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Genetic diversity, paraphyly and incomplete lineage sorting of mtDNA, ITS2 and microsatellite flanking region in closely related *Heliopora* species (Octocorallia) ☆

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ABSTRACT

Examining genetic diversity and lineage sorting of different genes in closely related species provides useful information for phylogenetic analyses and ultimately for understanding the origins of biodiversity. In this study, we examined inter- and intraspecific genetic variation in internal transcribed spacer 2 (ITS2), partial mitochondrial gene (*mtMutS*), and nuclear microsatellite flanking region in two closely related octocoral species (*Heliopora coerulea*, HC-A and HC-B). These species were recently identified in a population genetic study using microsatellite markers. The two species have different reproductive timing, which ecologically promotes lineage sorting. In this study, we examined whether species boundaries could be detected by the commonly used nuclear ITS2 and *mtMutS*, as well as by possibly neutral microsatellite flanking sequences. Haplotype network analysis of microsatellite flanking region revealed that a possible ancestral haplotype was still shared between the two species, indicating on-going lineage sorting. Haplotype network analysis of ITS2 and microsatellite flanking region revealed shared haplotypes between the two lineages. The two species shared fewer ITS2 sequences than microsatellite flanking region sequences. The almost fixed point mutation at the tip of helix 3 of ITS2 was not associated with the secondary structure or compensatory base changes (CBCs). The phylogenetic tree of ITS2 showed paraphyly and that of the microsatellite flanking region indicated that lineage sorting for the two species may be incomplete. Much higher intra- and inter-individual variation of ITS2 was observed in HC-B than that in HC-A, highlighting the importance of examining ITS2 from multiple individuals to estimate genetic diversity. The mitochondrial *mtMutS* gene sequences from 39 individuals, including both species collected from Japan and Taiwan, showed no variation because of slow rates of mitochondrial nucleotide substitution. This study suggests caution is warranted when reciprocal monophyly in a phylogenetic tree is used as the criterion for delimiting closely related octocoral species based on ITS2 or *mtMtuS* sequences. Detection of boundaries between closely related species requires multi-locus analysis, such as genetic admixture analysis using multiple individuals.

1. Introduction

Examining genetic diversity and lineage sorting of different genes in closely related species provides significant information for species delimitation and phylogenetic analysis. Ultimately, such examination provides insights for understanding the origin

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of biodiversity. Species delimitation is important for assessing biodiversity and conserving natural resources. Misunderstanding of species boundaries can result in over or under estimation of geographic distribution, population abundance, and population risks (Kim et al., 2004; Prada et al., 2008). As genetic methods became more economical and accessible, phylogenetic analysis made it possible to reveal hidden species diversity that was undetectable using morphological analysis. Genetic identification techniques, such as DNA barcoding (Hebert et al., 2003) and reciprocal monophyly in phylogenetic trees, are increasingly popular tools for species determination. There are a number of advantages to using genetic methods of species delimitation when morphological identification is difficult, including simplicity and objectivity. The accuracy of genetic identification depends on the availability of fixed species-specific variations. Mitochondrial DNA, especially the partial cytochrome *c* oxidase subunit 1 (COI) mitochondrial region, is often used for DNA barcoding of higher animal species (Hebert et al., 2003; Waugh, 2007). Other genetic markers such as plant-specific matK, rbcLK, and psbA can be used for DNA barcoding of plant species (Hollingsworth et al., 2011; Yang et al., 2012). Second internal transcribed spacer (ITS2) and other mitochondrial genes (e.g., *mtMutS*) are used for basal animal species DNA barcoding (Dolan et al., 2013; McFadden et al., 2006; Mi-Hyun et al., 2007; Wörheide, 2006). However, there are challenges to species identification using genetic markers in closely related species (Moritz and Cicero, 2004). These challenges include incomplete lineage sorting (Funk and Omland, 2003), introgression (Chase et al., 2005), the presence of pseudogenes (Lorenz et al., 2005), intra-individual diversity in some multiple-copy families of genes (Dover, 1982), and low substitution rates of mitochondrial genes in some taxa, such as plants (Shearer et al., 2002; Waugh, 2007), sponges (Mi-Hyun et al., 2007; Wörheide, 2005), and anthozoans (Huang et al., 2008; Shearer et al., 2002).

As gene flow between incipient species becomes restricted, genetic drift causes loss of common ancestral alleles in neutral loci (Kingman, 1982). In multiple-copy gene families, concerted patterns of fixation (concerted evolution) further allow species discontinuities to establish in a manner not predicted by natural selection or genetic drift (Dover, 1982). However, if the time since speciation is too short, the common ancestral alleles remain in both species in the some loci, and incomplete lineage sorting will be observed. Similarly, hybridization between closely related species also hinders reciprocal monophyly. Because sorting of genes of different species always initiates from within-species genetic diversity, examination of both intra- and interspecific genetic variation in closely related species provides important information about molecular evolution that will be useful for species identification and for testing future phylogenetic analyses.

The anthozoan subclass Octocorallia is an invertebrate taxon for which morphological species delimitation is very challenging because of a low number of homologous characteristics, high plasticity, and intra- and interspecific variation. Thus, there is a practical need for genetic differentiation within this taxon (Bayer, 1961; Fabricius et al., 2001). Among several genetic markers, those used most often for Octocorallia are the *mtMutS* gene (Dolan et al., 2013; McFadden et al., 2010), which is the apparent homolog of a DNA mismatch repair gene and considered to evolve faster than other mitochondrial genes (France and Hoover, 2002), and the second internal ITS2, a multiple-copy gene family found within nuclear ribosomal RNA (rRNA) tandem arrays. Although the mitochondrial protein-coding gene *mtMutS* is useful for resolving many octocoral species (Dolan et al., 2013; McFadden et al., 2010), scleractinians generally have very slow rates of mitochondrial DNA evolution (Hellberg, 2006) and attempts to delimit species boundaries have sometimes had limited success (Dolan et al., 2013; France and Hoover, 2002; McFadden et al., 2011; McFadden et al., 2010). ITS2 has also been used to determine species boundaries and unique secondary structures of ITS2 with substantial sequence variations have been reported for Octocorallia species. ITS2 sequence alignment based on conserved secondary structure has been used for phylogenetic analysis of octocoral species (Sánchez and Dorado, 2008; Aguilar and Sanchez, 2009; Dorado and Sánchez, 2009). However, ITS2 may be invariant between some species and intraindividual variation sometimes obscures phylogenetic relationships (McFadden et al., 2010). Some octocorals have relatively low intraindividual ITS2 diversity (Aguilar and Sánchez, 2007; Grajales et al., 2007), which enables the use of direct sequencing methods. However, other species (e.g., temperate gorgonians (Calderon et al., 2006), Caribbean Sea octocorals (Torres-Suarez, 2014)), have relatively high levels of ITS2 variability, which hinders direct sequencing. Torres-Suarez (2014) used denaturing gradient gel electrophoresis (DGGE) to detect intraindividual ITS2 variation and found that the presence of compensatory base changes (CBCs) and hemi-CBCs is useful as a complementary tool for detecting species and population boundaries. Although imperfect, *mtMutS* and ITS2 are the most variable regions available for analysis and are commonly used as important genetic markers for phylogenetic analysis (Aguilar and Sanchez, 2007; Herrera et al., 2010; McFadden et al., 2010). Therefore, additional insights into intra- and interspecific variation in ITS2 and *mtMutS* among closely related octocoral species would be useful for understanding the robustness of these markers for delimitation of octocoral species. In addition to sequencing ITS2 and mtDNA in octocoral species, we sequenced a possibly neutral microsatellite flanking region for comparison.*Heliopora coerulea* is a shallow-water species found in warm tropical Indo-Pacific Oceans. Along with other corals, *H. coerulea* was recently listed as threatened by the International Union for Conservation of Nature and Natural Resources because of habitat degradation. Although *H. coerulea* was considered to be the sole surviving species of the *Helioporidae* (the only Helioporacea family) (Colgan, 1984), our recent population genetic analysis using microsatellite markers indicated that two closely related species, HC-A and HC-B, coexist along Kuroshio Current (Yasuda et al., 2014). Significant isolation resulting from geographic distance has been observed in these closely related species, and each population consists of either one or the other species (Yasuda et al., 2014). The two species have different reproductive timing, as indicated by sympatric populations of HC-A and HC-B. The brooding period of HC-A population seems to be different from sympatric HC-B population in southwest Japan (Saito et al., 2015) and the brooding period of HC-A is almost a month earlier than that of HC-B in the Philippines (Villanueva in review). Such ecological reproductive isolation mechanisms are considered to promote lineage sorting of the genes of the two *Heliopora* species.

Here, we examined intra- and interspecific sequence variation of ITS2, a nuclear microsatellite flanking region and *mtMutS* in HC-A and HC-B from geographically separated populations (approximately 1800 km apart, from the Philippines, Taiwan, and Japan) to determine whether the ITS2, microsatellite flanking region and *mtMutS* sequences can be used to detect species boundaries.

The goal of this study was to reveal genetic diversity of ITS2, a microsatellite flanking region and *mtMutS* in order to examine whether species boundaries of closely related octocorals are detectable by examining lineage sorting of the genes that are commonly used in phylogenetic analysis. Because the species boundary was originally detected through genetic admixture analysis using microsatellite data (Yasuda et al., 2014), we also sequenced a potentially neutral microsatellite flanking region for comparison with the commonly used markers described above.

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