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Microsatellite allele sizes alone are insufficient to delineate species boundaries in *Symbiodinium*

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Running Title: Microsatellites and *Symbiodinium* species boundaries

Abstract:

Symbiodinium is a diverse group of unicellular dinoflagellates that are important nutritional symbionts of reef-building corals. *Symbiodinium* putative species (“types”) are commonly identified with genetic markers, mostly nuclear and chloroplast encoded ribosomal DNA regions. Population genetic analyses using microsatellite loci have provided insights into *Symbiodinium* biogeography, connectivity, and phenotypic plasticity, but are complicated by: (1) a lack of consensus criteria used to delineate inter- versus intra-genomic variation within species; and (2) the high density of *Symbiodinium* in host tissues, which results in single samples comprising thousands of individuals. To address this problem, Wham & LaJeunesse (2016) present a method for identifying cryptic *Symbiodinium* species from microsatellite data based on correlations between allele size distributions and non-geographic genetic structure. Multilocus genotypes that potentially do not recombine in sympatry are interpreted as secondary “species” to be discarded from downstream population genetic analyses. However, *Symbiodinium* species delineations should ideally incorporate multiple physiological, ecological and molecular criteria. This is because recombination tests may be a poor indicator of species boundaries in *Symbiodinium* because of its predominantly asexual mode of reproduction. Furthermore, discontinuous microsatellite allele sizes in sympatry may be explained by secondary contact between previously isolated populations and by mutations that occur in a non-stepwise manner. Limitations of using microsatellites alone to delineate species are highlighted in earlier studies that demonstrate occasional bimodal distributions of allele sizes within *Symbiodinium* species and considerable allele size sharing among *Symbiodinium* species. We outline these issues and discuss the validity of reinterpretations of our previously published microsatellite data from *Symbiodinium* populations on the Great Barrier Reef (Howells *et al.* 2013).

Main Text:

Despite decades of research into the ecology, physiology and genetics of *Symbiodinium*, there is no consensus on what constitutes a species within this dinoflagellate genus. Genetic diversity is currently partitioned into a hierarchy of clades, types, putative species, populations and clones that are described with alphanumeric systems (reviewed in Coffroth & Santos 2005). Variation at the nuclear ribosomal internal spacer regions (ITS1 and ITS2 rDNA) has often been used to approximate species-level diversity in *Symbiodinium* (LaJeunesse 2001; Sampayo *et al.* 2009; van Oppen *et al.* 2001) and occasionally, closely related groups have been distinguished using additional markers such as chloroplast genes and microsatellites (Hume *et al.* 2015; LaJeunesse *et al.* 2014; Santos *et al.* 2004; Thornhill *et al.* 2014). However, delineations between *Symbiodinium* groups at finer scales (i.e., type to clone) are often blurred and inconsistent due to the application of different makers and techniques (e.g. direct sequencing, cloning, gels), different naming systems, and sometimes unknown origins of variation that could be inter- versus intra-genomic (Correa & Baker 2009; Sampayo *et al.* 2009; Stat *et al.* 2012; Thornhill *et al.* 2007), as samples are typically made up of pools of individual cells. Despite these challenges, binomial species naming of *Symbiodinium* has increased in the past few years (Hume *et al.* 2015; Jeong *et al.* 2014; LaJeunesse *et al.* 2015; LaJeunesse *et al.* 2012; LaJeunesse *et al.* 2014; Lee *et al.* 2015; Parkinson *et al.* 2015). In most instances, new *Symbiodinium* species names have been assigned using a combination of phylogenetic and ecological criteria (De Queiroz 2007), where fixed genetic variation corresponds with either specific host taxa, habitats and/or physiological tolerances (Hume *et al.* 2015; LaJeunesse *et al.* 2012; LaJeunesse *et al.* 2014). In this issue, Wham and LaJeunesse (2016) advocate the use of microsatellite data to apply a biological species concept to delineate closely related *Symbiodinium* (De Queiroz 2005; Mayr 1942). Specifically, they interpret that fine scale genetic structure in sympatry arises from reproductive isolation, “where individuals reproduce within their species to the exclusion of the other”. For *Symbiodinium*, a purely biological species concept is at odds with what is known about their reproductive systems and symbiotic lifestyle. *Symbiodinium* are believed to be predominantly asexual within the host environment and many studies have demonstrated that the millions of

Symbiodinium cells within a host are comprised of one to several individual genotypes or clones (Andras *et al.* 2011; Howells *et al.* 2009; Howells *et al.* 2013; Kirk *et al.* 2009; Magalon *et al.* 2006; Pettay *et al.* 2011; Santos *et al.* 2003; Thornhill *et al.* 2009). Meiosis genes indicate that cryptic sexual reproduction occurs in the *Symbiodinium* life cycle (Chi *et al.* 2014). However, as sexual stages have not been directly observed in symbiotic or free-living *Symbiodinium*, the frequency of sex remains unknown. Thus, population genetic signals of incompatible breeding systems between species (i.e. linkage disequilibrium or non-random association of alleles) could alternatively be explained by conspecific genotypes that frequently reproduce asexually but only very rarely sexually (i.e., limited recombination and genetic reshuffling) (Tibayrenc *et al.* 1991). A further source of genetic structure in sympatry is mixing of conspecific *Symbiodinium* populations that previously diverged in isolation from one another. Most coral hosts acquire *Symbiodinium* from the environment during a brief period as larvae or juveniles and then maintain this cohort in symbiosis for their lifespan of up to decades to centuries (e.g. Abrego *et al.* 2009; Coffroth *et al.* 2010). An outcome of this symbiotic lifestyle is that the cohort of *Symbiodinium* within each coral colony is isolated from that of adjacent hosts, with a predominantly one-way input of *Symbiodinium* back into the free-living environment (i.e. symbiont cells released from hosts; Jones & Yellowlees 1997). This is consistent with observations of significant genetic differentiation of *Symbiodinium* populations between sympatric coral colonies of different ages (B1 microsatellite multilocus genotypes; Andras *et al.* 2011). Moreover, *Symbiodinium* populations may diverge in allopatry (e.g. following bottlenecks and local extinctions) and then undergo secondary contact (e.g. during range expansions, introductions and/or hybridisation). Such processes are expected to be common on corals reefs which are exposed to relatively frequent environmental disturbances and population size variations (Gardner 1997). For instance, *Symbiodinium* populations experience population size reductions during bleaching events (Weis 2008; discussed in Howells *et al.* 2013), are prone to secondary contact across large distances via vectored introductions (Pettay *et al.* 2015; Stat & Gates 2008; Thornhill *et al.* 2013), and exhibit evidence of hybridisation between divergent lineages (Wilkinson *et al.* 2015).

Wham and LaJeunesse (2016) illustrate their approach to delineating cryptic *Symbiodinium* species by presenting analyses of simulated and real microsatellite datasets, each comprising mixed populations belonging to distinct species. They make numerous assumptions to generate alleles in simulated *Symbiodinium* populations, including the frequency of asexual and sexual recombination, associated linkage equilibrium, and a stepwise mutation rate. Given the many unknowns for *Symbiodinium* populations, it is impossible to fully evaluate whether these assumptions are representative, and little to no justification of the parameters selected was provided by the authors. The possibility for mutations to be multi-step was not considered by the authors, despite the fact that they can account for a significant proportion of mutational events in microsatellites (i.e. 5-75% of mutations, reviewed in Ellegren 2004). In their real microsatellite dataset, alleles were analysed in two divergent types of *Symbiodinium* from a mixed set of cultures and host species. Given that these *Symbiodinium* types are readily distinguished with several markers, including ITS2 (C1 vs. C3, LaJeunesse *et al.* 2004) and ITS1 (C1 vs. C2, van Oppen *et al.* 2001), it is not at all surprising that they can also be delineated with microsatellite loci. Unfortunately, Wham and LaJeunesse (2016) have not demonstrated the success of their method on more closely related “species” that may occur within a single ITS type, as this would be more representative of the microsatellite datasets they reevaluate (Howells *et al.* 2013; Wirshing *et al.* 2013). Notably, Wham and LaJeunesse (2016) highlight that their approach is less effective at resolving discrete multilocus genotypes when the degree of divergence between putative species is low, as might be expected within a single ITS type. For instance, closely related species of clade D *Symbiodinium* share a considerable number of microsatellite alleles of identical size at multiple loci (LaJeunesse *et al.* 2014), which may result in an inability to delineate species without additional criteria. A further issue is the treatment of intragenomic diversity. Wham and LaJeunesse (2016) treat some datasets as haploid based on microsatellite data that show that *Symbiodinium* from clade B are haploid (Santos & Coffroth 2003), however others are handled as “diploid” based on their assumed duplication of loci within *Symbiodinium* clade C genomes (Wham *et al.* 2014). The duplication of some loci does not necessarily equate to whole genome duplication (Lin *et al.* 2015) and it is misleading to use these terms interchangeably. A single-copy microsatellite in diploid cells ($2n$) would result in two alleles expected to exhibit independent assortment in haploid stages (n). Yet, a duplicated microsatellite

in haploid cells would also produce two alleles but these would be more prone to non-independent assortment.

In Howells *et al.* (2013), we examined the population genetic structure of *Symbiodinium* in the central Great Barrier Reef using eight microsatellite loci (Bay *et al.* 2009; Howells *et al.* 2009). The ~400 samples we genotyped were from a single host species, *Acropora millepora*, and were prescreened for *Symbiodinium* ITS1 rDNA variation to ensure that all samples included in the microsatellite analyses only contained the target *Symbiodinium* type, C2 (i.e., C3 *sensu* LaJeunesse *et al.* 2004). We treated this allelic data set as conspecific based on the common assumption that *Symbiodinium* types represent species. This approach is consistent with other population genetic studies of *Symbiodinium* to date (e.g. Andras *et al.* 2011; Howells *et al.* 2009; Kirk *et al.* 2009; Pettay *et al.* 2011; Pettay *et al.* 2015; Santos *et al.* 2003; Thornhill *et al.* 2009; Thornhill *et al.* 2013; Wirshing *et al.* 2013). Within our samples, we recovered multiple alleles at each locus, which prevented the assignment of multilocus *Symbiodinium* C2 genotypes and has implications for the types of analyses possible. These issues appear to be a repeated feature of *Symbiodinium* clade C microsatellite data (Bay *et al.* 2009; Howells *et al.* 2009; Howells *et al.* 2013; Magalon *et al.* 2006) and are discussed in our original manuscript. While we commend Wham and LaJeunesse (2016) for their innovative approach to attempt to improve the way in which *Symbiodinium* population data are generated and analysed, we have concerns about the applicability of their approach to all datasets. With regard to our data specifically, Wham and LaJeunesse (2016) violate their own criteria for delineating *Symbiodinium* species, as multilocus genotypes cannot be established in our dataset. Despite this violation of their own criterion, they apply ad hoc reasoning of allele numbers and allele size distributions to infer that ~27% of our data is from a second species of *Symbiodinium* C2. Based on recovered allele numbers, Wham and LaJeunesse (2016) interpret that most of our loci amplify two *Symbiodinium* C2 species, while two loci are specific to one species only. This interpretation is contradicted by the amplification of these “specific” loci across more divergent clade C types (Bay *et al.* 2009; Howells *et al.* 2009). An alternative interpretation is that microsatellites vary in their level of polymorphism (e.g. Andras *et al.* 2009; Bay *et al.* 2009; Wham *et al.* 2014; Wham *et al.* 2011). Additionally, discontinuous allele sizes at some loci, while potentially diagnostic of separate

species (Santos *et al.* 2004; Wham & LaJeunesse 2016), could also occur under scenarios of secondary contact, hybridisation and locus duplication (outlined above) or from genotyping artefacts (see Howells *et al.* 2013; Pinzon *et al.* 2011). Indeed, bimodal microsatellite allele size distributions have been documented in a range of species, including humans (Valdes *et al.* 1993), marine fish (*Gadus morhua*, Brooker *et al.* 1994; *Makaria nigricans*, Buonaccorsi *et al.* 2001), fruit flies (wild *Drosophila melanogaster*, England *et al.* 1996) and even other *Symbiodinium* types (LaJeunesse *et al.* 2014; Santos & Coffroth 2003). For example, Santos and Coffroth (2003) observed a bimodal microsatellite allele size distribution in the host-specific *Symbiodinium* B1 of *Antillologorgia elisabethae* in the Bahamas (Santos *et al.* 2004).

Wham and LaJeunesse (2016) conclude that spatiotemporal patterns of genetic differentiation in Howells *et al.* (2013) reflect niche partitioning of two species of *Symbiodinium* C2 across the ~28 km² study area rather than the sum of historical gene flow, disturbance and selection on population genotypes. Wham and LaJeunesse (2016) state that we interpret spatial differentiation as arising from limited active and passive dispersal of *Symbiodinium*. We would like to clarify that while dispersal potential is important, we have discussed at length the roles of environmental disturbance type and frequency in structuring *Symbiodinium* associations (Howells *et al.* 2013). Notably, the bleaching events, cyclones and floods that differentially impacted sites in our study are likely to have had both neutral (e.g. bottlenecks) and non-neutral (e.g. selection) effects and thus would have impacted the relative abundance of *Symbiodinium* genotypes. For the host coral in our study, *A. millepora*, association with a range of *Symbiodinium* types is well documented across the Great Barrier Reef (e.g. Cooper *et al.* 2011). However, further research is required to determine whether this plasticity extends to physiologically distinct *Symbiodinium* C2 variants and whether these constitute distinct species. Ideally, investigations of species borders within *Symbiodinium* should target multiple lines of evidence, including ecological and physiological attributes, as well as several molecular markers to avoid erroneous species assignments (LaJeunesse *et al.* 2012; Stat *et al.* 2012). Based on current knowledge of *Symbiodinium* molecular markers, suggested starting points to assess the occurrence of cryptic species include the cp psbA non-coding region and microsatellite flanking regions (Hume *et al.* 2015; Santos *et al.* 2004; Thornhill *et al.* 2014).

The decision to exclude samples with mixed genotypes from population genetic datasets is best evaluated on a case-by-case scenario considering the objectives of the study. While a key benefit of the exclusion of samples is conformity to traditional and routine population genetic analyses (Wham & LaJeunesse 2016), assumptions of such analyses may be violated by haploid and asexual traits of *Symbiodinium*. Notably, excluding genotypes and samples from datasets may come at a cost of underestimating genetic diversity in coral host species and geographic locations. There are inherent limitations of genotyping a “population within a population” (i.e., thousands to millions of cells within a coral host colony), especially when more than one *Symbiodinium* genotype is in symbiosis and marker copy number is unknown. Working towards overcoming these obstacles will likely require alternative or supplementary methods to microsatellite profiling, in addition to the statistical methods proposed by Wham and LaJeunesse (2016). Suggested novel approaches include single cell genotyping (Wilkinson *et al.* 2015) and/or quantitative genotyping of fast evolving regions in mixed samples (e.g. qPCR, deep-sequencing) (Arif *et al.* 2014; Mieog *et al.* 2007; Quigley *et al.* 2014; Silverstein *et al.* 2012) - both of which are already being successfully used to investigate *Symbiodinium* genetic diversity at higher taxonomic levels. The recent (and upcoming) availability of genome sequences for *Symbiodinium* (Lin *et al.* 2015; Shoguchi *et al.* 2013; Voolstra *et al.* 2015) is anticipated to facilitate the development of improved sets of markers for addressing population genetics and speciation hypotheses within this diverse genus.

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