Misool, e) *E. bandanus* from Cendrawasih Bay, f) *E. randalli* from Halmahera, g) *E. similis* from Anambas, h) *E. trilineatus* from Timor Leste, and i) *E. trilineatus* from Raja Ampat.

within this genus, which also confirmed by the recent study of wild birds' identification in Medellin, Colombia (Marín-Villa et al., 2025). Moreover, this study enhances evolutionary resolution in Blenniids, showing that molecular data reveal genetic structures beyond morphology and highlight cryptic diversity within *Ecsenius*, emphasizing the need for integrative taxonomic approaches.

Conclusions

Ecsenius is often underestimated in visual reef fish surveys, yet this study demonstrates that mitochondrial DNA reveals substantial genetic variation, including cryptic and recognized taxonomic unit, that are not apparent morphologically. The occurrence of these lineages underscores the underestimated diversity of the genus and highlights its role as a significant component of Indo-West Pacific reef biodiversity. While most clades were monophyletic, low genetic divergence in some morphospecies suggests that not all diversity patterns can be generalized. This study emphasized that mitochondrial DNA is a powerful initial screening tool but requires integrative support to refine species delimitation and resolve taxonomic complexity. The integration of nuclear markers, genomic approaches, and expanded sampling across species and localities will be essential. Collectively, our findings confirm the value of molecular approaches in uncovering hidden diversity, strengthening reef fish inventories, and advancing evolutionary and conservation studies of Blenniidae.

Ethical Statement

Specimen collection was conducted in accordance with animal care and preservation protocols established by the Indonesian Ministry of Forestry (Government Regulation No. 7/1999).

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Author Contribution

First Author: Conceptualization, Formal Analysis, Investigation, Methodology, Visualization, Writing - original draft, Writing -review and editing; Second Author: Conceptualization, Investigation, Data Curation, and Writing -review and editing; Third Author: Funding

Acquisition, Project Administration, Resources, Writing -review and editing.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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