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# Identifying coral reef fish larvae through DNA barcoding: A test case with the families Acanthuridae and Holocentridae<sup>1</sup>

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## Abstract

A reference collection of COI barcode (650 bp) for the Pacific Society Islands has been constituted for 22 species of Acanthuridae and 16 species of Holocentridae. Divergence between congeneric species was on average 20-fold to 87-fold higher than divergence between conspecific sequences and this set of DNA-identifiers was used to identify 40 larvae of both families. All larvae sequenced could be identified to species using DNA-barcodes. Pools of larvae constitute multi-specific assemblages and no additional species compared to adult reef communities were sampled in larval pools, suggesting that the larval assemblages originated from adult communities on neighboring reefs.

**Keywords:** Coral reef, Barcoding, Larvae, Identification, Mitochondrial DNA

## 1. Introduction

DNA barcoding seeks to develop automated DNA-based identifications using molecular species tags based on short, standardized gene regions (Hebert et al., 2003; Hebert and Gregory, 2005). The primary goal of DNA barcoding is to create reference DNA-barcode libraries for known species used as DNA-identifiers (e.g. Kerr et al., 2007; Hubert et al., 2008). Mitochondrial DNA (mtDNA) has been widely used in evolutionary studies owing to its higher mutation rate and lower effective population size than nuclear DNA (Brown et al., 1979; Birky et al., 1989), and efforts have converged on a 650-bp portion of the mitochondrial cytochrome c oxidase I gene (COI) that can be readily recovered from a vast array of lineages with a limited set of primers. For a barcoding approach to succeed, within species DNA sequences need to be more similar to one another than those between species and recent studies confirmed that the majority of species examined are well delineated by a tight cluster of very similar sequences (Ward et al., 2005; Clare et al., 2006; Robins et al., 2007; Kerr et al., 2007; Hubert et al., 2008; Foottit et al., 2009; Sheffield et al., 2009). Nevertheless, some pitfalls have been identified due to the presence of pseudogenes, introgressive hybridization, and retention of ancestral polymorphism (Zhang and Hewitt, 1996;

Funk and Omland, 2003; Meyer and Paulay, 2005). The occurrence of mixed genealogies among closely related species were estimated to reach 20% (Funk and Omland, 2003), although recent barcoding surveys suggest that it may not exceed 5–10% (Kerr et al., 2007; Hubert et al., 2008). Coral reefs are among the most diverse ecosystems and the Indo-Pacific region alone hosts 10,490 fish species, nearly 32 percent of Earth's ichthyofauna (Froese and Pauly, 2000). In ecosystems with no obvious physical barriers, assessing the determinants of connectivity is a priority for conservation practices (Mora et al., 2006; Claudet et al., 2008). In marine systems, connectivity is widely assessed through analysis of gene flow (e.g. Doherty et al., 1995; Jones et al., 1999; Almany et al., 2007). However, community level processes such as competitive exclusion, assortative settlement and habitat selection may strongly influence species distribution and thereby, communities connectivity (Loreau and Mouquet, 1999; Mouquet and Loreau, 2002; Webb et al., 2002; Leibold et al., 2004). Given their high diversity and dramatic phenotypic changes during development, coral reef fish species identification is no easy task and only feasible up to the genera at best for early ontogenetic stages based on diagnostic morphological characters (e.g. Leis and Carson-Ewart, 2004). Species interactions, however, are likely to vary largely depending on ontogenetic stages through which interactions occur (e.g. Webb,

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2000). Here, we explore the efficacy of the barcoding approach in the identification of coral reef fish larvae to the species level in order to address the following questions. First, larvae are aggregated in patches and schools (Doherty, 1987) and are often collected in pools of several phylogenetically-related individuals, e.g. several individuals from one family or one genus. Do these pools host multi-specific assemblages or correspond to single-species schools? Second, without additional knowledge, larvae collected are assumed to come from the neighboring reefs. With a more precise species-level identification, one can ask whether larvae samples contain species that are not present in adult communities? In such context, we first assessed the genetic variability at COI for two of the most abundant coral reef fish families, namely Acanthuridae and Holocentridae, and further explored its use in a species-level tagging for the identification of early ontogenetic stages up to the species level.

## 2. Material and Methods

### 2.1. Sampling reef fishes larvae in the pelagic realm

Fish larvae were sampled during an oceanographic campaign aboard the N.O. Alis in May 2006 all around the atoll of Tetiaroa in the Society archipelago (17°S, 149°55W). Owing to its relative isolation by surface currents, the atoll of Tetiaroa is likely to be an autonomous system where self-recruitment sustains most of the local populations. In order to describe the spatial distribution of larvae, thirteen stations laid in a radiating pattern around the atoll were sampled twice. Samples were obtained by trawling with a Multiple Opening-Closing Net and Environmental Sampling System (MOCNESS) with an 800  $\mu$ m mesh and 4 m<sup>2</sup> opening. Trawling was conducted between the surface and 100 m where the majority of coral reef fish larvae are found (Boehlert et al., 1992). Sampling was stratified vertically: net M0 sampled from 0 down to 100 m and nets M1 to M4 sampled back up to the surface, in 25 m layers. M0 samples were preserved in 90% ethanol to allow the use of DNA-based identification. M1–M4 samples were preserved in a 4% buffered formaldehyde-sea water solution, which preserves pigmentation, for morphological identification and further oceanographic analysis. In the M0 net, larvae were identified to the lowest taxonomic level possible using a stereo-microscope to look for morphological criteria described in previous systematic surveys (e.g. Moser, 1996; Leis and Carson-Ewart, 2004; Miller and Tsukamoto, 2004) such as general shape, fin rays and spines count, head spine location, and pigmentation. Equivocal identifications were confirmed by using an online photography database developed for this purpose

(<http://cbetm.univ-perp.fr/larvae>). Over all dataset, morphological identification discriminated 82 families, with a number of larvae ranking from 4 to 231 individuals per station. The families Acanthuridae and Holocentridae were the most abundant in the formaldehyde-preserved samples, particularly in stations 9 and 10 during the first round of sampling since 87 out of the 158 Acanthuridae and 78 out of the 120 Holocentridae were sampled there (Irisson, 2008). Likewise, Acanthuridae and Holocentridae were the most abundant families in ethanol-preserved samples and stations 9 and 10 of the first sampling round also contained high concentrations of larvae, with 15 Acanthuridae and 20 Holocentridae at stations 9 and 11 Acanthuridae at station 10. Finally, a total of 46 larvae (sum of Holocentridae and Acanthuridae of stations 9 and 10) were used for DNA-based identifications.

### 2.2. Adult sampling, reference library and

**barcoding** Fish adults used as DNA-identifiers for larvae in the reference library were sampled apart in the context of the Moorea biocode project and FISH-BOL campaign (Ward et al., 2009) in 2006 all around the atoll of Moorea in the Society Archipelago. The reference library of adult sequences was built from ethanol-preserved fin clips on specimens identified by experts using morphological criteria (Randall, 2005). For each species, reference specimens were deposited as vouchers in publicly available collections, namely the 'Muséum National d'Histoire Naturelle' (MNHN) in Paris and the Moorea Biocode collection (MBIO) in Berkeley. In order to fit with the BARCODE criteria in GenBank (Hubert et al., 2008), sequences were bi-directionally sequenced for at least 500 bp and electropherogram trace files were made accessible in the NCBI Trace Archive as well as forward and reverse PCR amplification primers. In addition, a link with the Barcode of Life Data System (BOLD) has been created to provide access to detailed voucher data including collection record and photographs. These data are also publicly available in the Moorea Biocode databases (<http://biocode.berkeley.edu>) (Table 1).

Genomic DNA was extracted using the Genra System Puregene DNA Purification Kit according to manufacturer specifications. A 650-bp segment was amplified from the 5' region of the mitochondrial COI gene using the primers FF2d-5'TTCTCCACCAACCACAARGAYATYGG3' and FR1d-5'CACCTCAGGGTGTCCGAARAAYCARAA3' (Ivanova et al., 2007). PCR amplifications were performed in 27  $\mu$ l including 10.7  $\mu$ l of ultrapure water, 2.5  $\mu$ l of 10 PCR buffer, 3  $\mu$ l of MgCl<sub>2</sub> (25 mM), 2.5  $\mu$ l of each primers (10 mM), 3  $\mu$ l of each dNTP (2 mM), 0.3  $\mu$ l of Taq DNA polymerase (5U/ $\mu$ l), and 4

$\mu\text{l}$  of template DNA. The PCR conditions consisted of 94°C (5 min), 10 cycles of 94°C (1 min), 60–50°C decreasing 1°C per cycle (1 min), 72°C (1 min 30 s) followed by 25 cycles of 94°C (1 min), 50°C (1 min), 72°C (1 min 30 s), with a final extension at 72°C (5 min). All sequences have been deposited in GenBank (Accession Numbers XX–XX). Accession Numbers for the barcodes, specimen and collection data, sequences, trace files, and primers details are available within the project “in progress” in BOLD (<http://www.barcodinglife.org>). Sequence divergence was calculated using the JC69 model (Jukes and Cantor, 1969) and ultrametric trees were computed using the UPGMA algorithm as implemented in \*PAUP 4.0b10 (Swofford, 2002) to provide a graphic representation of species divergence. Finally, several metrics were computed from the pairwise distance matrix using the package APE for R (Paradis et al., 2004), namely the mean, minimum and maximum of the distance within species ( $D_{\text{within species}}$ ), the distance to the nearest neighbor ( $D_{\text{NN}}$ ) and the distance between species ( $D_{\text{between species}}$ ).

### 3. Results and discussion

All adult specimens were successfully amplified using the primers FF2d and FR1d. Four genera and 22 species were discriminated among the 53 adults of Acanthuridae. Another four genera and 16 species were characterized from 53 adults of Holocentridae (Table 1). Those 106 sequences constituted the reference library of DNA-identifiers used to assign larvae to known species. Among the 46 larvae collected in this study, only three Acanthuridae and three Holocentridae failed to amplify using FF2d and FR1d. The 40 remaining larvae provided COI consensus established through bi-directional sequencing. A total of 146 COI barcodes of 650-bp were thus obtained for 38 species and eight genera. No insertions, deletions or codon stops were found, supporting that all amplified sequences constitute functional mitochondrial COI. All the amplified sequences were larger than 600-bp, the maximum size limit observed for non-functional nuclear copies of mtDNA genes (Zhang and Hewitt, 1996).

The distribution of pairwise differences among COI barcodes of adults revealed little to no overlap in the distribution of divergence within and between species (Figure 1). The majority of COI sequences were identical within species, while reaching up to four differences in Acanthuridae and two differences in Holocentridae. The minimum number of inter-specific differences was eight for Acanthuridae and two for Holocentridae, hence only slightly overlapping with intra-specific differences for the

latter. The divergence of sequences within species ( $D_{\text{within species}}$ ) was relatively homogeneous between genera, ranging from 0.001 to 0.004 in both families (Table 2). By contrast, divergence between species ( $D_{\text{between species}}$ ) greatly varied among genera from an average of 0.063 in *Myripristis* to 0.11 on average in *Acanthurus*. Overall, the divergence was 20-fold to 87-fold higher among congeneric species than among conspecific sequences. The average distance to the nearest neighbor ( $D_{\text{NN}}$ ), i.e. the minimum genetic distance between a species and its closest congeneric relative, was lower than the average distance between species while remaining 13-fold to 85-fold higher than the mean divergence within species. Unexpectedly,  $D_{\text{NN}}$  was more homogeneous between genera than the average congeneric distance between species, averaging 0.05 in *Naso*, *Zebrasoma*, *Myripristis*, and *Sargocentron* while reaching 0.08 in *Acanthurus* and *Ctenochaetus*.

The distribution of pairwise differences within and among species slightly overlapped in Holocentridae, but all species from both families were monophyletic and formed clusters of tightly related sequences (Table 2, Figure 2). It has been recently proposed that the efficacy of the barcoding approach relies on the existence of a ‘barcoding gap’, between intra- and inter-specific divergence (Meyer and Paulay, 2005). This gap is created by the fact that mutation is more frequent than speciation, therefore lineages diversify more quickly between species than within species (Pons et al., 2006). Provided that speciation is stochastic, sampling large pools of species increases the probability of sampling species pairs that diverged recently. Nevertheless, overlap in the distribution of divergence will only lead to erroneous molecular identification if sister species remain in the lineage sorting period and still share polymorphism through common ancestry (Funk and Omland, 2003). This was not the case here since the sister species examined were all reciprocally monophyletic, even the pair *Sargocentron caudimaculatum* and *S. spiniferum* that diverged only by 0.007 on average. This result reinforces the view that no canonical threshold applies to the frontier separating populations and species in fishes (e.g. Hubert et al., 2008).

The pattern of divergence together with the topology of species’ genealogies support that barcoding with COI is effective for the Acanthuridae and Holocentridae communities from the Society Islands. Thus, this set of DNA-identifiers was used for the molecular identification of the 40 larvae sequenced for both families (Figure 2). The 40 larval sequences all branched unambiguously within a species’ genealogy. At station 9 (MOCNESS M9/1), three *Acanthurus* and one *Naso* species were identified (*A. glaucopareius*,



**Table 1:** Details of the capture and registration of specimens. The columns are: voucher catalog number either in the Muséum National d'Histoire Naturelle (MParis) or in Moorea Biocode Databases (MBIO), MBIO specimen numbers, Barcode of Life Database (BOLD) specimen and sequence numbers, GenBank Accession Numbers, and geographic location. Stars ( ) denote species names assigned to an early stage specimen by the present molecular analysis.

Family	Species	Voucher	Biocode ref	BOLD				Island	Island group	Latitude/Longitude
				Specimen ref	Sequence ref	Genbank	Island			
Acanthuridae	<i>Acanthurus achilles</i>	MNHN2008-953	MBIO1277	MBIO1277.4	MBFA764-07	HM034179	Moorea	Society Islands	-17.48440/-149.91580	
Acanthuridae	<i>Acanthurus achilles</i>	MNHN2008-954	MBIO1278	MBIO1278.4	MBFA765-07	HM034178	Moorea	Society Islands	-17.48440/-149.91580	
Acanthuridae	<i>Acanthurus achilles</i>	MBIO18242	MOH101	MBIO18242	MBFC008-10	HM034177	Mohotani	Marquesas Islands	-9.95/-138.832	
Acanthuridae	<i>Acanthurus blochii</i>	MNHN2008-891	MBIO1159	MBIO1159.4	MBFA681-07	HM034180	Moorea	Society Islands	-17.51330/-149.84960	
Acanthuridae	<i>Acanthurus glaucopareius</i>	MBIO18213	MOH172	MBIO18213	MBFC003-10	HM034184	Mohotani	Marquesas Islands	-9.96/-138.838	
Acanthuridae	<i>Acanthurus glaucopareius</i>	MNHN2009-1628	MOCNESS M9/1-M0-2	MOCNESS M9.1-M0-2	PFPL001-09	HM034137	Tetiaroa	Society Islands	-16.96977/-149.56289	
Acanthuridae	<i>Acanthurus lineatus</i>	MNHN2008-762	MBIO0918	MBIO0918.4	MBFA565-07	HM034183	Moorea	Society Islands	-17.48900/-149.85800	
Acanthuridae	<i>Acanthurus lineatus</i>	MBIO0919	MBIO0919	MBIO0919.4	MBFA566-07	HM034182	Moorea	Society Islands	-17.48900/-149.85800	
Acanthuridae	<i>Acanthurus nigricans</i>	MNHN2008-239	MBIO136	MBIO136.4	MBFA077-07	HM034186	Moorea	Society Islands	-17.48240/-149.88300	
Acanthuridae	<i>Acanthurus nigricans</i>	MNHN2008-240	MBIO137	MBIO137.4	MBFA078-07	HM034185	Moorea	Society Islands	-17.48240/-149.88300	
Acanthuridae	<i>Acanthurus nigricauda</i>	MNHN2008-888	MBIO1154	MBIO1154.4	MBFA679-07	HM034187	Moorea	Society Islands	-17.51330/-149.84960	
Acanthuridae	<i>Acanthurus nigricauda</i>	MNHN2008-454	MBIO041	MBIO041.4	MBFA263-07	HM034189	Moorea	Society Islands	-17.50270/-149.92500	
Acanthuridae	<i>Acanthurus nigricauda</i>	MNHN2008-761	MBIO0917	MBIO0917.4	MBFA564-07	HM034188	Moorea	Society Islands	-17.48900/-149.85800	
Acanthuridae	<i>Acanthurus nigrofuscus</i>	MNHN2008-230	MBIO126	MBIO126.4	MBFA068-07	HM034190	Moorea	Society Islands	-17.48240/-149.88300	
Acanthuridae	<i>Acanthurus nigrofuscus</i>	MNHN2008-168	MBIO041	MBIO041.4	MBFA013-07	HM034192	Moorea	Society Islands	-17.48240/-149.88300	
Acanthuridae	<i>Acanthurus nigrofuscus</i>	MNHN2008-169	MBIO042	MBIO042.4	MBFA014-07	HM034191	Moorea	Society Islands	-17.48240/-149.88300	
Acanthuridae	<i>Acanthurus nigrofuscus</i>	MNHN2009-1629	MOCNESS M9/1-M0-1	MOCNESS M9.1-M0-1	PFPL002-09	HM034138	Tetiaroa	Society Islands	-16.96977/-149.56289	
Acanthuridae	<i>Acanthurus nigrofuscus</i>	MNHN2009-1630	MOCNESS M9/1-M0-3	MOCNESS M9.1-M0-3	PFPL003-09	HM034139	Tetiaroa	Society Islands	-16.96977/-149.56289	
Acanthuridae	<i>Acanthurus nigrofuscus</i>	MNHN2009-1631	MOCNESS M9/1-M0-4	MOCNESS M9.1-M0-4	PFPL004-09	HM034140	Tetiaroa	Society Islands	-16.96977/-149.56289	
Acanthuridae	<i>Acanthurus nigrofuscus</i>	MNHN2009-1632	MOCNESS M9/1-M0-6	MOCNESS M9.1-M0-6	PFPL005-09	HM034141	Tetiaroa	Society Islands	-16.96977/-149.56289	
Acanthuridae	<i>Acanthurus nigrofuscus</i>	MNHN2009-1633	MOCNESS M9/1-M0-7	MOCNESS M9.1-M0-7	PFPL006-09	HM034142	Tetiaroa	Society Islands	-16.96977/-149.56289	
Acanthuridae	<i>Acanthurus nigrofuscus</i>	MNHN2009-1634	MOCNESS M9/1-M0-11	MOCNESS M9.1-M0-11	PFPL007-09	HM034143	Tetiaroa	Society Islands	-16.96977/-149.56289	
Acanthuridae	<i>Acanthurus nigrofuscus</i>	MNHN2009-1635	MOCNESS M9/1-M0-12	MOCNESS M9.1-M0-12	PFPL008-09	HM034144	Tetiaroa	Society Islands	-16.96977/-149.56289	
Acanthuridae	<i>Acanthurus nigrofuscus</i>	MNHN2009-1636	MOCNESS M9/1-M0-13	MOCNESS M9.1-M0-13	PFPL009-09	HM034145	Tetiaroa	Society Islands	-16.96977/-149.56289	
Acanthuridae	<i>Acanthurus nubilus</i>	MNHN2008-788	MBIO0953	MBIO0953.4	MBFA591-07	HM034193	Moorea	Society Islands	-17.48430/-149.86940	
Acanthuridae	<i>Acanthurus olivaceus</i>	MBIO1233	MBIO1233	MBIO1233.4	MBFA725-07	HM034197	Moorea	Society Islands	-17.48240/-149.89330	
Acanthuridae	<i>Acanthurus olivaceus</i>	MNHN2008-763	MBIO0920	MBIO0920.4	MBFA567-07	HM034198	Moorea	Society Islands	-17.48900/-149.85800	
Acanthuridae	<i>Acanthurus olivaceus</i>	MBIO18257	MOH116	MBIO18257	MBFC011-10	HM034196	Mohotani	Marquesas Islands	-9.95/-138.832	
Acanthuridae	<i>Acanthurus olivaceus</i>	MBIO18261	MOH120	MBIO18261	MBFC012-10	HM034195	Mohotani	Marquesas Islands	-9.95/-138.832	
Acanthuridae	<i>Acanthurus olivaceus</i>	MBIO18328	MOH187	MBIO18328	MBFC014-10	HM034194	Mohotani	Marquesas Islands	-9.97/-138.842	
Acanthuridae	<i>Acanthurus pyroferus</i>	MBIO1316	MBIO1316	MBIO1316.4	MBFA793-07	HM034200	Moorea	Society Islands	-17.47140/-149.77280	
Acanthuridae	<i>Acanthurus pyroferus</i>	MNHN2008-757	MBIO0909	MBIO0909.4	MBFA559-07	HM034202	Moorea	Society Islands	-17.48900/-149.85800	
Acanthuridae	<i>Acanthurus pyroferus</i>	MNHN2008-758	MBIO0910	MBIO0910.4	MBFA560-07	HM034201	Moorea	Society Islands	-17.48900/-149.85800	
Acanthuridae	<i>Acanthurus pyroferus</i>	MBIO18263	MOH122	MBIO18263	MBFC013-10	HM034199	Mohotani	Marquesas Islands	-9.95/-138.832	
Acanthuridae	<i>Acanthurus thompsoni</i>	MBIO1620	MBIO1620	MBIO1620.4	MBFA936-07	HM034203	Moorea	Society Islands	-17.48350/-149.85860	
Acanthuridae	<i>Acanthurus thompsoni</i>	MNHN2008-808	MBIO0987	MBIO0987.4	MBFA612-07	HM034204	Moorea	Society Islands	-17.48260/-149.89900	
Acanthuridae	<i>Acanthurus thompsoni</i>	MNHN2008-161	MBIO033	MBIO033.4	MBFA007-07	HM034205	Moorea	Society Islands	-17.48240/-149.88300	
Acanthuridae	<i>Acanthurus triostegus</i>	MNHN2008-774	MBIO0934	MBIO0934.4	MBFA578-07	HM034207	Moorea	Society Islands	-17.48430/-149.86940	
Acanthuridae	<i>Acanthurus triostegus</i>	MBIO0935	MBIO0935	MBIO0935.4	MBFA579-07	HM034206	Moorea	Society Islands	-17.48430/-149.86940	
Acanthuridae	<i>Acanthurus triostegus</i>	MNHN2009-1637	MOCNESS M10/1-M0-37	MOCNESS M10.1-M0-37	PFPL010-09	HM034146	Tetiaroa	Society Islands	-16.95403/-149.56125	
Acanthuridae	<i>Acanthurus triostegus</i>	MNHN2009-1638	MOCNESS M10/1-M0-38	MOCNESS M10.1-M0-38	PFPL011-09	HM034147	Tetiaroa	Society Islands	-16.95403/-149.56125	
Acanthuridae	<i>Acanthurus triostegus</i>	MNHN2009-1639	MOCNESS M10/1-M0-39	MOCNESS M10.1-M0-39	PFPL012-09	HM034148	Tetiaroa	Society Islands	-16.95403/-149.56125	
Acanthuridae	<i>Acanthurus triostegus</i>	MNHN2009-1640	MOCNESS M9/1-M0-5	MOCNESS M9.1-M0-5	PFPL013-09	HM034149	Tetiaroa	Society Islands	-16.96977/-149.56289	
Acanthuridae	<i>Acanthurus triostegus</i>	MNHN2009-1641	MOCNESS M9/1-M0-8	MOCNESS M9.1-M0-8	PFPL014-09	HM034150	Tetiaroa	Society Islands	-16.96977/-149.56289	
Acanthuridae	<i>Acanthurus triostegus</i>	MNHN2009-1642	MOCNESS M9/1-M0-9	MOCNESS M9.1-M0-9	PFPL015-09	HM034151	Tetiaroa	Society Islands	-16.96977/-149.56289	
Acanthuridae	<i>Acanthurus triostegus</i>	MNHN2009-1643	MOCNESS M9/1-M0-10	MOCNESS M9.1-M0-10	PFPL016-09	HM034152	Tetiaroa	Society Islands	-16.96977/-149.56289	
Acanthuridae	<i>Acanthurus triostegus</i>	MNHN2009-1644	MOCNESS M9/1-M0-14	MOCNESS M9.1-M0-14	PFPL017-09	HM034153	Tetiaroa	Society Islands	-16.96977/-149.56289	
Acanthuridae	<i>Acanthurus xanoptentus</i>	MNHN2008-887	MBIO1153	MBIO1153.4	MBFA678-07	HM034208	Moorea	Society Islands	-17.51330/-149.84960	
Acanthuridae	<i>Ctenochaetus flavicauda</i>	MNHN2008-805	MBIO0982	MBIO0982.4	MBFA608-07	HM034212	Moorea	Society Islands	-17.48260/-149.89900	
Acanthuridae	<i>Ctenochaetus flavicauda</i>	MNHN2008-806	MBIO0983	MBIO0983.4	MBFA609-07	HM034211	Moorea	Society Islands	-17.48260/-149.89900	
Acanthuridae	<i>Ctenochaetus flavicauda</i>	MBIO18256	MOH115	MBIO18256	MBFC010-10	HM034209	Mohotani	Marquesas Islands	-9.95/-138.832	
Acanthuridae	<i>Ctenochaetus striatus</i>	MNHN2008-242	MBIO140	MBIO140.4	MBFA079-07	HM034214	Moorea	Society Islands	-17.48240/-149.88300	
Acanthuridae	<i>Ctenochaetus striatus</i>	MNHN2008-243	MBIO141	MBIO141.4	MBFA080-07	HM034213	Moorea	Society Islands	-17.48240/-149.88300	
Acanthuridae	<i>Naso annulatus</i>	MNHN2008-1130	MBIO1798	MBIO1798.4	MBFA998-07	HM034241	Moorea	Society Islands	-17.53500/-149.77110	
Acanthuridae	<i>Naso annulatus</i>	MNHN2009-1645	MOCNESS M10/1-M0-41	MOCNESS M10.1-M0-41	PFPL024-09	HM034154	Tetiaroa	Society Islands	-16.95403/-149.56125	
Acanthuridae	<i>Naso annulatus</i>	MNHN2009-1646	MOCNESS M10/1-M0-46	MOCNESS M10.1-M0-46	PFPL025-09	HM034155	Tetiaroa	Society Islands	-16.95403/-149.56125	
Acanthuridae	<i>Naso lituratus</i>	MNHN2008-820	MBIO1002	MBIO1002.4	MBFA624-07	HM034242	Moorea	Society Islands	-17.48260/-149.89900	
Acanthuridae	<i>Naso lituratus</i>	MNHN2008-271	MBIO177	MBIO177.4	MBFA105-07	HM034246	Moorea	Society Islands	-17.48240/-149.88300	
Acanthuridae	<i>Naso lituratus</i>	MNHN2008-272	MBIO178	MBIO178.4	MBFA106-07	HM034245	Moorea	Society Islands	-17.48240/-149.88300	
Acanthuridae	<i>Naso lituratus</i>	MNHN2008-158	MBIO029	MBIO029.4	MBFA005-07	HM034248	Moorea	Society Islands	-17.48240/-149.88300	
Acanthuridae	<i>Naso lituratus</i>	MNHN2008-159	MBIO30	MBIO30.4	MBFA006-07	HM034247	Moorea	Society Islands	-17.48240/-149.88300	
Acanthuridae	<i>Naso lituratus</i>	MNHN2008-725	MBIO0861	MBIO0861.4	MBFA525-07	HM034244	Moorea	Society Islands	-17.57840/-149.87250	
Acanthuridae	<i>Naso lituratus</i>	MNHN2008-726	MBIO0862	MBIO0862.4	MBFA526-07	HM034243	Moorea	Society Islands	-17.57840/-149.87250	
Acanthuridae	<i>Naso unicornis</i>	MNHN2008-292	MBIO206	MBIO206.4	MBFA124-07	HM034249	Moorea	Society Islands	-17.50700/-149.82240	
Acanthuridae	<i>Naso unicornis</i>	MNHN2009-1647	MOCNESS M9/1-M0-15	MOCNESS M9.1-M0-15	PFPL026-09	HM034156	Tetiaroa	Society Islands	-16.96977/-149.56289	
Acanthuridae	<i>Naso unicornis</i>	MNHN2009-1648	MOCNESS M10/1-M0-42	MOCNESS M10.1-M0-42	PFPL027-09	HM034157	Tetiaroa	Society Islands	-16.95403/-149.56125	
Acanthuridae	<i>Naso unicornis</i>	MNHN2009-1649	MOCNESS M10/1-M0-44	MOCNESS M10.1-M0-44	PFPL018-09	HM034158	Tetiaroa	Society Islands	-16.95403/-149.56125	
Acanthuridae	<i>Naso unicornis</i>	MNHN2009-1650	MOCNESS M10/1-M0-45	MOCNESS M10.1-M0-45	PFPL019-09	HM034159	Tetiaroa	Society Islands	-16.95403/-149.56125	
Acanthuridae	<i>Naso vlamingii</i>	MNHN2008-921	MBIO1214	MBIO1214.4	MBFA711-07	HM034251	Moorea	Society Islands	-17.48230/-149.89330	
Acanthuridae	<i>Naso vlamingii</i>	MBIO1215	MBIO1215	MBIO1215.4	MBFA712-07	HM034250	Moorea	Society Islands	-17.48230/-149.89330	
Acanthuridae	<i>Zbrasoma rostratum</i>	MBIO18243	MOH102	MBIO18243	MBFC009-10	HM034282	Mohotani	Marquesas Islands	-9.95/-138.832	
Acanthuridae	<i>Zbrasoma scopas</i>	MNHN2008-257	MBIO160	MBIO160.4	MBFA094-07	HM034286	Moorea	Society Islands	-17.48240/-149.88300	
Acanthuridae	<i>Zbrasoma scopas</i>	MNHN2008-258	MBIO161	MBIO161.4	MBFA095-07	HM034285	Moorea	Society Islands	-17.48240/-149.88300	
Acanthuridae	<i>Zbrasoma scopas</i>	MBIO0442	MBIO0442	MBIO0442.4	MBFA264-07	HM034284	Moorea	Society Islands	-17.50270/-149.92500	
Acanthuridae	<i>Zbrasoma scopas</i>	MBIO18386	MOH030	MBIO18386	MBFC015-10	HM034283	Moorea	Society Islands	-17.481/-149.903	
Acanthuridae	<i>Zbrasoma veliferum</i>	MNHN2008-839	MBIO1033	MBIO1033.4	MBFA641-07	HM034288	Moorea	Society Islands	-17.48260/-149.89900	
Acanthuridae	<i>Zbrasoma veliferum</i>	MBIO1034	MBIO1034	MBIO1034.4	MBFA642-07	HM034287	Moorea	Society Islands	-17.48260/-149.89900	
Holocentridae	<i>Myripristis adusta</i>	MNHN2008-903	MBIO1181	MBIO1181.4	MBFA691-07	HM034216	Moorea	Society Islands	-17.51330/-149.84960	
Holocentridae	<i>Myripristis adusta</i>	MBIO1182	MBIO1182	MBIO1182.4	MBFA692-07	HM034215	Moorea	Society Islands	-17.51330/-149.84960	
Holocentridae	<i>Myripristis amaena</i>	MNHN2008-1075	MBIO1598	MBIO1598.4	MBFA921-07	HM034220	Moorea	Society Islands	-17.59220/-149.84030	
Holocentridae	<i>Myripristis amaena</i>	MNHN2008-1094	MBIO1634	MBIO1634.4	MBFA946-07	HM034218	Moorea	Society Islands	-17.49220/-149.92530	
Holocentridae	<i>Myripristis amaena</i>	MBIO1635	MBIO1635	MBIO1635.4	MBFA947-07	HM034217	Moorea	Society Islands	-17.49220/-149.92530	
Holocentridae	<i>Myripristis amaena</i>	MNHN2009-1651	MOCNESS M9/1-M0-33	MOCNESS M9.1-M0-33	PFPL020-09	HM034160	Tetiaroa	Society Islands	-16.96977/-149.56289	
Holocentridae	<i>Myripristis berndti</i>	MNHN2008-1092	MBIO1630	MBIO1630.4	MBFA942-07	HM034222	Moorea	Society Islands	-17.49220/-149.92530	
Holocentridae	<i>Myripristis berndti</i>	MBIO1631	MBIO1631	MBIO1631.4	MBFA943-07					

Table 1 (continued)

Holocentridae	Neoniphon sammara	MNHN2008-457	MBIO446	MBIO446.4	MBFA268-07	HM034255	Moorea	Society Islands	-17.50270/-149.92500
Holocentridae	Neoniphon sammara	MNHN2008-458	MBIO447	MBIO447.4	MBFA269-07	HM034254	Moorea	Society Islands	-17.50270/-149.92500
Holocentridae	Neoniphon sammara	MNHN2008-1064	MBIO1574	MBIO1574.4	MBFA913-07	HM034253	Moorea	Society Islands	-17.59220/-149.84030
Holocentridae	Neoniphon sammara	MBIO1575	MBIO1575	MBIO1575.4	MBFA914-07	HM034252	Moorea	Society Islands	-17.59220/-149.84030
Holocentridae	Plectrypops lima	MNHN2008-266	MBIO171	MBIO171.4	MBFA101-07	HM034256	Moorea	Society Islands	-17.48240/-149.88300
Holocentridae	Sargocentron caudimaculatum	MBIO609	MBIO609	MBIO609.4	MBFA369-07	HM034272	Moorea	Society Islands	-17.48240/-149.88300
Holocentridae	Sargocentron caudimaculatum	MBIO18217	MOH76	MBIO18217	MBFC004-10	HM034257	Mohotani	Marquesas Islands	-9.96/-138.838
Holocentridae	Sargocentron caudimaculatum	MNHN2009-1655	MOCNESS M9/1-M0-21	MOCNESS M9.1-M0-21	FPFL028-09	HM034164	Tetiaroa	Society Islands	-16.96977/-149.56289
Holocentridae	Sargocentron caudimaculatum	MNHN2009-1656	MOCNESS M9/1-M0-23	MOCNESS M9.1-M0-23	FPFL029-09	HM034165	Tetiaroa	Society Islands	-16.96977/-149.56289
Holocentridae	Sargocentron diadema	MNHN2008-915	MBIO1206	MBIO1206.4	MBFA707-07	HM034259	Moorea	Society Islands	-17.51330/-149.84960
Holocentridae	Sargocentron diadema	MNHN2008-916	MBIO1207	MBIO1207.4	MBFA708-07	HM034258	Moorea	Society Islands	-17.51330/-149.84960
Holocentridae	Sargocentron melanospilos	MNHN2008-914	MBIO1205	MBIO1205.4	MBFA706-07	HM034260	Moorea	Society Islands	-17.51330/-149.84960
Holocentridae	Sargocentron melanospilos	MNHN2008-710	MBIO839	MBIO839.4	MBFA508-07	HM034261	Moorea	Society Islands	-17.57840/-149.87250
Holocentridae	Sargocentron microstoma	MNHN2008-262	MBIO166	MBIO166.4	MBFA098-07	HM034265	Moorea	Society Islands	-17.48240/-149.88300
Holocentridae	Sargocentron microstoma	MNHN2008-263	MBIO167	MBIO167.4	MBFA099-07	HM034264	Moorea	Society Islands	-17.48240/-149.88300
Holocentridae	Sargocentron microstoma	MNHN2008-651	MBIO751	MBIO751.4	MBFA449-07	HM034263	Moorea	Society Islands	-17.60630/-149.83400
Holocentridae	Sargocentron microstoma	MNHN2008-652	MBIO752	MBIO752.4	MBFA450-07	HM034262	Moorea	Society Islands	-17.60630/-149.83400
Holocentridae	Sargocentron punctatissimum	MNHN2008-305	MBIO223	MBIO223.4	MBFA137-07	HM034270	Moorea	Society Islands	-17.48250/-149.88210
Holocentridae	Sargocentron punctatissimum	MNHN2008-306	MBIO224	MBIO224.4	MBFA138-07	HM034269	Moorea	Society Islands	-17.48250/-149.88210
Holocentridae	Sargocentron punctatissimum	MNHN2008-699	MBIO824	MBIO824.4	MBFA498-07	HM034268	Moorea	Society Islands	-17.57840/-149.87250
Holocentridae	Sargocentron punctatissimum	MNHN2008-700	MBIO825	MBIO825.4	MBFA499-07	HM034267	Moorea	Society Islands	-17.57840/-149.87250
Holocentridae	Sargocentron punctatissimum	MBIO18192	MOH51	MBIO18192	MBFC002-10	HM034266	Mohotani	Marquesas Islands	-9.96/-138.838
Holocentridae	Sargocentron spiniferum	MNHN2008-455	MBIO443	MBIO443.4	MBFA265-07	HM034276	Moorea	Society Islands	-17.50270/-149.92500
Holocentridae	Sargocentron spiniferum	MBIO444	MBIO444	MBIO444.4	MBFA266-07	HM034275	Moorea	Society Islands	-17.50270/-149.92500
Holocentridae	Sargocentron spiniferum	MNHN2008-514	MBIO530	MBIO530.4	MBFA321-07	HM034274	Moorea	Society Islands	-17.50270/-149.92500
Holocentridae	Sargocentron tierie	MNHN2008-288	MBIO200	MBIO200.4	MBFA120-07	HM034279	Moorea	Society Islands	-17.48240/-149.88300
Holocentridae	Sargocentron tierie	MNHN2008-289	MBIO201	MBIO201.4	MBFA121-07	HM034278	Moorea	Society Islands	-17.48240/-149.88300
Holocentridae	Sargocentron tierie	MNHN2008-752	MBIO902	MBIO902.4	MBFA122-07	HM034277	Moorea	Society Islands	-17.48900/-149.85800
Holocentridae	Sargocentron tierie	MNHN2009-1657	MOCNESS M9/1-M0-17	MOCNESS M9.1-M0-17	FPFL030-09	HM034166	Tetiaroa	Society Islands	-16.96977/-149.56289
Holocentridae	Sargocentron tierie	MNHN2009-1658	MOCNESS M9/1-M0-18	MOCNESS M9.1-M0-18	FPFL031-09	HM034167	Tetiaroa	Society Islands	-16.96977/-149.56289
Holocentridae	Sargocentron tierie	MNHN2009-1659	MOCNESS M9/1-M0-19	MOCNESS M9.1-M0-19	FPFL032-09	HM034168	Tetiaroa	Society Islands	-16.96977/-149.56289
Holocentridae	Sargocentron tierie	MNHN2009-1660	MOCNESS M9/1-M0-22	MOCNESS M9.1-M0-22	FPFL033-09	HM034169	Tetiaroa	Society Islands	-16.96977/-149.56289
Holocentridae	Sargocentron tierie	MNHN2009-1661	MOCNESS M9/1-M0-24	MOCNESS M9.1-M0-24	FPFL034-09	HM034170	Tetiaroa	Society Islands	-16.96977/-149.56289
Holocentridae	Sargocentron tierie	MNHN2009-1662	MOCNESS M9/1-M0-25	MOCNESS M9.1-M0-25	FPFL035-09	HM034171	Tetiaroa	Society Islands	-16.96977/-149.56289
Holocentridae	Sargocentron tierie	MNHN2009-1663	MOCNESS M9/1-M0-26	MOCNESS M9.1-M0-26	FPFL036-09	HM034172	Tetiaroa	Society Islands	-16.96977/-149.56289
Holocentridae	Sargocentron tierie	MNHN2009-1664	MOCNESS M9/1-M0-27	MOCNESS M9.1-M0-27	FPFL037-09	HM034173	Tetiaroa	Society Islands	-16.96977/-149.56289
Holocentridae	Sargocentron tierie	MNHN2009-1665	MOCNESS M9/1-M0-29	MOCNESS M9.1-M0-29	FPFL038-09	HM034174	Tetiaroa	Society Islands	-16.96977/-149.56289
Holocentridae	Sargocentron tierie	MNHN2009-1666	MOCNESS M9/1-M0-30	MOCNESS M9.1-M0-30	FPFL039-09	HM034175	Tetiaroa	Society Islands	-16.96977/-149.56289
Holocentridae	Sargocentron tierieoides	MNHN2008-751	MBIO901	MBIO901.4	MBFA553-07	HM034280	Moorea	Society Islands	-17.48900/-149.85800
Holocentridae	Sargocentron tierieoides	MBIO658	MBIO658	MBIO658.4	MBFA391-07	HM034281	Moorea	Society Islands	-17.49460/-149.86190
Holocentridae	Sargocentron tierieoides	MNHN2009-1667	MOCNESS M9/1-M0-20	MOCNESS M9.1-M0-20	FPFL040-09	HM034176	Tetiaroa	Society Islands	-16.96977/-149.56289

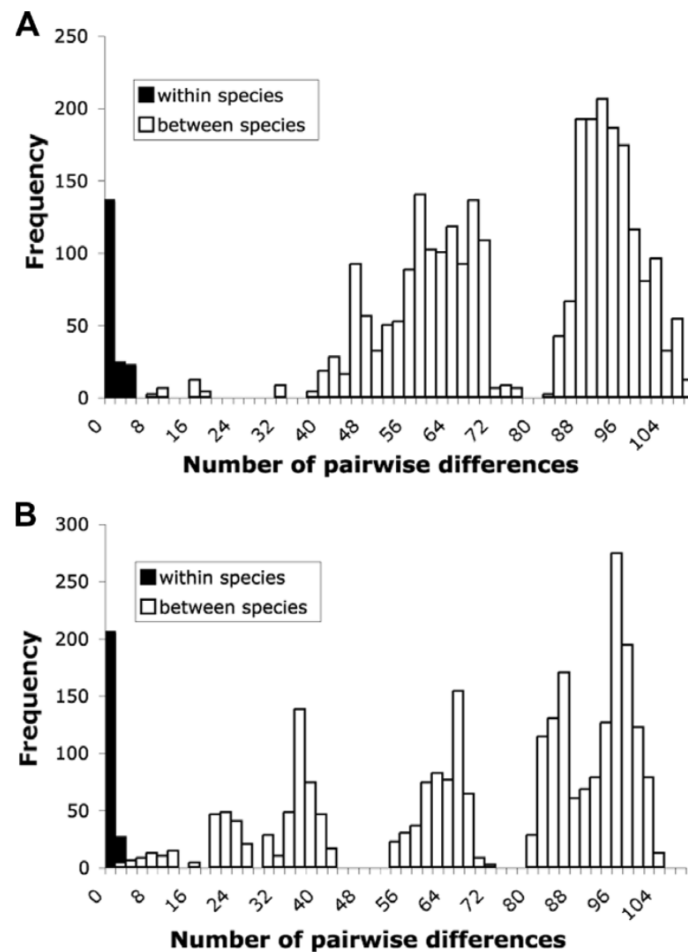
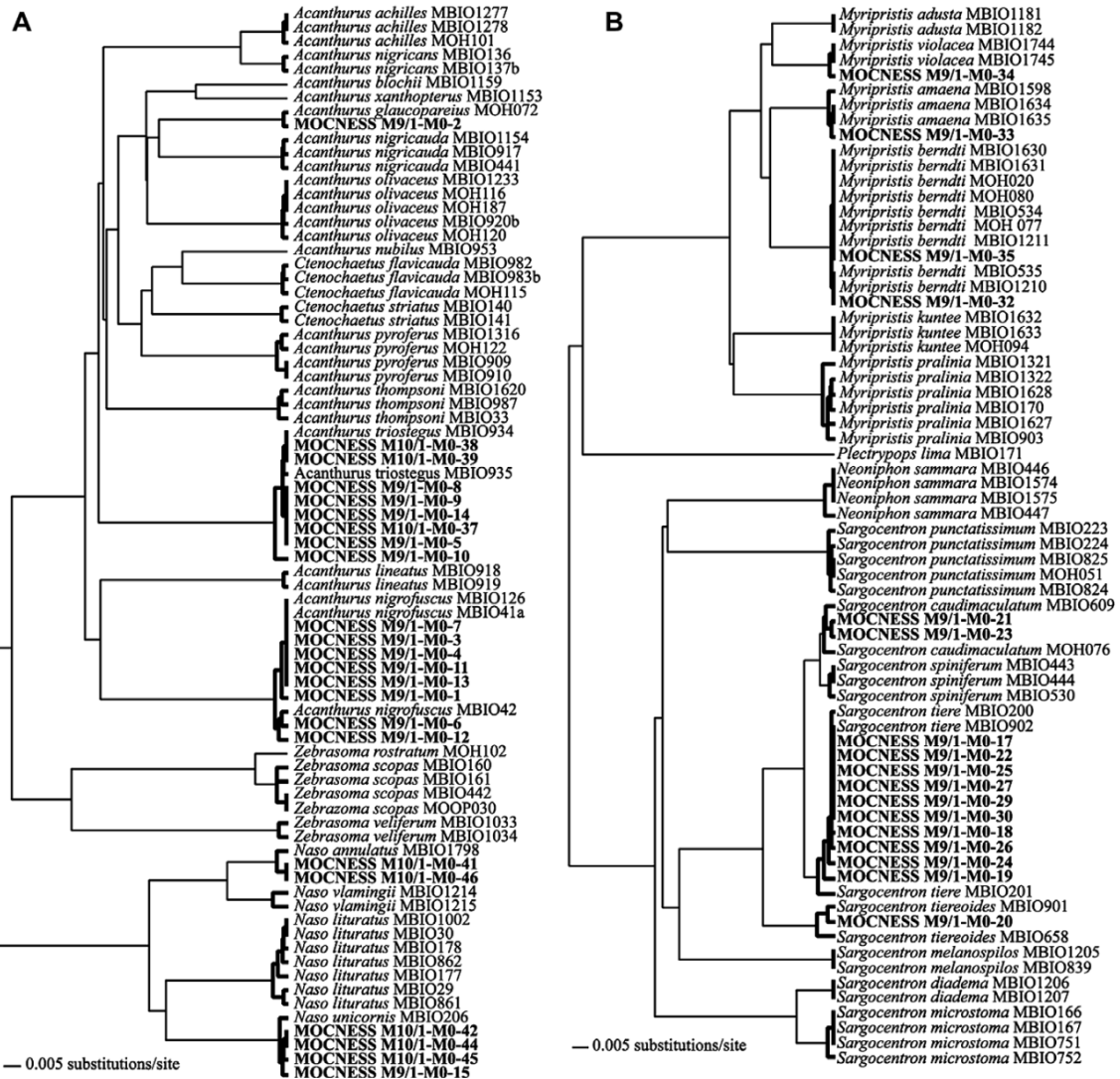


Figure 1: Distribution of the number of pairwise differences among COI sequences between and within species for Acanthuridae (A) and Holocentridae (B).

**Table 2:** Summary of genetic divergences (JC69 model used for computing distances) at COI for the 38 species of the 8 genera analyzed here. Early stage specimens are not included. N, number of pairwise comparisons;  $D_{within\ species}$ , distance between conspecific haplotypes;  $D_{NN}$ , distance to the nearest neighbor (i.e. the minimum of  $D_{between\ species}$ ),  $D_{between\ species}$ , distance between two heterospecific haplotypes; M, percent of monophyletic species.

Family	Genus	N	Species	$D_{within\ species}$			$D_{NN}$			$D_{between\ species}$			M
				min	mean	max	min	mean	max	min	mean	max	
Acanthuridae	Acanthurus	465	13	0	0.002 ± 0.002	0.008	0.028	0.075 ± 0.026	0.104	0.110 ± 0.018	0.134	100	
	Ctenochaetus	6	2	0	0.001 ± 0.001	0.003	0.085	0.085	0.085	0.087 ± 0.002	0.090	100	
	Naso	55	4	0	0.004 ± 0.004	0.010	0.034	0.052 ± 0.021	0.072	0.081 ± 0.014	0.093	100	
	Zebrasoma	21	3	0	0.003 ± 0.003	0.006	0.017	0.056 ± 0.067	0.134	0.104 ± 0.054	0.140	100	
Holocentridae	Myripristis	300	6	0	0.001 ± 0.002	0.009	0.040	0.050 ± 0.013	0.050	0.063 ± 0.012	0.080	100	
	Neoniphon	6	1	0	0.002 ± 0.003	0.006	-	-	-	-	-	-	
	Plectrypops	1	1	-	-	-	-	-	-	-	-	-	
	Sargocentron	253	8	0	0.002 ± 0.002	0.009	0.007	0.052 ± 0.047	0.117	0.102 ± 0.038	0.138	100	



**Figure 2:** UPGMA dendrograms based on a JC69 model of sequence evolution for Acanthuridae (A) and Holocentridae (B). Thick branching represent within species genetic variability. COI barcodes obtained from larvae are labeled MOCNESS and printed in bold.

*A. triostegus*, *A. nigrofuscus*, and *N. unicornis*) while three *Myripristis* and three *Sargocentron* species were identified (*M. violacea*, *M. amaena*, *M. berndti*, *S. caudimaculatum*, *S. tiere*, and *S. tiereoides*). By contrast, station 10 (MOCNESS M10/1) was dominated by the genus *Naso* with two species (*N. annulatus*, *N. unicornis*) and a single-species of *Acanthurus* was detected (*A. triostegus*).

Compared to morphological diagnostic characters, the present barcoding approach provided an unprecedented level of resolution in the identification of early stages of fish. Among the 26 of Acanthuridae larvae, identifications to the sub-family level were feasible for all specimens due to the convex profile of the head in Acanthurinae and a pigmentation spot on the caudal peduncle in Nasinae. Within the Nasinae, the eight specimens were assigned unambiguously to *Naso*, the only genus of Nasinae in the region, while only two specimens in the Acanthurinae were unambiguously identified as *Acanthurus* due to the presence of nine dorsal fin spines. Likewise, identification in Holocentridae was possible to the sub-family level due to the shape of the rostrum (long and simple in Holocentrinae, short and bifurcated in Myripristinae). A single specimen was large enough to count anal and dorsal fins rays which allowed to assign it unambiguously to the genus *Sargocentron*. Species-level meristic characters are not even available for those families in the region. Overall, identifications up to the genus were not feasible for 16 out of 26 Acanthuridae larvae and 19 out of 20 Holocentridae larvae, due to overlap in meristic characters or to the small size of the specimens prohibiting unambiguous counts of spines and fin rays. Morphological identification and molecular assignment were in agreement, although DNA-barcoding allowed unambiguous identifications up to the species level for all 40 specimens.

Placement of larval COI barcodes in the COI tree provided insights into our first concern, 'Do these pools host multi-specific assemblages or correspond to single-species schools?'. The two samples MOCNESS M9/1 and MOCNESS M10/1 yielded multi-specific assemblages of the most specious genera, namely *Acanthurus*, *Naso*, *Myripristis*, and *Sargocentron*. This result suggests that larvae from several species may be found concomitantly in a limited volume of water (i.e. 1000 m<sup>3</sup> of water sampled on average per trawling), and further evidence that conspecific larvae does not necessarily aggregate in large school as previously suggested (Doherty, 1987). For example, samples from MOCNESS M9/1 yielded 32 larvae from 10 species suggesting that conspecific larvae do not necessarily aggregate in large schools but rather mix in multi-specific assemblages. In addition,

the two samples of Acanthuridae showed considerable heterogeneity since *Acanthurus* dominated in MOCNESS M9/1 while *Naso* was more common in MOCNESS M10/1. Finally, the question regarding the potential occurrence of species caught as larvae but undetected in adult reef communities remains open since no additional species compared to adult communities were sampled in larvae at these two stations.

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