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DNA barcoding of coral reef fishes from Chuuk State, Micronesia

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ABSTRACT

The fish diversity of Chuuk Micronesia is currently under threat due to rapid changes in the coral reef ecosystem. Thus, accurate fish identification using DNA barcodes is fundamental for exploring species biodiversity and resource protection. In this study, we analyzed 162 fish mitochondrial DNA cytochrome *c* oxidase I (COI) barcodes from Chuuk Micronesia. Consequently, we identified 95 species from 53 genera in 26 families and seven orders. The average Kimura 2-parameter genetic distances within species, genera, families, and orders were calculated as 0.17%, 11.78%, 15.63%, and 21.90%, respectively. Also, we have utilized DNA barcodes to perform genetic divergence and phylogenetic analysis of families recognized as dominant groups in Chuuk State. Our findings confirm that DNA barcodes using COI are an effective approach in identifying coral reef fish species. We anticipate that the results of this study will provide baseline data for the protection of coral reef fish biodiversity at Chuuk Micronesia.

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Coral reef fish; mitochondrial DNA COI; DNA barcode; identification; Chuuk State; Micronesia

Introduction

Micronesia, which is located in the Western Pacific Ocean, consists of four states (Yap, Chuuk, Pohnpei, and Kosrae) that collectively have a coral reef area exceeding 6000 km^2 (Andréfouët et al. 2006). As growth and spawning grounds for a wide range of marine organisms, coral reefs are often characterized by their high biodiversity (Reaka-Kudla 1997). The reefs of Micronesia have served as a habitat for many species of corals, fishes, and invertebrates. Chuuk State consists of 18 major volcanic islands, many smaller and uninhabited islands, and a diversity of tropical marine reefs, ranging in size from 0.4 to 4.6 km^2. Recently, population expansion, economic growth, and indiscriminate fishing have threatened the biodiversity of the region (Edward 2002). Further, global climate change is causing ocean acidification, rising sea levels, and rising water temperatures, changes that have been considered detrimental to the coral reef ecosystems and thus creating a crisis of marine biodiversity (Hoegh-Guldberg et al. 2007; Baker et al. 2008; Thompson and Van Woesik 2009).

Effective conservation and management of fish biodiversity require reliable baseline estimates of fish species diversity based on accurate species identification. Identification of fish species is traditionally based on morphology (Dayrat 2005; Triantafyllidis et al. 2011). However, morphological identification requires considerable expertise, given that the morphology of fish varies and often changes concomitantly with developmental stage (Leis and Carson-Ewart 2000; Wang et al. 2018). These issues can be addressed by DNA barcoding, which is based on pattern analysis of standardized gene regions. This approach has been identified to be more reliable for species identification (Hebert et al. 2003; Hebert and Gregory 2005). A 655-bp fragment of the mitochondrial COI gene is widely used for species-level identifications. Mitochondrial DNA shows a high mutation rate and large copy numbers. Organisms with small effective population sizes often provide genomes that are useful for analyses of evolutionary patterns and processes (Brown et al. 1979; Birky et al. 1989). Numerous previous studies around the world, including studies in Taiwan (Bingpeng et al. 2018), Pacific Canada (Steinke et al. 2009), Australia (Ward et al. 2005), the Philippines (Abdulmalik-Labe and Quilang 2019), China (Wang et al. 2018), India (Lakra et al. 2011), Turkey (Keskin and Atar 2013), and Japan (Zhang and Hanner 2011), have demonstrated the utility of COI barcodes in fish species identification.

We used mitochondrial DNA COI barcodes to identify some coral reef fish species from Chuuk State, Micronesia. These species can be difficult to identify by morphological identification.

Materials and methods

Sample collection

The research area is along the northeastern coast of Weno Island in Chuuk State (7°27′N, 151°51′E), where coral reefs

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are well developed. Fishes were collected by diving and netting or were purchased from a local market in 2006, 2007, 2008, and 2011.

DNA isolation

Genomic DNA was extracted from tissue pieces using a Qiagen DNeasy Blood & Tissue Kits (QIAGEN, Valencia, CA, USA), following the manufacturer's protocol. All gDNAs extracted from whole samples were stored at −20 °C at the Marine Ecosystem Research Center, Korea Institute of Ocean Science and Technology, Busan, Korea. The quality and quantity of extracted DNA were measured using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

Amplification and sequencing

PCR amplification was performed using combinations of primers for fish 655-bp COI barcoding region (Ward et al. 2005). Thermal amplification reactions were performed in 25 μL reaction mixtures, which contained 1× PCR buffer, 2 mM $MgCl_2$, 10 pmol of each primer, 0.25 mM of each dNTP, 0.25 U of Taq polymerase, and 100 ng of DNA template. The thermocycling program consisted of an initial step of 94 °C for 1 min; followed by 35 cycles of 94 °C for 30 s, 50 °C for 40 s, and 72 °C for 1 min; a final extension at 72 °C for 10 min; and a final hold at 4 °C. PCR products were then checked using 2% agarose gel electrophoresis. PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA), following the manufacturer's protocol. Sequencing reactions were performed in an MJ Research PTC-225 Peltier Thermal Cycler using ABI PRISM BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems), following the protocols provided by the manufacturer.

Sequence analysis

All sequences were aligned and integrated using MEGA X (Kumar et al. 2018). Obtained sequences were then compared with sequences from NCBI GenBank databases. Samples with similarity indices greater than 97% compared with available database sequences were considered to be the same species. Nucleotide composition, transition(si)/transversion(sv) pair ratios, and K2P genetic distances, including intra- and interspecific divergences, were calculated using MEGA X. Neighbor-joining (NJ) phylogenetic tree (Saitou and Nei 1987) was constructed based on K2P genetic distance using MEGA X with bootstrap tests of 1000 replications were generated to verify the robustness of the tree. The K2P can be rapidly calculated, which in turn can provide consistent results for many species that show required differences between intra- and interspecies variability (Kimura 1980; Shen et al. 2016). The K2P model is commonly used in DNA barcoding (Zhang and Hanner 2011; Keskin and Atar 2013; Bingpeng et al. 2018; Wang et al. 2018).

Results and discussions

Analysis of 162 COI DNA barcodes was able to identify 95 species, 53 genera, 26 families, and seven orders (Anguilliformes, Beloniformes, Beryciformes, Mugiliformes, Ophidiiformes, Perciformes, and Tetraodontiformes) among fishes from Chuuk State. We then obtained the NCBI accession numbers for all the specimens (Table 1). The COI barcode used in the analyses comprised 655 nucleotide base pairs per taxon, and no contamination, insertions, deletions, or stop codons were determined in any obtained sequence. Average K2P genetic distances within species, genera, families, and orders were determined to be 0.17%, 11.78%, 15.63%, and 21.90%, respectively. The average interspecific genetic distance increased concomitant with an increase in genetic variation at progressively higher taxonomic levels. DNA barcoding efficiency is then verified by intraspecific and interspecific distances (Lievens et al. 2001). Average intraspecific genetic distance is 0.3% in BOLD (Barcode of Life Data System) fish databases, and congeneric distance is at least 30-fold higher than conspecific distances (Zhang and Hanner 2011). Intraspecific distance and congeneric distance were determined to be 69-fold higher than conspecific distance in the current study. Our study confirmed that DNA barcodes are useful in identifying coral reef fish species. Moreover, we found that intraspecific genetic distances determined in this present study are less than the previously reported distances; in contrast, interspecific genetic distance was found to be greater.

Average nucleotide composition of the 162 DNA barcodes was T = 29.08%, C = 28.39%, A = 24.18%, and G = 18.35%. The average GC and AT contents were 46.74% and 53.26%, respectively. The highest (52.76%) and lowest (38.51%) GC values were detected in COI barcodes of *Fibramia thermalis* and *Zenarchopterus dispar*. Further, the average ratio (si/sv) of all specimens has been determined to be 1.38. Divergence time among specimens was analyzed in terms of transition(si)/transversion(sv) ratio and genetic distance. The former is considered a general property of DNA sequence evolution. This ratio provides a reliable estimate of sequence distance and can be further used in phylogeny reconstruction. A high si/sv ratio is indicative of a small genetic distance, and vice versa (Yang and Yoder 1999). We were able to analyze the divergence times among families, for example, Acanthuridae, Labridae, Scaridae, and Serranidae, which are dominant in Chuuk Micronesia using DNA barcodes of the fish collected in this study. Average si/sv ratios for these families were 2.10, 1.56, 3.5, and 1.8, respectively. Further, the mean genetic distances among species within families were 16.08%, 20.25%, 11.15%, and 18.80%, respectively. Scaridae family displays the highest si/sv ratio (3.5) and the lowest genetic distance among species within families (11.15%). Scaridae appears to be a recently diverged group and is youngest among dominant families in Chuuk State, Micronesia. Moreover, compared with other families with similar divergence times, we collected a larger number of species in the Scaridae. It is predicted that Scaridae is well adapted to the rich coral reef found at Chuuk State. In contrast,

Table 1. List of species analyzed for DNA barcodes and sequence information.

Order	Family	Genus/Species	GenBank accession no.	Voucher ID	*N*	Reference accession no.	Similarity (%)
Perciformes	Acanthuridae	*Acanthurus lineatus*	MN733529	CKF003	1	HM034183	100
		Acanthurus nigricauda	MN733530, MN733650	CKF004, CKF121	2	HM034188	100
		Acanthurus triostegus	MN733531, MN733532	CKF005, CKF006	2	JQ349668	100
		Ctenochaetus striatus	MN733528, MN733569, MN733570	CKF002, CKF042, CKF043	3	MK658679	99
		Naso brevirostris	MN733610, MN733665	CKF082, CKF134	2	KF930171	100
		Naso lituratus	MN733611, MN733612	CKF083, CKF084	2	HM034244	100
		Naso unicornis	MN733613, MN733614, MN733615	CKF085, CKF086, CKF087	3	KF714984	99
		Naso vlamingii	MN733616	CKF088	1	HQ564379	100
		Zebrasoma velifer	MN733649	CKF120	1	MK657444	100
	Ambassidae	*Ambassis miops*	MN733533, MN733678, MN733702	CKF007, CKF146, CKF160	3	HQ654651	99
	Apogonidae	*Cheilodipterus quinquelineatus*	MN733703	CKF161	1	KP194469	99
		Fibramia lateralis	MN733537, MN733538	CKF010, CKF011	2	KP194856	99
		Sphaeramia orbicularis	MN733639, MN733640, MN733641	CKF111, CKF112, CKF113	3	AP018927	100
		Fibramia thermalis	MN733539	CKF012	1	AB890041	99
	Blenniidae	*Blenniella paula*	MN733593	CKF066	1	MK658217	100
	Caesionidae	*Caesio caerulaurea*	MN733670	CKF138	1	KF009569	99
	Carangidae	*Carangoides plagiotaenia*	MN733651	CKF122	1	KC970456	100
		Caranx melampygus	MN733542	CKF015	1	KC970375	100
		Selar boops	MN733673	CKF141	1	KF009659	100
	Chaetodontidae	*Chaetodon ephippium*	MN733546, MN733547, MN733548 MN733549, MN733550, MN733551 MN733552, MN733553, MN733554 MN733555, MN733556, MN733557	CKF019, CKF020, CKF021 CKF022, CKF023, CKF024 CKF025, CKF026, CKF027 CKF028, CKF029, CKF030	12	JF434773	100
		Chaetodon lunulatus	MN733558	CKF031	1	KJ967960	100
		Chaetodon ornatissimus	MN733559	CKF032	1	JF434807	99
		Chaetodon ulietensis	MN733560	CKF033	1	FJ583101	99
	Gobiidae	*Amblygobius phalaena*	MN733700	CKF158	1	AF391369	99
		Asterropteryx ensifera	MN733541, MN733699, MN733679	CKF014, CKF157, CKF147	3	JX483981	100
	Kyphosidae	*Kyphosus cinerascens*	MN733594, MN733689	CKF067, CKF153	2	JQ350079	100
	Labridae	*Cheilinus chlorourus*	MN733562	CKF035	1	KF714912	99
		Cheilinus trilobatus	MN733561	CKF034	1	KF009582	100
		Coris batuensis	MN733568	CKF041	1	KP194597	100
		Halichoeres margaritaceus	MN733590	CKF063	1	JQ839484	99
		Halichoeres marginatus	MN733591	CKF064	1	AY850781	100
		Halichoeres melanurus	MN733589	CKF062	1	KP194607	98
		Halichoeres trimaculatus	MN733592	CKF065	1	KP194873	100
		Oxycheilinus celebicus	MN733617	CKF089	1	HQ564433	99
		Oxycheilinus digramma	MN733618, MN733619	CKF090, CKF091	2	KP194504	100
		Stethojulis bandanensis	MN733643	CKF115	1	KP194849	100
	Lethrinidae	*Lethrinus erythropterus*	MN733598, MN733660	CKF071, CKF130	2	HM902431	100
		Lethrinus obsoletus	MN733595, MN733596	CKF068, CKF068	2	AP009165	99
		Lethrinus olivaceus	MN733597	CKF070	1	KJ968135	99
		Lethrinus xanthochilus	MN733659, MN733661	CKF129, CKF131	2	KP194924	100
		Monotaxis grandoculis	MN733604, MN733605	CKF077, CKF078	2	AP009166	99
		Monotaxis heterodon	MN733606, MN733663	CKF079, CKF133	2	MK657454	100
	Lutjanidae	*Lutjanus fulvus*	MN733599, MN733600	CKF072, CKF073	2	KF009613	99
		Lutjanus decussatus	MN733601	CKF074	1	MN870144	100
		Macolor macularis	MN733602, MN733686	CKF075, CKF150	2	EF609403	100
		Macolor niger	MN733662	CKF132	1	KF489639	100
	Monodactylidae	*Monodactylus argenteus*	MN733603	CKF076	1	AP009169	100
	Mullidae	*Mulloidichthys flavolineatus*	MN733607, MN733608	CKF080, CKF081	2	MN870473	100

(*continued*)

Table 1. Continued.

Order	Family	Genus/Species	GenBank accession no.	Voucher ID	*N*	Reference accession no.	Similarity (%)
		Parupeneus barberinus	MN733620	CKF092	1	AP018401	100
		Parupeneus cyclostomus	MN733667	CKF136	1	MK658446	100
		Parupeneus insularis	MN733666	CKF135	1	JQ431985	99
		Parupeneus multifasciatus	MN733621	CKF093	1	AP012314	99
	Pomacentridae	*Abudefduf vaigiensis*	MN733527	CKF001	1	AP006016	99
		Amblyglyphidodon curacao	MN733535, MN733536	CKF008, CKF009	2	KF929588	100
		Chromis viridis	MN733676	CKF144	1	MT199208	100
		Chrysiptera glauca	MN733625, MN733692	CKF097, CKF154	2	JQ707144	98
		Neopomacentrus azysron	MN733626	CKF098	1	KP194962	100
	Scaridae	*Cetoscarus bicolor*	MN733544, MN733545	CKF017, CKF018	2	AY662758	99
		Chlorurus bleekeri	MN733563, MN733655	CKF036, CKF125	2	MN870261	100
		Chlorurus frontalis	MN733653	CKF124	1	JQ431617	100
		Chlorurus sordidus	MN733565, MN733566, MN733567	CKF038, CKF039, CKF040	3	AP006567	99
		Chlorurus microrhinos	MN733564	CKF037	1	JN313047	99
		Scarus chameleon	MN733628, MN733629	CKF100, CKF101	2	FJ237915	100
		Scarus ghobban	MN733656	CKF126	1	FJ449707	99
		Scarus niger	MN733672	CKF140	1	JQ432105	99
		Scarus oviceps	MN733631	CKF103	1	JQ432106	100
		Scarus psittacus	MN733630, MN733632	CKF102, CKF104	2	MK658527	100
		Scarus rubroviolaceus	MN733633	CKF105	1	FJ227899	99
		Scarus schlegeli	MN733671	CKF139	1	JQ432114	100
		Hipposcarus longiceps	MN733695	CKF155	1	KF929973	100
	Scombridae	*Thunnus albacares*	MN733644, MN733645	CKF116, CKF117	2	KP259550	99
	Serranidae	*Aethaloperca rogaa*	MN733698	CKF156	1	KC593376	100
		Cephalopholis argus	MN733543	CKF016	1	MF185407	100
		Epinephelus polyphekadion	MN733585, MN733586, MN733571 MN733572, MN733573, MN733574 MN733575, MN733576, MN733577 MN733578, MN733579, MN733580 MN733581, MN733582	CKF058, CKF059, CKF044 CKF045, CKF046, CKF047 CKF048, CKF049, CKF050 CKF051, CKF052, CKF053 CKF054, CKF055	14	MH707787	100
		Epinephelus howlandi	MN733583, MN733657	CKF056, CKF127	2	MH707757	100
		Epinephelus merra	MN733584	CKF057	1	KC970471	99
		Epinephelus spilotoceps	MN733658	CKF128	1	MH707800	100
		Plectropomus areolatus	MN733668	CKF137	1	KC262636	100
		Plectropomus laevis	MN733622	CKF094	1	KP194704	100
		Plectropomus oligacanthus	MN733623, MN733624	CKF095, CKF096	2	HM422409	99
		Variola louti	MN733647, MN733648	CKF118, CKF119	2	KC593369	100
	Siganidae	*Siganus argenteus*	MN733675	CKF143	1	MN870479	100
		Siganus guttatus	MN733635, MN733674	CKF107, CKF142	2	KJ420577	99
		Siganus virgatus	MN733634	CKF106	1	KF715023	99
		Siganus stellatus	MN733636, MN733637	CKF108, CKF109	2	KT997948	100
		Siganus vulpinus	MN733638	CKF110	1	FJ584115	100
	Sphyraenidae	*Sphyraena jello*	MN733642	CKF114	1	HM422420	99
		Sphyraena qenie	MN733677	CKF145	1	MK657164	100
Tetraodontiformes	Tetraodontidae	*Arothron manilensis*	MN733540	CKF013	1	AP011929	99
Beloniformes	Zenarchopteridae	*Zenarchopterus dispar*	MN733682, MN733704	CKF148, CKF162	2	KP194857	99
Ophidiiformes	Carapidae	*Carapus mourlani*	MN733652	CKF123	1	KU681392	100
Beryciformes	Holocentridae	*Sargocentron spiniferum*	MN733627	CKF099	1	KP194463	100
		Neoniphon sammara	MN733685, MN733701	CKF149, CKF159	2	MG816708	100
Mugiliformes	Mugilidae	*Moolgarda engeli*	MN733687	CKF151	1	MG816710	100
Anguilliformes	Muraenidae	*Gymnothorax pictus*	MN733587, MN733588, MN733688	CKF060, CKF061, CKF152	3	KP194043	99

All COI reference databases were derived from GenBank. (*N*: Number of individuals).

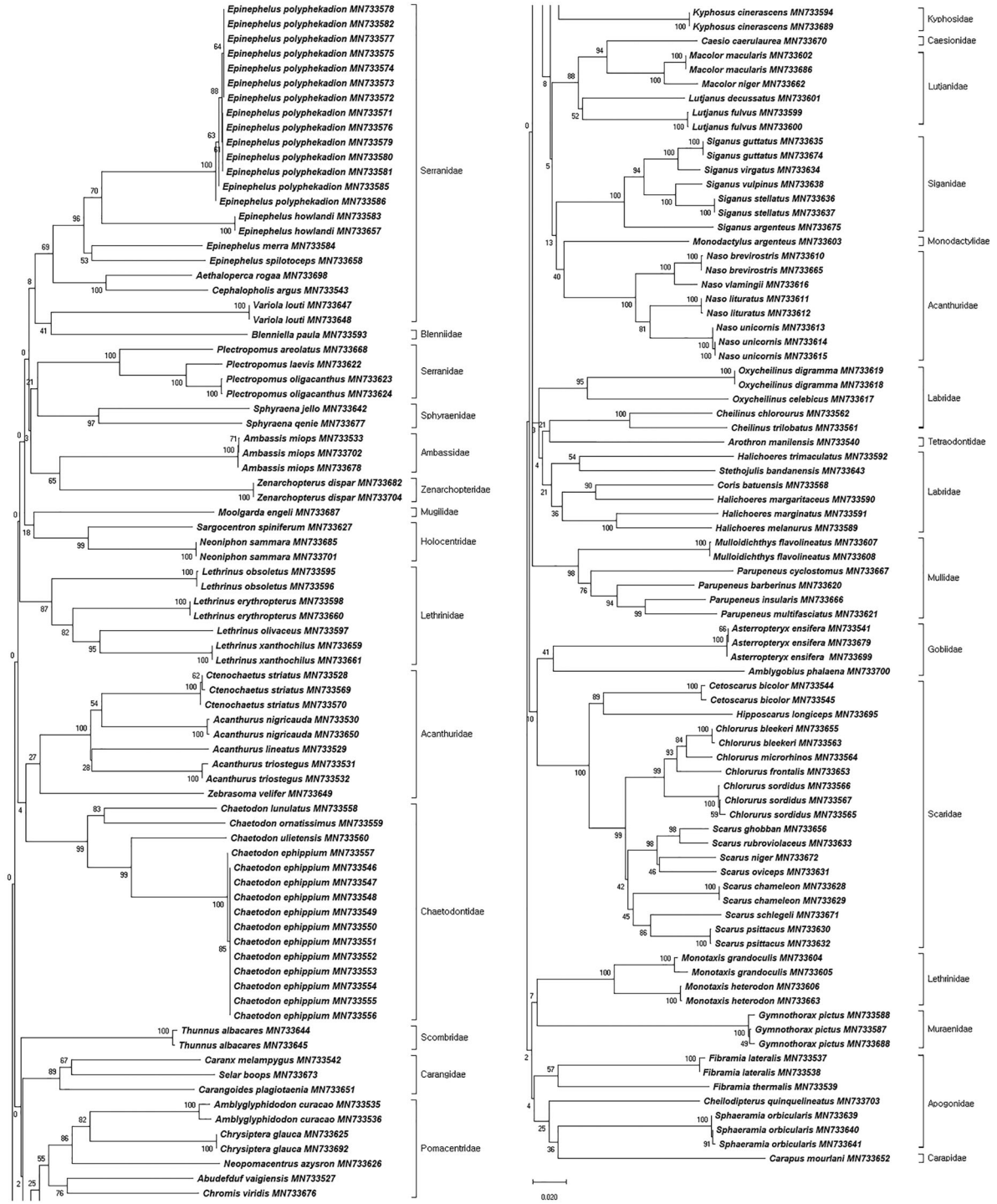

Figure 1. Neighbor-joining (NJ) tree of 162 COI barcodes using K2P distances.

the Labridae family has showed the highest genetic distance (20.25%) and lowest si/sv ratio (1.74) among major groups. This result may reflect an early divergence of species in the Labridae.

The NJ tree from 162 specimens was constructed based on K2P distances (Figure 1). We used this tree to confirm that all species were clustered monophyletic. Thus, DNA barcode analysis is effective in identifying species known to be similar based on morphological observation. Confamilial species are then classified and grouped as independent clades in general phylogenetic analysis. However, some families in this study (Acanthuridae, Serranidae, and Labridae) were not grouped together. Mitochondrial DNA evolves faster than nuclear DNA and is characterized by larger numbers of variable and informative sites. Rapid substitution rates of mitochondrial DNA also make it useful for analyses at species and genus levels. However, deeper branching may then reduce saturation, which can result in homoplasy, as the phylogenetic signal has been reduced (Caterino et al. 2001; Rubinoff and Sperling 2002; Rubinoff and Holland 2005). A previous study (Ward et al. 2005) suggests that phylogenetic analysis using single mitochondrial DNA is suitable for simpler studies, not for deep phylogenetic analysis. Therefore, we confirmed that mitochondrial DNA COI barcodes are effective for

identification of coral reef fish species and analysis of phylogenetic relationships at the species and genus level.

This study, to the best of our knowledge, is the first in which mitochondrial DNA COI barcodes have been used in analyzing coral reef fishes in Chuuk, Micronesia. We identified 95 species, 53 genera, 26 families, and seven orders based on DNA barcoding of 162 fish specimens. Furthermore, we have analyzed divergence time and phylogenetic relationships of fish families that are dominant groups in Chuuk State. Our results confirm that the mitochondrial COI DNA barcodes are an effective tool for the identification of coral reef fish. We predict that similar analyses using larger sample sizes would yield more accurate results given the high marine biodiversity of the study area. We thus anticipate that DNA barcode information obtained in this study will provide baseline data for the protection of coral reef fish biodiversity in Chuuk State, Micronesia.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data that support the findings of this study are openly available in NCBI at https://www.ncbi.nlm.nih.gov/, all reference numbers in Table 1.

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