Metabolic differentiation facilitates coexistence in two coral reef fish species

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Appendix S1

*Gut content DNA metabarcoding*

Library preparation and sequencing were performed by Jonah Ventures (Boulder, Colorado, USA). We targeted the mitochondrial cytochrome *c* oxidase subunit I (COI) gene region, which performs well across metazoans, and the chloroplast 23S rRNA gene region, which performs well across autotrophs. For the COI markers, we used the m1COIintF forward primer (Leray et al. 2013) and the jgHCO2198 reverse primer (Geller et al. 2013). For the 23S markers, we used the p23SrV\_f1 and Diam23Sr1 23S primers (Sherwood and Presting 2007, Hamsher et al. 2011, Cannon et al. 2016). We ran two-step PCR reactions at 25 μl according to the Promega PCR Master Mix guidelines (Promega, Madison, Wisconsin, USA): 12.5 μl Master Mix, 0.5 μM of each primer, 1 μl gDNA, and 10.5 μl DNase/Rnase-free water. We followed the following conditions for PCR amplification for the COI marker: denaturation at 94 °C for 2 minutes, followed by 45 cycles of 15 seconds at 94 °C, 30 seconds at 50 °C, and 1 minute at 72 °C, then a final elongation at 72 °C for 10 minutes. We used the following conditions for PCR amplification for the 23S marker: denaturation at 94 °C for 3 minutes, followed by 40 cycles of 30 seconds at 94 °C, 45 seconds at 55 °C, and 1 minute at 72 °C, then a final elongation at 72 °C for 10 minutes. Following the PCR amplifications, we visually inspected each reaction with a 2% agarose gel.

The following library preparation and sequencing protocols apply to both the COI and 23S gene. We performed clean-ups by incubating amplicons with Exo1/SAP for 30 minutes at 37 °C, followed by inactivation at 95 °C for 5 minutes. We then stored the products at -20 °C. Afterwards, we ran a second indexing PCR to bind a unique 12-nucleotide index sequence, which included Promega Master mix, 0.5 μM of each primer, and 2 μl of template DNA under the following conditions: denaturation at 95 °C for 3 minutes, followed by 8 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds. Each reaction was visually inspected with a 2% agarose gel to ensure successful amplification.

We cleaned and normalized 25 μl of each indexed amplicon with the SequalPrep Normalization Kit (Life Technologies, Carlsbad, California, USA), pooled 5 μl of each sample, and sent the library pools to the Genohub service provider (Austin, Texas, USA). Bead cleaning with Agencourt AMPure XP beads (Beckman Coulter, Brea, California, USA) was performed to remove <200 bp amplicons, samples were quantified with a Qubit Fluorometer (Invitrogen, Carlsbad, California, USA), and amplicon average size was analyzed with an Agilent TapeStation 4200 (Agilent, Santa Clara, California, USA). Finally, sequencing was performed on an Illumina HiSeq using the HiSeq Rapid SBS Kit v2, 500-cycles (Illumina, San Diego, California, USA).

*Sequence bioinformatics*

We processed all raw COI and 23S sequences with the JAMP pipeline (<https://github.com/VascoElbrecht/JAMP>). After demultiplexing, forward and reverse reads were pair-end merged with USEARCH v11.0.667 (Edgar 2010), and primers were trimmed from both ends using Cutadapt v1.18 (Martin 2011). Quality filtering was performed with expected error filtering through USEARCH (Edgar and Flyvbjerg 2015), and all sequences affected by sequencing and PCR error were removed using the UNOISE algorithm (Edgar 2016). Exact sequence variants (ESVs) were then compiled into ESV tables, and taxonomy was assigned to each ESV using the basic local alignment search tool (BLAST) hit when query coverage and percent identity exceeded 95% and 80%, respectively, or by mapping them against a 23S database from Silva (Yilmaz et al. 2013), specifying zero deviations to ensure mapping accuracy. Consensus taxonomy was generated from the hit tables, first considering 100% matches, then decreasing by 1% until hits were available for each ESV. Taxonomy that was present in at least 90% of the hits was reported; otherwise, an “NA” was assigned when several different taxa matched the ESV. For error reduction due to misidentified taxa, the bracket was increased to 2% when matches of 97% and higher were present, but no family-level or lower taxonomy was assigned. Finally, we removed all self-hits from the COI dataset, which likely resulted from sequencing the host tissue.

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