Metabarcoding dietary analysis of coral dwelling predatory fish demonstrates the minor contribution of coral mutualists to their highly partitioned, generalist diet

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Understanding the role of predators in food webs can be challenging in highly diverse predator/prey systems composed of small cryptic species. DNA based dietary analysis can supplement predator removal experiments and provide high resolution for prey identification. Here we use a metabarcoding approach to provide initial insights into the diet and functional role of coral-dwelling predatory fish feeding on small invertebrates. Fish were collected in Moorea (French Polynesia) where the BIOCODE project has generated DNA barcodes for numerous coral associated invertebrate species. Pyrosequencing data revealed a total of 292 Operational Taxonomic Units (OTU) in the gut contents of the arc-eye hawkfish (Paracirrhites arcatus), the flame hawkfish (Neocirrhites armatus) and the coral croucher (Caracanthus maculatus). 149 (51%) of them had species-level matches in reference libraries (>98% similarity) while 76 additional OTUs (26%) could be identified to higher taxonomic levels. Decapods that have a mutualistic relationship with Pocillopora and are typically dominant among coral branches, represent a minor contribution of the predators' diets. Instead, predators mainly consumed transient species including pelagic taxa such as copepods, chaetognaths and siphonophores suggesting non random feeding behavior. We also identified prey species known to have direct negative interactions with stony corals, such as Hapalocarcinus sp, a gall crab considered a coral parasite, as well as species of vermetid snails known for their deleterious effects on coral growth. Pocillopora DNA accounted for 20.8% and 20.1% of total number of sequences in the guts of the flame hawkfish and coral croucher but it was not detected in the guts of the arc-eye hawkfish. Comparison of diets among the three fishes demonstrates remarkable partitioning with nearly 80% of prey items were consumed by only one predator. Overall, the taxonomic resolution provided by the metabarcoding approach highlights a highly complex interaction web and demonstrates that levels of trophic partitioning among coral reef fishes have likely been underestimated. Therefore, we strongly encourage further empirical approaches to dietary studies prior to making assumptions of trophic equivalency in food web reconstruction.

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contribution of coral mutualists to their highly partitioned, generalist diet.

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Introduction

17 Anthropogenic stressors are impacting all ecosystems on Earth, causing both drastic changes in

18 the structure of communities and a reduction in biodiversity (Wright, 2005; Hoegh-Guldberg &

19 Bruno, 2010). Predators are among the most vulnerable trophic group, and have long been

20 known to play a crucial role in stabilizing ecosystems by generating top-down forces and trophic

21 cascades (Paine, 1966, 1969). Yet because all predator species are not functionally equivalent,

22 understanding how species partition their diet and their ecological role in food webs have

23 become a major focus to help predict the consequences of their decline on ecosystem services

24 (Harley, 2011).

25   A detailed knowledge of a predator's diet is a key element for deciphering its

26 ecological function. Among the numerous techniques used in the literature to characterize a

27 predator's diet, PCR-based molecular analysis of gut contents is among the most powerful

28 because species-diagnostic DNA fragments can be detected even after several hours of digestion

29 (Symondson, 2002). Moreover, the availability of versatile PCR primers targeting short

30 hypervariable DNA regions combined with a high-throughput sequencing platform now offer the

31 possibility to characterize the dietary breadth of any predator (Pompanon et al., 2012; Leray et

32 al., 2013a). The ecological influence of a predator may then be inferred from its dietary

33 selectivity as well as the traits and functional role of prey consumed (Chapman et al., 2013). On

34 land, this tool is already proving invaluable for understanding the biological control potential of

35 insect predators (Mollot et al., 2014) and the ecological effects of large herbivores (Kowalczyk et

36 al., 2011) and carnivores (Shehzad et al., 2012). On the other hand, the use of high-throughput

37 sequencing for understanding trophic links in marine systems has been more limited to date

38 (Leray et al., 2013a).

39   On coral reefs, one of the most diverse and threatened ecosystems, predatory fish

40 feeding on benthic invertebrates are the dominant trophic category. They often dwell within the

41 reef framework where they feed upon diverse communities of small cryptic species that are

42 known to perform a variety of functions including direct positive or negative interactions with

43 stony corals, the foundation species of the coral reef ecosystem (reviewed by Stella et al., 2011).

44 Some invertebrate taxa benefit the survival and growth of corals by slowing the progression of

45 coral diseases (Pollock et al., 2012), protecting corals against corallivores (Glynn 1980, 1983;

46 McKeon and Moore 2014; Rouzé et al., 2014), removing sediments from their coral host

47 (Stewart et al., 2006; Stier et al., 2012) and alleviating detrimental effects of coral competitors or

48 parasites (Stier et al., 2010). Other invertebrates have deleterious effects on corals as they are

49 known vectors of coral diseases (Sussman et al., 2003; Williams & Miller, 2005), are parasites of

50 stony corals (Humes, 1985; Shima, Osenberg & Stier, 2010) or feed upon coral polyps (Turner,

51 1994; Rotjan & Lewis, 2008; Rawlinson et al., 2011) sometimes causing extensive and

52 widespread coral mortality (Leray et al., 2012a; Kayal et al., 2012). As a consequence, the

53 feeding behavior of these predatory fish may have significant cascading effects on the dynamics

54 of stony corals and subsequently the dynamics of the whole coral reef ecosystem, but it has

55 proven challenging to understand their ecological role.

56 The flame hawkfish (*Neocirrhites armatus*), arc-eye hawkfish (*Paracirrhites arcatus*) and

57 coral croucher (*Caracanthus maculatus*) are common predatory fish species on Indo-Pacific

58 coral reefs. They co-occur among the branches of Pocilloporids (genus *Stylophora* and

59 *Pocillopora*), one of the most important reef building corals, along with a wide diversity of

60 invertebrates (Patton, 1974; Coles, 1980; Odinetz, 1983; Stella, Jones & Pratchett, 2010). These

61 invertebrates include both coral mutualistic (family: Trapeziidae and some Alpheidae) and

62 parasitic (family: Cryptochiridae) decapod species (Simon-Blecher & Achituv, 1997), which are

63 potential prey for coral dwelling fish. A field manipulation of the two *Pocilloporid* obligate

64 species, the flame hawkfish and the coral croucher (habitat specialists), highlighted that their

65 presence among the branches of *Pocillopora eydouxi* reduced total abundance and diversity of

66 decapod recruits by 34% and 20% respectively (Stier & Leray, 2014). These predators modified

67 the composition and abundance of key mutualists (coral crabs, genus: *Trapezia*), whose benefits

68 to *Pocillopora* are known to be both density- and diversity- dependent (Stier et al., 2012).

69 Predator removal experiments have also shown that the presence of arc-eye hawkfish decreases

70 the density of coral associated mutualist damselfish (Holbrook, Schmitt & Brooks, 2011).

71 Preliminary molecular dietary analysis using traditional cloning showed the presence of coral

72 mutualists in the gut contents of both hawkfish species (Leray et al., 2013b), but sampling and

73 sequencing effort were too limited to understand their contribution to each species' diets.

74 In the present study, we use a high throughput sequencing approach targeting the

75 mitochondrial Cytochrome c. Oxidase subunit I gene (COI) (also referred to as metabarcoding

76 approach, Taberlet et al., 2012) to describe the dietary breadth of these predators. The study was

77 conducted in Moorea, French Polynesia, where an extensive library of COI DNA barcodes,

78   including all *Pocillopora* associated species, has been built by the BIOCODE project (Leray et

79   al., 2012b). Implications of each predator's feeding behavior are further discussed in light of our

80   findings.

81

Methods

82

83   *Predator and prey collections*

84   Twenty-five adult specimens of each of the three predator fish species were speared after sunset,

85   which corresponds to peak feeding time for all three species (M Leray, personal observation), in

86   the lagoon of the North shore of Moorea on the 8th, 10th and 15th of July 2010. We limited our

87   collections to a single site (17°28'40S; 149°50'25W, Fig. 1) where coral populations have been

88   little impacted by the recent outbreak of the corallivorous seastar, *Acanthaster planci* (Adjeroud

89   et al., 2009; Kayal et al., 2011; Rouzé et al., 2015). Adults of the flame hawkfish and coral

90   croucher always co-occurred among *Pocillopora* branches, whereas adult arc-eye hawkfish were

91   occasionally present. Fish were individually preserved in cold 50% ethanol *in situ* after which

92   their digestive track was dissected within 3 hours and preserved in eppendorf tubes containing

93   80% ethanol. Approval was granted from our institutional animal ethics committee, le Centre

94   National de la Recherche Scientifique (CNRS), for sacrificing and subsequently dissecting fish

95   (Permit Number: 006725). None of the fish species are on the endangered species list and no

96   specific authorization was required from the French Polynesian government for collection.

97

98   *Laboratory protocol*

99   The total content of the digestive track of each fish was dissected and used for total genomic

100 DNA extraction using the QIAGEN DNeasy Blood & Tissue kit. Genomic DNA was then

101 purified using the PowerClean DNA clean-up kit (MO BIO) to remove potential PCR inhibitors.

102 We used a single set of versatile PCR primers (mlCOIintF/jgHCO2198, Geller et al., 2013;

103 Leray et al., 2013a) known to perform well across the diversity of marine invertebrates, to

104 amplify a 313bp region of the mitochondrial Cytochrome c. Oxidase subunit I (COI) region from

105 each gut content sample. Moreover, this primer set was recently shown to provide reliable

106 estimates of relative abundance for metabarcoding benthic samples (Leray & Knowlton, 2015).

107 Because predator DNA co-amplification is known to impede prey detection (Vestheim &

108 Jarman, 2008), predator-specific annealing blocking primers were included at ten times the

109   concentration of versatile primers during PCR reactions as in Leray et al. (2013a). All primer

110   sequences are provided in Table 1. The PCR cocktail and touchdown temperature profile used in

111   this study can both be found in Leray et al. (2013a). Three PCR replications per sample were

112   generated, pooled, gel excised to ensure that all primer dimers were screened away, purified

113   using QIAGEN® MinElute columns and eluted in 12 µl of elution buffer. PCR product

114   concentration was measured with the Qubit® Fluorometer (Invitrogen).

115   We pooled equimolar amounts of the combined amplicons per individual gut content for

116   each predator species (e.g. 25 flame hawkfish gut content samples were pooled together) and 500

117   ng of PCR product was used per species for library preparation for Roche 454 FLX sequencing.

118   Amplicons were end-repaired and dA-tailed using the NEBNext Quick DNA Sample Prep

119   Reagent Set 2 chemistry (New England BioLabs). We then performed a ligation of 454

120   Multiplex Identifiers (a total of three, each one containing a recognizable sequence tag) using the

121   FLX Titanium Rapid Library MID Adaptors Kit (Roche). Finally, the ligated PCR product of

122   each sample was purified using Agencourt AMPure beads (Beckman Coulter Genomics), eluted

123   in 40µl of TE buffer, and the three samples pooled together for sequencing at the Duke Institute

124   for Genome Sciences and Policy (Duke University, NC, USA). Note that the three samples of the

125   present study were combined with several other samples in the same 454 run.

126

127   *Analysis of FLX sequencing data*

128   We followed a sequence data analysis pipeline optimized for analyzing large COI datasets. The

129   pipeline detailed in Leray et al. (2013a) takes advantage of the coding properties of the barcoding

130   region to discard all dubious sequences.

131   An initial step denoised flowgrams using Pyronoise (Quince et al., 2011) implemented in

132   Mothur (Schloss et al., 2009). We then further quality filtered the dataset by removing any reads

133   with the following criteria: shorter than 200bp; more than two mismatches in the primers

134   sequence; any ambiguous base calls (e.g. "N"); or with any homopolymer regions longer than

135   8bp. Remaining sequences were subsequently aligned to a high quality reference dataset

136   (Moorea BIOCODE barcode library) based on amino acid translations using the option

137   "enrichAlignment" in MACSE (Ranwez et al., 2011) and all sequences with any of the following

138   were also discarded: stop codon; frame shift; insertion; or more than three deletions. Finally,

139 potential chimeric sequences identified using UCHIME (Edgar et al., 2011) were removed to

140 obtain a high quality sequence dataset for downstream analysis.

141   To evaluate prey richness and composition, sequences were clustered in Operational

142 Taxonomic Units (OTUs) using a Bayesian algorithm implemented in CROP (Hao, Jiang &

143 Chen, 2011). This program delineates OTUs based on the natural distribution of sequence

144 dissimilarity in the data and within a range of sequence similarity values defined by the user.

145 This approach performs better for clustering sequences obtained from environmental samples

146 than a fixed dissimilarity cutoff (e.g. 5%) because they contain a diversity of phyla that differ in

147 their rate of COI evolution. The lower and upper bound variance were set to 3 and 4 respectively

148 (which corresponds to 6% and 8%) as they were shown to provide the best results for marine

149 invertebrates (Leray et al., 2013a,b). Following OTU delineation, a representative sequence per

150 OTU was used for taxonomic identification using BLAST searches in the local BIOCODE

151 database and in GENBANK. We considered that there was a species level match when sequence

152 similarity was at least 98% (Machida et al., 2009; Plaisance et al., 2009). Whenever sequence

153 similarity was lower than 98%, we used a Bayesian approach implemented in the Statistical

154 Assignment Package (SAP, Munch et al., 2008) to assign OTUs to a higher taxonomic group.

155 SAP conducts assignments by building 10,000 unrooted phylogenetic trees from a collection of

156 homologue sequences retrieved from a sequence database. It then calculates the probability that a

157 query sequence belongs to a monophyletic group within that set of homologues. Here, we

158 allowed SAP to retrieve 50 homologues from GENBANK with >70% sequence similarity to

159 each query sequence (i.e. each OTU representative sequence) and accepted taxonomic

160 assignments at an 80% posterior probability cutoff. Importantly, SAP can only assign sequences

161 to taxonomic groups that are represented in the database, as is also the case with other

162 assignment methods. Therefore, to minimize misidentification at lower taxonomic levels, we

163 only report assignments to the phylum, class and order levels (Appendix 1).

164

165 **Results**

166 We obtained 69,663 reads of which 54,283 high quality reads were retained after alignment

167 based on amino acid translation (arc-eye hawkfish: 24,629; flame hawkfish: 13,536; coral

168 croucher: 16,118). The Bayesian clustering algorithm delineated 292 OTUs in the gut contents of

169 the three predatory fish species (Appendix 1). The number of dietary items was much lower in

170   the gut contents of the coral croucher (64 taxa) than in both arc-eye (147 taxa) and flame

171   hawkfish (149 taxa). BLAST searches provided high-resolution taxonomic assignments (>98%

172   similarity) for 149 OTUs (51%) (Appendix 1) and the statistical assignment approach enabled

173   the identification of 76 additional OTUs to a higher taxonomic level (26%). 67 OTUs (22.9%)

174   remained unidentified (labeled as "Unidentified" in Appendix I). None of the rarefaction curves

175   reached a plateau (Fig. 2) which indicates that further sequencing effort would be necessary for a

176   more exhaustive dietary analysis of these predatory fish.

177   The diversity of dietary items spanned 25 classes belonging to 17 phyla. Malacostraca

178   was the dominant taxonomic prey group (36.7%, 21.5% and 43.7% for the arc-eye hawkfish, the

179   flame hawkfish and the coral croucher, respectively). The arc-eye hawkfish also consumed

180   numerous species of Actinopterygii (17.7% total OTUs) and Maxillopoda (10.9% total OTUs). A

181   significant proportion of the flame hawkfish and coral croucher's diet was represented by

182   Maxillopoda (12.1% and 6.2% total OTUs, respectively) and Gastropoda (9.4% and 4.7% total

183   OTUs, respectively). Eighteen OTUs (28%) detected in the gut contents of the coral croucher

184   remained unidentified. Direct matches to reference barcodes (>98% similarity) were more

185   prevalent among Actinopterygii (94.1%), Malacostraca (74.1%) and Gastropoda (79.2%)

186   compared to Maxillopoda (40%). Moreover, direct matches were more prevalent for OTUs

187   represented by large numbers of sequences (Fig. 3). Almost nine of ten OTUs (86.7%) matched

188   reference barcodes if they were common in the amplicon libraries (>1000 sequences), whereas

189   only a third (33.8%) of the single sequences matched a reference sequence. Probability of a

190   match increased as the number of sequences increased (1: 33.8%; [2-9]: 43.7%; [10-99]: 52.2%;

191   [100-999]: 70%; >1000: 86.7%; Fig. 3).

192   Most Malacostraca OTUs were decapods (81.5%, 46.9% and 78.6% for the arc-eye

193   hawkfish, the flame hawkfish and the coral croucher respectively – Appendix 1). All three

194   predatory fish fed upon *Pocillopora* obligate decapod species, but they represent a minor fraction

195   of the total diversity of the prey they consumed (arc-eye hawkfish: 2%; flame hawkfish: 4%,

196   coral croucher: 9.3%). Among them, we detected five coral crab species of the genus *Trapezia*

197   that are mutualists of *Pocillopora* (*Trapezia bidentata*, *T. serenei*, *T. rufopunctata*, *T. areolata*

198   and *T.* spp). These mutualists also represented a minor proportion of sequences in the gut

199   contents of the arc-eye and flame hawkfish (proportion of total sequences: 5.6% and 2.4%;

200   proportion of decapod sequences: 9.1 % and 12.7%, respectively). *Pocillopora* mutualists

201   represented a higher proportion of the coral croucher's diet with 15.3% of the total number of

202   sequences and 47.9% of the total number of decapod sequences..

203   Additional trophic links involving non-decapod prey are of particular interest for

204   understanding the effect of predators on coral and its associated communities. Predatory fish had

205   fed upon coral associated planktivorous damselfishes of the family Pomacentridae (*Dascyllus*

206   *flavicaudus*: 0.02%, 0% and 0.12%, *Chromis viridis*: 0.01%, 0.69% and 0% of total sequences in

207   the diet of the arc-eye hawkfish, the flame hawkfish and the coral croucher, respectively) that

208   benefit the growth of the coral host (Holbrook et al., 2008). Interestingly, Anthozoa were

209   represented by two OTUs among which the host *Pocillopora* itself accounted for 20.8% and

210   20.1% of total number of sequences in the guts of flame hawkfish and coral croucher, but was

211   completely absent from the gut of the arc-eye hawkfish. On the other hand, *Hapalocarcinus sp*, a

212   gall crab considered a coral parasite, was recovered in the diet of both the arc-eye and flame

213   hawkfish. Both hawkfish had also consumed vermetid snails known for their deleterious effects

214   on coral growth (Shima, Osenberg & Stier, 2010). *Harpiliopsis beaupresii*, a caridean shrimp

215   associated with *Pocillopora* but whose function is unknown, was also detected in the gut

216   contents of the coral croucher. Almost 10 percent (8.3%) of the coral croucher's diet is

217   composed of two snails (*Drupa ricinus* and *Pascula muricata*). Finally, predators had also

218   consumed pelagic taxa including members of Maxillipoda, Chaetognatha and Hydrozoa

219   (Appendix 1).

220   Prey species were remarkably partitioned among predators (Fig. 4). Almost eighty

221   percent (79.5%) of prey species had been consumed by only one predator species (232 of 292).

222   Eighteen percent (N=52) were found in two predator diets and only eight prey species (>3%) had

223   been ingested by all three predatory fish species analyzed. Of the shared components, the arc-eye

224   hawkfish and the coral croucher had consumed 14 taxa in common among which six were

225   Malacostraca. The arc-eye and flame hawkfish shared 29 prey taxa with a majority of

226   Actinopterygii and Malacostraca. Prey sharing was lowest (nine OTUs; of which six were

227   Malacostraca) between the two species that were always found co-occuring together in the coral

228   host, the flame hawkfish and coral croucher. Analyses that included only prey OTUs consisting

229   of >1% of either of the three species diets according to the relative abundance of reads

230   demonstrate even greater partitioning (Fig. 4). Only six of the sixty-six prey items were shared at

231   a proportion greater than 1% in any two fish species diets, and no prey species were shared

232   among all three. Of the 66 prey items making up at least 1% of any diet, nine out of ten were

233   consumed by only one predator. An alignment of all OTU representative sequences is provided

234   in Appendix 2 and the raw sequence dataset was deposited in the Dryad Repository

235   doi:XXXXXXXXXXXXX).

236

237   **Discussion**

238   Dietary analysis can be a powerful approach to gain insights into the ecological role of reef-

239   dwelling predatory fish, but low taxonomic resolution in prey identification often obscures the

240   complexity of trophic links (Longenecker, 2007). For example, the diet of the arc-eye hawkfish,

241   flame hawkfish and coral croucher previously described from morphological identification of

242   prey remains in gut contents was considered to be simply composed of small benthic crustaceans

243   (class: Malacostraca) (Bachet, Zysman & Lefevre, 2007). Preliminary DNA analysis using

244   traditional cloning revealed a breadth of prey species in the guts of the arc-eye and flame

245   hawkfish, the majority of which were crustaceans 18 of 24 (75%) and 21 of 31 (68%)

246   respectively (Leray et al., 2013b). This study highlights that a metabarcoding approach

247   significantly increases the taxonomic scope by documenting an even broader taxonomic

248   distribution of species consumed by hawkfishes (Appendix 1). The coral croucher diet also

249   includes a wide spectrum of prey demonstrating that all three predatory fish feed broadly across

250   community diversity. Our results highlight the importance of collecting empirical dietary data to

251   understand processes of species coexistence in this high diversity marine ecosystem.

252   The ecological influence of a predator is contingent upon the prey it consumes. Their

253   feeding behavior may induce cascading effects that will depend on the type of association that

254   the prey they consume (or interfere with) have with keystone species. For example, in terrestrial

255   ecosystems where up to 90% of flowering plant species use animal pollinators for reproduction

256   (Bushmann & Nabhan, 1996), a predator's effect on plant reproductive success, growth and

257   survival will depend on its relative consumption of pollinators and phytophageous insects

258   (Dukas, 2005; Knight et al., 2006). Similarly, some coral reef dwelling predatory fish may either

259   disrupt benefits to corals if they derive a significant proportion of their diet from coral mutualists

260   or alternatively alleviate deleterious effects on corals if they consume coral parasites.

261   Invertebrate communities occurring among the branches of live *Pocillopora* corals in Moorea or

262   elsewhere in the Pacific are typically composed of a preponderance of decapod mutualists (>80%

263   of diversity and abundance in live *Pocillopora*– see Patton 1974, Coles 1980, Odinetz 1983,

264   Stella et al., 2010, Leray et al., 2012a). Based on previous cloning studies (Leray et al., 2013b)

265   only the arc-eye hawkfish consumed functionally important prey (*Trapezia tigrina*). With

266   increased sequencing depth herein, we demonstrate that while many other mutualist decapod

267   species do occur in the diets (Fig. 1, Appendix 1), they represent a much smaller proportion of

268   the diet of the arc-eye hawkfish, flame hawkfish and coral croucher than exist in the natural

269   communities (5.6%, 2.4% and 15.3% of sequence abundance, respectively). Interestingly, we

270   found evidence of the *Pocillopora* obligate pontoniid shrimp *Harpiliopsis beaupressi* but no

271   detection of congeneric *Harpiliopsis depressa* and *H. spinigera* in the predators' gut contents*,*

272   despite their very high abundance reported on head-size *Pocillopora* in Moorea (Leray et al.,

273   2012a). It is also surprising not to discover *Alpheus lottini* in the diets of the three species

274   although this is a common species found in all living *Pocillopora* observed and known to have

275   beneficial effects on coral survivorship (Stier et al., 2012). Overall, our data indicate a non

276   random pattern of prey consumption atypical of an opportunistic feeding behavior (where prey

277   would be consumed in proportion to their abundance - Heinlein et al., 2010) which suggests the

278   outcome of coevolutionary dynamics between *Pocillopora* associated predator and prey.

279   Nevertheless, while our metabarcoding dietary analysis suggests limited predation

280   pressure on mutualists, a four-month recruitment experiment conducted on the North shore of

281   Moorea in 2009 showed a lower abundance of mutualists in corals where the coral croucher and

282   the flame hawkfish occurred (Stier & Leray, 2014), a pattern that may be driven by non-

283   consumptive effects of predators. For example, competent larvae may preferentially settle on

284   corals where predators are absent. Regardless of the mechanisms, such predator effects have

285   important implications for coral performance, because density and composition of mutualist

286   assemblages are known to be important for the quality of the services provided to their host

287   (Stier et al., 2012; Rouzé et al., 2014).

288   In addition, our metabarcoding analyses of gut contents revealed for the first time

289   predation on a gall crab (*Hapalocarcinus sp*) and vermetid snails (genus: *Dendropoma*), which

290   are considered detrimental to the coral host (Simon-Blecher & Achituv, 1997; Shima, Osenberg

291   & Stier, 2010). Vermetid snails are particularly prevalent in Moorea where they can reduce coral

292   growth by up to 81% and survival by up to 52% (Shima, Osenberg & Stier, 2010). Predation on

293   parasites may compensate for the negative effects of the reduction in density of decapod

294 mutualists in corals facing environmental stressors. We also recovered a significant proportion of

295 sequences belonging to *Pocillopora* from the flame hawkfish and the coral croucher gut contents,

296 which suggest that these predatory fish also feed on mucus released by their biogenic habitat.

297 The absence of *Symbiodinium* COI sequences from our dataset also supports the consumption of

298 mucus rather than coral polyps. Alternatively, *Pocillopora* DNA may have been sufficiently

299 abundant and well preserved in the gut contents of mucus feeding prey (e.g. Trapeziidae) to be

300 co-amplified (Harwood et al., 2001; Sheppard et al., 2005). Importantly though, *Pocillopora* was

301 completely absent from the arc-eye hawkfish diet which also includes Trapeziid species,

302 suggesting minimal secondary consumption or associated eDNA amplification. Overall, high-

303 resolution dietary data are revealing a highly complex interaction web with very specialized

304 functional roles played by each species. This highlights the shortcomings of the functional

305 groups approach commonly used to evaluate redundancy and complementarity among coral reef

306 species (Naeem & Wright, 2003; Micheli & Halpern, 2005).

307   Fine-scale spatial partitioning commonly occurs among coral reef fish species

308 (Robertson & Lassig, 1980; Waldner & Robertson, 1980; Ebersole, 1985; Bouchonnavaro, 1986;

309 Munday, Jones & Caley, 1997; Depczynski & Bellwood, 2004; Gardiner & Jones, 2005) but the

310 extent of food partitioning remains controversial (Longenecker, 2007). In fact, most early work

311 investigating differences in diet among reef fish species showed high levels of diet overlap (Hiatt

312 & Strasburg, 1960; Randall, 1967; Hobson, 1974; Talbot, Russell & Anderson, 1978; Harmelin-

313 Vivien, 1979; Anderson et al., 1981; Bouchonnavaro, 1986; Ross, 1986; Depczynski &

314 Bellwood, 2003; Kulbicki et al., 2005; Longenecker, 2007; Castellanos-Galindo & Giraldo,

315 2008) which has led many to the conclusion that trophic partitioning was not a mechanism

316 promoting species coexistence on coral reefs. However, these studies, which rely on

317 morphological identification of semi-digested prey remains in gut contents grouped food items

318 into broad categories therefore impeding accurate measures of partitioning (Longenecker, 2007).

319 Alternative strategies such as field observations of feeding behavior (Pratchett, 2005, 2007;

320 Pratchett & Berumen, 2008) or a combination of gut content and stable isotope analyses (Ho et

321 al., 2007; Nagelkerken et al., 2009) helped describe dietary differences between closely related

322 species, but generalizations about the importance of trophic partitioning for the maintenance of

323 coral reef diversity remain difficult. In the present study, high-resolution molecular data

324 highlight an unexpected level of dietary partitioning among the three study species. While both

325   hawkfish species are from the same family (Cirrhitidae), they share only a single prey item at

326   greater than 1% of either of their diets (*Trapezia serenei*). There is also a minor dietary overlap

327   between the coral croucher (family Caracanthidae) and the flame hawkfish that were always

328   found co-occurring in *Pocillopora* and are known to rarely venture outside the branching

329   structure provided by their host coral (Hiatt & Strasburg, 1960; Stier & Leray, 2014). These

330   results demonstrate that levels of trophic partitioning have likely been underestimated. We

331   strongly encourage further empirical approaches to dietary studies prior to making assumptions

332   of trophic equivalency in food web reconstruction (Leibold & McPeek, 2006).

333   The extent to which secondary prey co-amplification could lead to errors in food web

334   analysis has not been quantified in marine systems (see Sheppard et al., 2005 for an example in

335   terrestrial system). In the present dataset, numerous prey species identified in fish gut contents

336   are either grazers or detritivores (e.g. isopods, amphipods, decapods, ophiuroids and gastropods)

337   and are therefore unlikely to consume each other. Some fish species detected in the gut contents

338   are higher-level predators (e.g. *Caranx melampygus*) that could consume benthic grazers and

339   detritivores as adults, but they were most likely fed upon at a younger developmental stage (egg,

340   larva or juvenile) given the size of predators. Demospongiae, Ascidiacea and Gymnolaemata

341   represented by few or a single sequence in the dataset were, however, possibly ingested

342   unintentionally as secondary prey or epiphytes on the carapace of spider crabs (e.g. *Menaethius*

343   *monoceros* and *Perinia tumida*). Parasites of prey (e.g. parasitic isopods of coral crabs of the

344   genus *Trapezia*, Appendix 1) and parasites of a predator's digestive track (e.g. Trematoda and

345   Cestoda) may also confound food web reconstructions and care should be taken to consider the

346   targeted roles these fish predators have on various parasites. The recovery of secondary prey may

347   artificially inflate dietary partitioning if those lower levels are also partitioned. However, we

348   expect the amount of DNA that these secondary prey items represent in the guts of our target

349   predators should be minor and highly digested in comparison to primary prey. A recent

350   metabarcoding analysis of benthic samples (Leray & Knowlton, 2015) showed evidence of a

351   correlation between amount of DNA and number of reads. Thus if secondary prey is quickly

352   degraded, those taxa should be represented by one or few reads only. The present dataset shows

353   minor dietary overlap both with and without rare OTUs (<1% of total OTUs, fig. 4), further

354   supporting our conclusions regarding the extent of trophic partitioning among all three fish

355   species.

356   Importantly, our analysis shows that in-depth sequencing would enable a more
357   comprehensive representation of trophic links in this multi-faceted ecosystem. Additional reads
358   would provide more OTUs matching reference barcodes (in GENBANK, BOLD or BIOCODE)
359   but also a higher proportion of unidentified OTUs represented by a single sequence ("singleton",
360   Fig. 3) that are likely to be either (1) small taxa underrepresented in DNA barcode libraries
361   (Leray et al., 2013a), or (2) the product of sequencing artifacts despite our very stringent quality
362   filtering based on amino-acid translation. Further barcoding initiatives aiming to catalogue small
363   life forms (e.g. meiofauna) will be crucial to advance our understanding of food webs.
364   Systematic removal of singletons may also be used as a conservative measure, although most of
365   them likely represent valid taxa (Huse et al., 2010). As coral reef ecosystems decline worldwide,
366   understanding the role of predator species in a dominant, yet largely understudied trophic
367   category, is essential. Our study highlights the tremendous potential of metabarcoding as an
368   approach to provide unprecedented taxonomic resolution in the diet of coral dwelling predatory
369   fish. We encourage that further work should be conducted to understand the ecological role of
370   reef dwelling fish and invertebrates.
371

372   ## Acknowledgements

377

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579

Table 1(on next page)

List of primers used in this study

Primer label	Sequence (5 '- 3')	Reference
mlCOIintF	GGWACWGGWTGAACWGTWTAYCCYCC	Leray et al. 2013a
jgHCO2198	TAIACYTCIGGRTGICCRAARAAYCA	Geller et al. 2013
Narmatus_Blocking	CAAAGAATCAAAACAGGTGTTGATAAAGA-C3	Leray et al. 2013b
Parcatus_Blocking	CAAAGAATCAGAACAGATGTTGGTAAAGA-C3	Leray et al. 2013b
Cmaculatus_Blocking	CAAAGAATCAGAATAGGTGTTGGTACAGA-C3	Herein

2

Figure 1(on next page)

Map of the study location

French Polynesia

Moorea

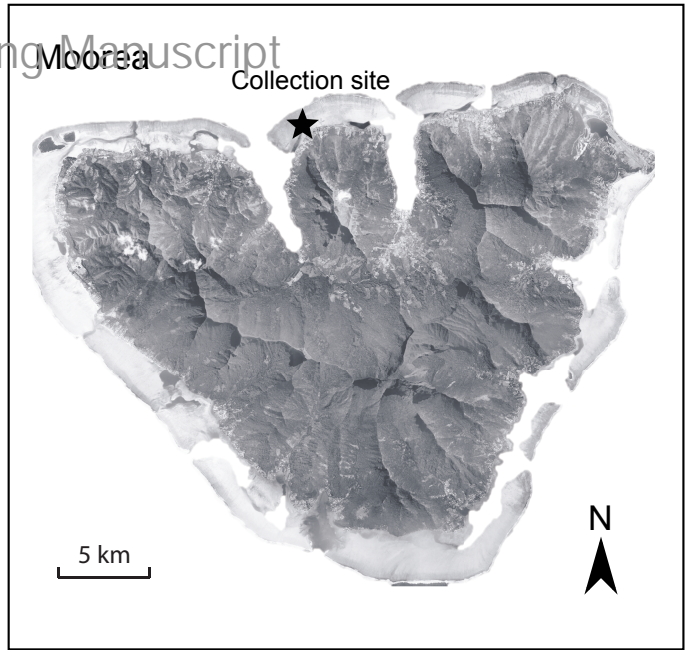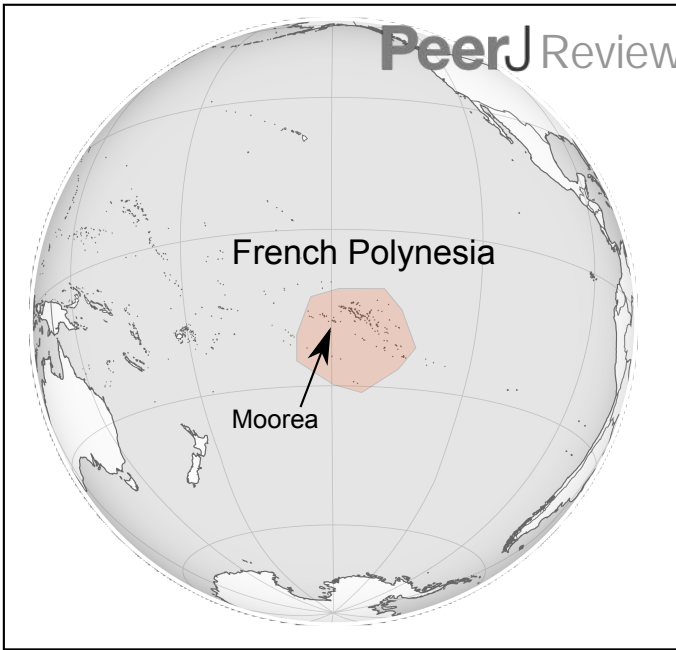Moorea

Collection site

5 km

N

Figure 2(on next page)

Rarefaction curves to evaluate the completeness of the sequencing effort at describing the diversity of dietary items in the gut contents of three coral reef fish species
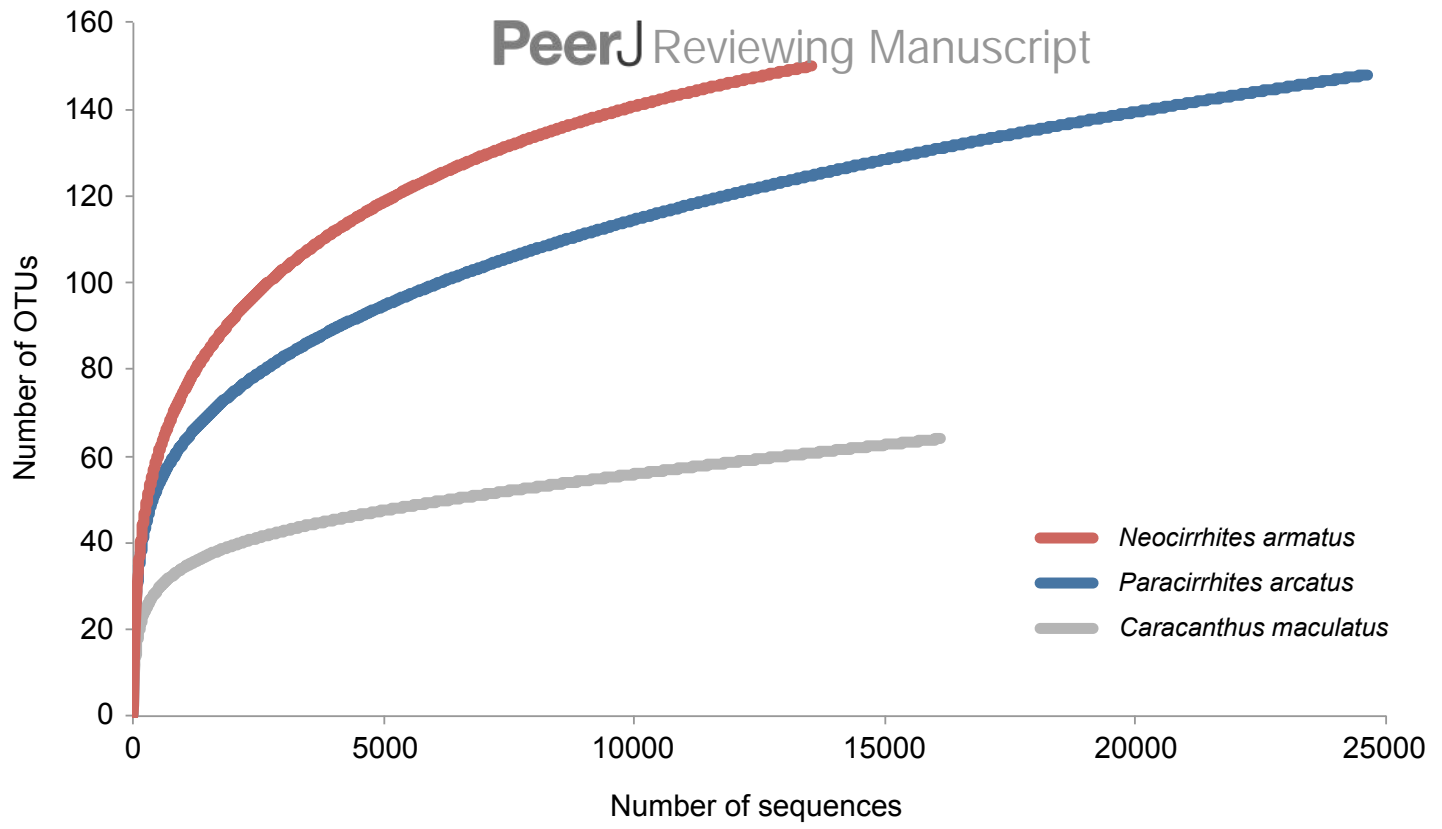
Figure 3(on next page)

Proportion of identified OTUs in relation to the number of sequences they represent

Whenever OTU sequence similarity to a reference barcode was < 98%, we used the Phylogenetic Bayesian assignment tool implemented in SAP to assign OTUs to a higher taxonomic group
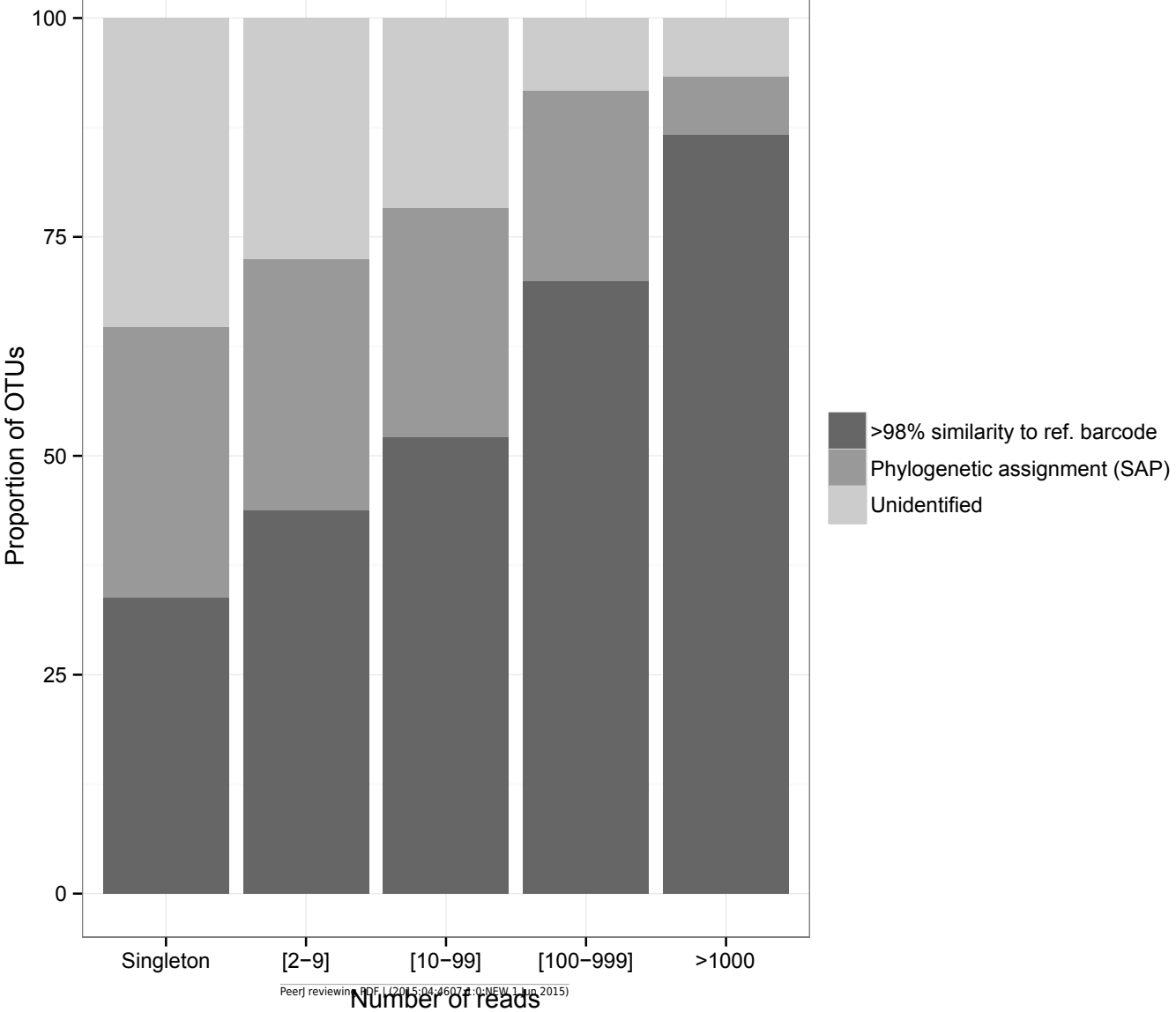
Figure 4(on next page)

Dietary partitioning among the three predatory fish species

Left neighbor-joining phylogeny using LogDet distance model based on a constraint topology of major clades represents relationship among the 66 prey OTUs that comprise >1% of any one species diet. Thickness of linkages to right represents relative proportion of predatory diets. Six shared species are highlighted with circles. Fish images courtesy of D. Liittschwager. The 66 OTUs are highlighted in Appendix I

demosponge
cnidarians
cestode
polychaetes
sipunculid
gastropods
copepods
isopods
amphipods
carids
anomurans
brachyurans
ophiuroid
fishes

Caracanthus maculatus

Neocirrhites armatus

Paracirrhites arcatus

● shared species in diets

thickness corresponds to % of total diet
(only prey >1% of diet represented)