Multi-gene incongruence consistent with hybridisation in Cladocopium (Symbiodiniaceae), an ecologically important genus of coral reef symbionts

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Coral reefs rely on their intracellular dinoflagellate symbionts (family Symbiodiniaceae) for nutritional provision in nutrient-poor waters, yet this association is threatened by thermally stressful conditions. Despite this, the evolutionary potential of these symbionts remains poorly characterised. In this study, we tested the potential for divergent Symbiodiniaceae types to sexually reproduce (i.e. hybridise) within Cladocopium, the most ecologically prevalent genus in this family. With sequence data from three organelles (cob gene, mitochondria; psbA(ncr) region, chloroplast; and ITS2 region, nucleus), we utilised the Incongruence Length Difference test, Approximately Unbiased test, tree hybridisation analyses and visual inspection of raw data in stepwise fashion to highlight incongruences between organelles, and thus provide evidence of reticulate evolution. Using this approach, we identified three putative hybrid Cladocopium samples among the 158 analysed, at two of the seven sites sampled. These samples were identified as the common Cladocopium types C40 or C1 with respect to the mitochondria and chloroplasts, but the rarer types C3z, C3u and C1# with respect to their nuclear identity. These five Cladocopium types have previously been confirmed as evolutionarily distinct and were also recovered in non-incongruent samples multiple times, which is strongly suggestive that they sexually reproduced to produce the incongruent samples. A concomitant inspection of Next Generation Sequencing data for these samples suggests that other plausible explanations, such as incomplete lineage sorting or the presence of co-dominance, are much less likely. The approach taken in this study allows incongruences between gene regions to be identified with confidence, and brings new light to the evolutionary potential within Symbiodiniaceae.
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Abstract

Coral reefs rely on their intracellular dinoflagellate symbionts (family Symbiodiniaceae) for nutritional provision in nutrient-poor waters, yet this association is threatened by thermally stressful conditions. Despite this, the evolutionary potential of these symbionts remains poorly characterised. In this study, we tested the potential for divergent Symbiodiniaceae types to sexually reproduce (i.e. hybridise) within Cladocopium, the most ecologically prevalent genus in this family. With sequence data from three organelles (cob gene, mitochondria; psbA\textsuperscript{ncr} region, chloroplast; and ITS2 region, nucleus), we utilised the Incongruence Length Difference test, Approximately Unbiased test, tree hybridisation analyses and visual inspection of raw data in stepwise fashion to highlight incongruences between organelles, and thus provide evidence of reticulate evolution. Using this approach, we identified three putative hybrid Cladocopium samples among the 158 analysed, at two of the seven sites sampled. These samples were identified as the common Cladocopium types C40 or C1 with respect to the mitochondria and chloroplasts, but the rarer types C3z, C3u and C1# with respect to their nuclear identity. These five Cladocopium types have previously been confirmed as evolutionarily distinct and were also recovered in non-incongruent samples multiple times, which is strongly suggestive that they sexually reproduced to produce the incongruent samples. A concomitant inspection of Next Generation Sequencing data for these samples suggests that other plausible explanations, such as incomplete lineage sorting or the presence of co-dominance, are much less likely. The approach taken in this study allows incongruences between gene regions to be identified with confidence, and brings new light to the evolutionary potential within Symbiodiniaceae.
Introduction

Coral reefs are a highly diverse and important ecosystem, yet are significantly threatened by anthropogenically-driven climate change (Hughes et al. 2017). In order for coral reefs to survive the stresses of a changing climate, genetic adaptation over rapid evolutionary timescales has to occur. Adaptation in the coral itself may go some way to provisioning for the environmentally challenging conditions predicted to come (Rodriguez et al. 2009). However, given that the response of corals to environmental conditions is inextricably linked to the diversity and performance of their intracellular symbionts (dinoflagellates of the family Symbiodiniaceae, LaJeunesse et al. 2018) increasing attention is being focused on the evolutionary potential within this family.

Coral symbionts have been thought to be exclusively asexual in hospite (Trench 1997; LaJeunesse 2005), thanks to their isolated position sequestered inside host cells, and the hypothesis that endosymbiotic sex would encourage exploitation of the host (Law and Lewis 1983). However, previous work in other taxa has shown that intracellular symbionts can sexually reproduce (Chesnick and Cox 1987). In general, it is thought that many such organisms may have cryptic sexual cycles that have previously been unappreciated, in addition to the production of clonal populations via asexual reproduction (Heitman 2010). Now, there is significant evidence that Symbiodiniaceae also displays a mixed reproductive strategy, with periods of asexuality interspersed with occasional to frequent sex (Thornhill et al. 2017). While it has never been explicitly observed, there are distinct and observable traces of sex in their genomes (e.g. Baillie et al. 2000; LaJeunesse 2001; Santos and Coffroth 2003; Santos et al. 2004; Pettay et al. 2011; Baums...
et al. 2014; Chi et al. 2014; LaJeunesse et al. 2014; Thornhill et al. 2014; Levin et al. 2016). However, these studies have been largely focused on a micro-scale, population level (i.e., intraspecific sex). By contrast, sex between diverse symbiont lineages (‘hybridisation’) has received little attention in the literature (but see Wilkinson et al. 2015). Given the highly thermally stressful conditions predicted by the end of the century (Kirtman et al. 2013), the mechanism of hybridisation could potentially have significant and vital adaptive value. By mixing diverse pools of genetic material, hybridisation can allow for rapid adaptation, facilitating macro-evolutionary jumps (Willis et al. 2006; Dittrich-Reed and Fitzpatrick 2013). Introgressive hybridisation, where the F1 hybrids subsequently mate with one or both parent populations, can transfer a large quantity of genetic material between the two parent lineages in the space of a few generations. In addition, hybridisation can also produce offspring with elevated fitness (‘hybrid vigour’), which can even outcompete the parent species (Ellstrand and Hoffman 1990; Rhymer and Simberloff 1996). Importantly, instances of hybridisation have also been shown to increase in taxing conditions (Rhymer and Simberloff 1996; Moran and Alexander 2014). Therefore, the possibility of hybridisation in coral symbionts raises the potential for adaptation at the required pace and scale for survival.

Research on taxa with similar life-histories suggests that hybridisation is plausible. Hybridisation has previously been reported in a range of dinoflagellate genera, including Dinophysis, Protoperidinium, Preperidinium and Diplopsalis (Edvardsen et al. 2003; Gribble and Anderson 2007; Hart et al. 2007). There is also evidence from plant-fungi relationships that endosymbionts can successfully hybridise. In particular, the endophytes Epichloë spp. are pathogenic or mutualistic fungi that inhabit a wide range of grasses. Hybridisation appears to be a major
mechanism for diversification in this genus, and has been reported to occur inside the grasses
*Lolium perenne* (Schardl et al. 1994), *Festuca arundinacea* (Tsai et al. 1994), *Bromus laevipes*
(Charlton et al. 2014) and *Poa alsodes* (Shymanovich et al. 2017). In several instances, multiple
cases of hybridisation have been recorded, and evidence put forward that those hybrids are fitter
than non-hybrids (Schardl et al. 1994; Moon et al. 2004). While Symbiodiniaceae *in hospite* are
generally sequestered inside host cells (Davy et al. 2012), the extensive presence of background
symbiont populations inside hosts (Santos et al. 2001; Kemp et al. 2015), the observation that
corals themselves hybridise (Willis et al. 2006; Combosch and Vollmer 2015), and the existence
of a free-living state (Coffroth et al. 2006; Nitschke et al. 2016) mean that it is highly possible that
at some point diverse symbiont communities may interact, with the possibility for sexual
reproduction.

The evolutionary potential of hybridisation has not been targeted within Symbiodiniaceae.
However, several indirect observations are suggestive of its occurrence, all within *Cladocopium*,
the most prevalent genus. LaJeunesse et al. (2003) reported an ITS2 sequence variant they called
C1c and treated as an intragenomic variant, as it was only observed in DGGE profiles associated
with type C1. However, it was then discovered to be an independent type and called C45
(LaJeunesse 2005). Therefore, the additive DGGE pattern shown in LaJeunesse et al. (2003) could
have in fact resulted from the hybridisation of C1 and C45. LaJeunesse (2005) also defined type
C3m using the ITS2 region, which has co-dominant characteristics of both C1 and C3, a pattern
attributed to either sexual recombination or homoplasy. A similar scenario was also recorded in
symbiont type C3h, an apparent intermediary between C3 and C21 (LaJeunesse et al. 2004). This
time, the pattern was hypothesised to be due to incomplete lineage sorting or sexual recombination
between the two different types. Indeed, given the unambiguous existence of ‘pure’ C3 and C21 in the samples, sexual recombination is a credible explanation. Finally, Wilkinson et al. (2015) reported two symbiont types but three distinct symbiont populations inside a single *Pocillopora* colony: C100 symbionts, C109 symbionts, and symbionts having co-dominant C100 and C109 repeats in the same cell. Again, the extensive presence of the two ‘pure’ populations means incomplete lineage sorting is a less parsimonious explanation than hybridisation. However, it cannot be completely eliminated as a possibility. In addition, this study took place at Lord Howe Island, the world’s southern-most coral reef, and therefore may not be widely applicable across less marginal, low-latitude sites.

Hence, there is a body of indirect evidence for sexual recombination between diverse symbiont types (hybridisation *sensu lato*), and this warrants further study. The current study aimed to gather further defensible evidence as to whether hybridisation occurs in coral symbionts. Because it is very difficult to observe hybridisation directly, it is generally inferred through genetic signals. One of the most common of these is incongruence between gene regions. Because nuclear genes are typically inherited biparentally, while organelle genes are inherited uniparentally, sexual reproduction between different species will result in organelle genes resembling one parent only, while the nuclear genome will have clear traces of both parents (Rieseberg et al. 1996). In extreme cases, repeated backcrosses with a parent type can result in organelle capture, where novel, discordant nuclear-organellar combinations are observed (Folk et al. 2017). Following a hybridisation event, selection can also act to produce incongruence between gene regions: there may be elevated (or reduced) fitness of certain nuclear-cytoplasmic combinations, or selection pressure may be different for nuclear and cytoplasmic genomes (*e.g.* a greater selection pressure
acting on nuclear genes) (Rieseberg et al. 1996). Therefore, identifying incongruence between
gene regions is a common method for assessing potential hybridisation (Planet et al. 2006;
Govindarajulu et al. 2015), and was utilised in the current study.

The chosen location for this study, Atauro Island and the north coast of Timor, is in the Coral
Triangle and therefore widely applicable to other important reef systems. The hypothesis tested
was that hybridisation between distinct Cladocopium genotypes has occurred at these sites, as
evidenced by gene regions in separate organelles (cob, mitochondria; ITS2, nucleus; psbA\text{ncr},
chloroplast) having experienced different evolutionary histories. Defendable evidence of
hybridisation would be a significant step towards understanding the evolution of Symbiodiniaceae
and potential coral reef persistence in the future.

Materials and methods

Data acquisition

This study represents a novel analysis of the data presented in Brian et al. (2019). Briefly, 43 coral
genera were sampled from four sites at Atauro Island: BBR (Beloi Barrier Reef); BHB (Beloi
Harbour); BLS (Beloi Lagoon South); and BSP (Beloi Saddlepatch) (for a complete list of genera
see Table S1 of Brian et al. 2019). In addition, three sites were sampled on the northern coast of
Timor: HEW (Hera West); LIE (Lamsana Inlet East); and LIW (Lamsana Inlet West). In total, 650
samples were collected from the seven sites. The corals in this study were sampled with the
permission of the Ministerio da Agricultura e Pescas (permit number LNC-PC0012.VI.16).
Symbiont DNA was extracted using a guanidinium protocol, and amplification via PCR was carried out for the cob, ITS2 and psbA\textsubscript{ncr} regions of the symbiont DNA. For full details of PCR reactions and conditions, see the Supplementary Material. The cob amplification utilised either Dinocob1F/Dinocob1R (Zhang \textit{et al.} 2005) or Cob\textsubscript{f1}/Cob\textsubscript{r1} (Pochon \textit{et al.} 2012) primers, while the psbA\textsubscript{ncr} amplification utilised the primers 7.4-Forw/7.8-Rev (Moore \textit{et al.} 2003). Following purification with MagNA solution (Rohlhand and Reich 2012), the cob and psbA\textsubscript{ncr} were sequenced in the forward direction with traditional Sanger sequencing (Macrogen Inc., Seoul, South Korea).

In contrast, the ITS2 region was amplified via Next Generation Sequencing (NGS). Samples underwent an initial amplification with the primers ITSD (Pochon \textit{et al.} 2001) and ITS2Rev2 (Stat \textit{et al.} 2009), with Illumina adapters attached. Amplicons were purified using MagNA solution, and unique forward/reverse index primers (IDT) were annealed to the ends of each amplification using a second short PCR run of 8 cycles. Amplifications were quantified and checked for quality using qPCR (Applied Biosystems StepOne instrument), with the primers ITSD/ITS2Rev2. All samples were pooled, with different volumes of each sample added to achieve an equal concentration (final concentration of pooled library: 4 nM DNA). The pooled library was sequenced on a single lane of the Illumina MiSeq platform by the Centre for Genomics and Proteomics, University of Auckland, New Zealand. As the incongruence tests utilised (see below) require a single sequence \textit{per} sample, the most dominant ITS2 sequence from the NGS in each sample was extracted (an ‘ASV’ in Brian \textit{et al.} 2019). While this was necessary for analysis, it could lead to interpretational issues (see Discussion). Only samples that had successful sequences for all three gene regions were chosen, as the tests require exactly the same taxa lists for each tree or partition. Further, only samples that could be placed in an unambiguous alignment were used, which eliminated several
samples with highly divergent psbA\textsuperscript{ncr} sequences. This left between 18 and 28 samples per site (\(\bar{x} = 22.6\)), with a total of 158 samples used.

\textit{Incongruence tests}

Ideally, a statistical test would be able to test the null hypothesis ‘Dataset X and Dataset Y are not incongruent’, against an alternate hypothesis ‘Dataset X and Dataset Y are incongruent.’ A test with this explicit hypothesis does not exist for phylogenetic data, so other tests with slightly different hypotheses have been frequently employed as an approximation. Two of these tests were utilised in this study.

The Incongruence Length Difference (ILD) test (Farris et al. 1994) uses the criterion of maximum parsimony, and compares two data partitions (nucleotide alignments) X and Y, of arbitrary length. The null hypothesis is that the defined partitions (X, Y) are no more parsimonious (in terms of making a phylogeny) than random partitions generated from a combination of X and Y, while the alternate hypothesis is that the defined partitions are significantly more parsimonious than random partitions. Functionally, this can be used to test if two datasets have undergone separate evolutionary histories (Planet 2006). The implication is that if X and Y are indeed more parsimonious, they encode contrary evolutionary information that is lost when randomised.

The Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa 1999) is an explicit tree-based test using the criterion of maximum likelihood (ML), comparing how well phylogenetic trees explain alignment data. The null hypothesis is that all tested trees are equally good explanations
of the data, while the alternate is that some or all tested trees are not equally good explanations of
the data. In practice, this test identifies the best tree for a given dataset (i.e. a multiple sequence
alignment), and then presents output as to whether other candidate trees are statistically distinct
from that best tree. The output hence appears as pairwise comparisons between two trees. This
procedure can be used to test for incongruence in datasets X and Y, using trees \( T_X \) and \( T_Y \) made
from those datasets. If \( T_X \) and \( T_Y \) are equally likely for all or most characters in X and in Y (tested
in two separate tests), the test will find a p-value >0.05, and it can be concluded that X and Y are
not incongruent, as their trees do an equally good job of explaining each other’s data. If they are
incongruent, it is expected that \( T_X \) will be significantly better than \( T_Y \) when considering dataset X,
and vice versa for \( T_Y \) and Y. The Approximately Unbiased (AU) test was developed by Shimodaira
(2002) as a derivation of the SH test, and generally finds more accurate results when there are
many candidate trees, or some trees are particularly unlikely (Shimodaira 2002; Strimmer and
Rambaut 2002); the AU test was hence utilised for testing procedures.

To identify incongruence, these two tests (ILD, AU) in addition to other analyses described below
were conducted in stepwise fashion (Fig. 1).

Data assembly

Alignments were created and manually edited in Geneious v8.0.5 (Biomatters), using the built-in
Geneious alignment algorithm with all default settings (gap open penalty = 12, extension = 3).
Each site (BBR, BHB, BLS, BSP, HEW, LIE, LIW) had a separate alignment for each gene region
(\( \text{cob}, \text{psbA}\text{ncr}, \text{ITS2} \)), leading to 21 alignments. Additional holistic datasets for each gene region
were created for Atauro Island (92 samples) and Timor (66 samples), to facilitate broad-scale
island comparisons. All alignments had 787, 369 and 531 columns for the cob, ITS2 and psbA
regions, respectively. In total, 27 separate datasets were assembled (3 marker regions x (7 sites +
2 main islands)). Datasets can be accessed online at github.com/brianjosh/Cladocopium_alignments. Durusdinium glynnii (D1) was used as the
outgroup for these analyses (GenBank Accession Numbers: KY131780 (cob); JN558075 (ITS2);
MH329571 (psbA)). Gaps were coded as a fifth character state. All analyses described below
used the program PAUP* 4.0a161 (Swofford 2002) unless otherwise specified. Note that in
PAUP*, the ILD test is called the partition homogeneity test.

Incongruence Length Difference (ILD) tests

The three gene regions were concatenated for each site, with each region then treated as a separate
partition (cob: 1-787; ITS2: 788-1156; psbA 1157-1687; total of 1687 columns). This was
carried out for each site, plus for Atauro Island samples and Timor samples as above (total of nine
different concatenations). The individual site analyses were originally carried out with 100
replications, using a MaxTrees value (number of trees stored at any one time) of 1000. For results
that had p-values <0.2, a more thorough confirmatory analysis was run with 1000 replicates and a
MaxTrees value of 10000. In all cases, the p-values between the two sets of tests differed by
<0.015, and therefore the tests with original p-values >0.2 would be extremely unlikely to change
the result if the more extensive tests had been run on them. All other settings used for the tests
were the PAUP* defaults. Conclusions were drawn at $\alpha = 0.05$. The null hypothesis was that there
was no incongruence between the three partitions.
Approximately Unbiased (AU) tests

Maximum likelihood trees were generated for all gene regions by individual site (all possible combinations of \{cob, psbA\textsuperscript{ncr}, ITS2\} and \{BBR, BHB, BLS, BSP, HEW, LIE, LIW\} \textit{i.e.} 21 different trees). Trees were also made for each gene region for Atauro Island and Timor datasets \textit{(i.e.} six trees). The appropriate evolutionary model was determined for each of the 27 datasets individually by first making a neighbour-joining tree using a Jukes-Cantor distance measure and running the \texttt{automodel} command. The appropriate evolutionary model for each dataset was then employed when making the maximum likelihood trees (Table S1). A basic heuristic search was run to generate a base tree or trees, which was then bootstrapped. All bootstrapping procedures used a heuristic search with random sequence addition and had unlimited MaxTrees; all other settings were the \texttt{PAUP*} defaults. \textit{cob} datasets had 1000 bootstrap replicates, while the ITS2 and psbA\textsuperscript{ncr} datasets had 100 replicates. In addition, for the psbA\textsuperscript{ncr} datasets, the number of addition sequence replicates was set to 2 \textit{(versus} the default of 10), to limit computational burden. The exception is the Atauro Island and Timor datasets, which had 1000 replicates using the \texttt{faststep} search option for all three gene regions. Nodes with <50\% bootstrap support were collapsed into polytomies. This procedure yielded 27 maximum likelihood trees, one for each gene region for each of the nine datasets.

A set of 100 random trees was also generated for each dataset, using the \texttt{generate random} command employing an equiprobable model. These additional trees are necessary to gain an accurate p-value. In theory, every single possible tree topology of the data should be present, to
ensure that the ‘true’ maximum likelihood tree is available to be chosen by the test, and to allow
calculation of the null distribution for the test statistic (Goldman et al. 2000; Planet 2006).
However, given that the number of possible topologies increases exponentially with the addition
of taxa, this criterion is functionally impossible to meet for most modern studies. As such, a
random subset of all possible topologies is chosen instead (e.g. Robinson et al. 2005).

Because the AU test assesses whether competing trees are equally likely hypotheses of the data,
the choice of dataset will affect the conclusions of the test: it may be expected that for dataset X,
tree $T_X$ made from that dataset may be statistically better than another tree $T_Y$, even if they do not
inherently disagree. This would not be evidence for incongruence, just the test behaving in its
originally intended manner. Because of this, for each site, reciprocal AU tests were run. For
example, for site BBR, the $cob$ BBR alignment was initially used as the dataset for the test, and all
three trees (from the $cob$ BBR, ITS2 BBR and $psbA^{ncr}$ BBR alignments) were compared with the
AU test, to see: (a) which of the trees explained the dataset best, and (b) whether the other trees
were significantly worse than the best tree at explaining the dataset. This was then repeated using
the ITS2 BBR and $psbA^{ncr}$ BBR alignments as the dataset in question, to compare the same three
trees. 10000 RELL (resampling estimated log-likelihood, Kishino et al. 1990) bootstrap replicates
were used for calculation of p-values. Because there were six pairwise comparisons carried out for
each site (best tree vs. other two trees for $cob$, $psbA^{ncr}$ and ITS2 regions), a within-site Bonferroni
correction was applied ($\alpha = 0.0085$). The null hypothesis was that the two trees being compared
explained the sequence alignment equally well. The gene regions were considered incongruent if
there was reciprocal incongruence; for example, if the ITS2 tree was significantly worse than the
296  *cob* tree at explaining the *cob* dataset and the *cob* tree was significantly worse than the ITS2 tree at explaining the ITS2 dataset.

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299  Post hoc analyses

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301  Based on the original analyses, several datasets displayed consistent evidence of incongruence (see Results). To verify these results, further ILD tests were executed, using only two gene regions at a time (*e.g.* for a single site, the following concatenations were assembled and tested: *cob* vs. ITS2; *cob* vs. *psbA*; ITS2 vs. *psbA*). As there were three tests per site, conclusions were drawn at a Bonferroni-corrected $\alpha=0.017$. This allowed the location of incongruence to be established (in terms of between gene regions), as the original ILD tests could not say which partitions were incongruent, only that incongruence existed. An extra site which had consistently shown no evidence of incongruence (LIW) was used as a control.

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310  Following that, the datasets which continued to show incongruence had their ML trees input into Dendroscope 3.0 (Huson and Scornavacca 2012), and pairwise tanglegrams were constructed to identify the source of incongruence. In addition, tree hybridisation networks were created using the Autumn algorithm (Huson and Linz 2016), implemented in Dendroscope 3.0. This algorithm attempts to make a consensus tree from two input trees, and identifies the taxa that cannot be reconciled. Finally, raw sequence alignments were inspected to verify incongruence in the identified samples. All background sequences from populations related to putative hybrids were also inspected in an effort to assess the likelihood of possible alternate explanations.
Patterns of putative hybridisation (see Results) could potentially be verified by inspecting an additional non-intragenomically variable nuclear marker. Therefore, the actin gene (symbiont nuclear DNA) was sequenced for putative hybrid samples and closely related samples identified in this study. Samples were PCR-amplified and directly sequenced in the forward direction by the Macrogen Sequencing Service (Macrogen Inc., Seoul, South Korea) using the primer pair actin_f1/actin_r1 (Pochon et al. 2012). An initial PCR run used a 7 min denaturation at 95°C, followed by 40 cycles of 94°C (40 s), 58°C (40 s), 72°C (90 s) and a final denaturation of 10 min at 72°C. PCRs contained 1 × MyTaq HS Red Mix (Bioline, Randolph, MA, USA), ~20 ng sample DNA, 10 μg BSA, 0.25 μM each primer, and H2O to a total volume of 20 μl. All samples had multiple bands present (observed by running on a 1.5% agarose gel), so the PCR product was run on a 1% agarose gel for 1 h 30 min. Bands at the correct length (~900 bp) were excised with a pipette tip and reamplified using 20 cycles of the above conditions. Prior to sequencing, the samples were purified with MagNA PCR clean-up solution (Rohland and Reich 2012). Sequences were inspected visually, and no further steps were taken (see Discussion).

Results

Incongruence Length Difference tests

The cob, psbA\textsuperscript{nct} and ITS2 gene partitions for Timor sites (HEW, LIE, LIW) were not incongruent, a trend which was also seen in the overall Timor island analysis (ILD test, p = 1 for all). This p-value is not concerning; it simply indicates that among the replicates, the partitions were never more parsimonious than random partitions. The Atauro dataset as a whole did not show statistically
substantiated evidence of incongruence, though it approached significance (p = 0.0874). In this case, it is valid to use the term ‘approaching significance’, as the test statistic is directly correlated to the number of replicates for which the original partitions were found to vary from random data (Planet 2006). Looking at each Atauro site individually, BBR and BLS were not incongruent (p = 0.99), while BHB displayed an equivocal result (p = 0.129) and BSP was strongly incongruent between partitions (p = 0.001). However, these tests on three partitions could not identify where potential incongruences were located.

Approximately Unbiased (AU) tests

In 24 of the 27 tests conducted, the best tree chosen was the one that was made from that gene region originally (i.e. for a test with the ITS2 region as its base, the ITS2 tree was chosen as the best tree). The exceptions were sites BBR, HEW and LIE, where either the psbA\textsubscript{ncr} or ITS2 trees were chosen as the best explanation of the cob dataset. The test always found incongruence when using the psbA\textsubscript{ncr} region as a base; this is likely due to an issue with the test (see Discussion), and therefore the results for the cob and ITS2 gene regions are the major focus of these results.

There was a very clear island-wide partitioning of results when it came to the AU test (Table 1). All Timor sites (HEW, LIE, LIW) were not incongruent for either the cob or ITS2 gene regions; all three trees (cob, ITS2, psbA\textsubscript{ncr}) did an equally good job of explaining these two regions. While there was incongruence between cob and ITS2 trees using the ITS2 region as a base in the overall Timor analysis, this was not reciprocated (i.e. these two trees were not incongruent when considering the cob dataset). In contrast, the Atauro datasets showed high levels of reciprocal
incongruence. Overall, the ITS2 tree (but not the psbA\textsuperscript{ncr} tree) made from all Atauro samples was incongruent with the cob dataset, and both the cob and psbA\textsuperscript{ncr} trees were incongruent with the ITS2 dataset. Looking at individual sites, the same complete reciprocal incongruence exists for the BHB and BSP datasets. These three datasets (Atauro, BHB, BSP) correspond to the three lowest p-values returned by the ILD tests. In general, the tests reveal incongruence between the organellar (cob and psbA\textsuperscript{ncr}) and nuclear (ITS2) gene regions. In all cases, the AU test was unable to reject congruence between the cob and psbA\textsuperscript{ncr} regions. However, it did reject congruence between the ITS2 and psbA\textsuperscript{ncr} regions (using the ITS2 region as a base), and showed reciprocal incongruence between the cob and ITS2 region (using both the ITS2 and cob regions as a base). In addition, the ITS2 tree was incongruent with the cob dataset (but not the other way around) for site BLS. As such, these four datasets (complete Atauro, BHB, BSP, BLS) were carried forward to post hoc testing.

Post hoc analyses

Additional ILD tests were carried out using two partitions at a time. Site LIW was included as a control to ensure that the tests still successfully supported congruence where appropriate. These results strongly support the AU test (Table 2). There is clear incongruence between the nuclear ITS2 region and the other two organellar gene regions, which are not incongruent when considered together. Site LIW is clearly not incongruent at all regions. This shows that these two-way tests are functioning as expected. BLS is also not incongruent; while the AU test indicated potential incongruence, the other tests do not and so it was not carried forward as a candidate for hybridisation. Pairwise tanglegrams were made for BHB, BSP, and Atauro datasets, with
potentially incongruent branches verified by attempting to hybridise the two trees to create a consensus. Those branches and closely related sequences subsequently had their raw sequences inspected in an attempt to confirm incongruence.

The tanglegrams and tree hybridisation analyses for site BHB (Fig. 2) support the results of the statistical tests. Comparing the two organellar genes with the ITS2 region (Fig. 2a, 2b) reveals two incongruent samples, BHB146 and BHB148, while BHB148 is also incongruent between the cob and psbA(ncr) regions (Fig. 2c). Inspection of raw sequence alignments reveals BHB146 is an example of true incongruence (Fig. 3), whereas the incongruence in BHB148 is due to a highly divergent psbA(ncr) sequence, and does not show a reticulate pattern (Fig. S1). For the organellar gene regions, BHB146 belongs to the *Cladocopium* C1 radiation (symbiont types C42a and C1v respectively, see Brian et al. 2019). For the ITS2 region, it is identified as type C1#, which groups more closely with the *Cladocopium* C3 radiation.

The BSP tanglegrams (Fig. 4) also support the statistical analyses, with six potentially incongruent samples identified. After inspection of the raw sequence data, four of these were disregarded (BSP211, BSP320, BSP372, BSP387), as they were more likely caused by parsimony-uninformative mutations in a single sequence (Fig. S2). However, two samples were verified as incongruent (BSP343 and BSP364, Figs. 5 and 6). BSP364 belongs to two different previously defined subclades: a variant of *Cladocopium* type C40 for psbA(ncr), and type C3z for ITS2. BSP343 also shows clear incongruence between the organellar and nuclear genes regions. The psbA(ncr) is a variant of *Cladocopium* type C40, which groups it most closely with the psbA(ncr) C3z clade.
b), while the ITS2 region features type C3u, which places it as distinct from both the C3z and C40 groups.

Pairwise tanglegrams and hybridisation analyses were also executed for the whole Atauro Island dataset (sites BBR, BHB, BLS, BSP). Despite the inclusion of two more sites, the analyses showed that incongruence was caused by exactly the same samples as found by the individual site analyses, affirming BHB and BSP as sites with incongruent samples. Further, no other sites contributed any incongruent samples. The overall results are presented in Table 3, which demonstrates that ITS2 comparisons displayed the most incongruence, while any incongruences between cob and psbA\textsuperscript{ncr} regions were due to non-reticulate sequence variation. This is strongly supportive of the AU test results as well as Table 2, which all indicate that incongruence occurs between the organellar and nuclear genomes of Cladocopium. Of the three clearly incongruent samples (BHB146, BSP343, BSP364) there was no general clear pattern in coral host (host genera: Pavona, Symphyllia and Acropora respectively). The sequencing of the actin gene was uninformative, with only occasional non-parsimonious variation observed (i.e. polymorphisms in a single sequence only).

Background sequences for populations related to the putative hybrids were also analysed. This was particularly fruitful for sample BSP364 (which has incongruent C3z and C40 genetic signals). At site BSP, there were eight additional samples with C3z as the dominant ITS2 sequence (these were also C3z for psbA\textsuperscript{ncr} and cob regions), and thirteen with C40 as the dominant ITS2 sequence (these were also C40 for the psbA\textsuperscript{ncr} and cob regions) at site BSP (Fig. 7). Of the eight C3z samples (Fig 7a), seven had no C40 sequences in their genomes, while one had C40 traces at a frequency of 0.61% (Fig 7b). C40 samples had a low proportion of background sequences (Fig. 7c), and less
than half of them possessed C3z traces, which only appeared as the 3\textsuperscript{rd} most common background sequence in terms of average abundance, and 5\textsuperscript{th} most common in terms of frequency (Fig. 7d). The other two putative hybrid ITS2 types (C3u, C1#) did not occur frequently enough to conduct a similar analysis, but are mentioned in the Discussion.

**Discussion**

**Methodological approach taken**

There are many factors, such as character sampling and bias due to differential gene length, which can give false signals of incongruence (Som 2014). However, the approach taken in this study has been able to clearly display incongruence between organellar and nuclear regions in *Cladocopium*. In isolation, it is true that there are issues with the tests utilised. For example, the AU test presented an issue with most trees being incongruent for the psbA\textsuperscript{ncr} region. The psbA\textsuperscript{ncr} region is highly variable (LaJeunesse and Thornhill 2011; Thornhill *et al.* 2014), and hence a more complex tree is required to explain it. The cob and ITS2 trees with multiple polytomies could not do this as effectively, and hence a result of incongruence was returned. Therefore, the results from the cob and ITS2 datasets are likely more reliable, and were the focus of the Results. Further, the ILD test has been criticised for being overly sensitive, especially when comparing partitions of different resolutions (Barker and Lutzoni 2002). The refutation of this is simple: in all cases, it found congruence between the psbA\textsuperscript{ncr} and cob regions, the two most different in terms of resolution (Table 2), so this is clearly not contributing to the positive results between the organellar and nuclear partitions observed here. Indeed, it failed to reject congruence between the cob and psbA\textsuperscript{ncr...
regions for sites BSP and BHB despite the tree hybridisation analyses finding potential incongruence (Fig. 2c, 4c), and so appears to be reasonably conservative in this case. The results of the AU and ILD tests are also compelling because they are differential: they show consistently different patterns between datasets and are therefore likely responding to genuine phylogenetic signals. This was confirmed by looking at the raw sequence data, and shows the efficacy of the approach taken here. With such a wide range of samples, initially searching for incongruences in sequence data would be functionally impossible, as it would require comparing all possible combinations of sequences (in this study, this would require $1.17 \times 10^{278}$ comparisons). However, the stepwise use of analyses allowed the initial identification of which sites may host incongruent samples, and then visualisation on phylogenetic trees allowed simple alignments of appropriate samples to be generated, where incongruence could clearly be refuted or confirmed. In addition, given the issues with tests in isolation, the multiplicity of analyses used generates a far more convincing picture of reticulate evolution.

Hybridisation in Cladocopium?

Incongruence was comprehensively established for the samples BHB146, BSP343 and BSP364. However, this does not necessarily translate to hybridisation, as there are a range of analytical or biological factors that can cause incongruence in phylogenetic data. For example, one hypothesised to be quite common but insidious in its undetectable nature is heterotachy, shifts in site-specific evolutionary rates through time (Som 2014). While there is no particular way to identify heterotachy or exclude it as a cause, except with a very large number of sequences,
maximum likelihood methods in particular have been shown to be robust to even intermediate levels of heterotachy (Som 2014).

A more plausible explanation is incomplete lineage sorting (ILS), often considered the most common cause of incongruence (Degnan and Rosenberg 2009). This is due to polymorphisms not segregating fully during speciation events, leading to phylogenetic signals in gene trees that conflict with the overall species tree. This has been shown to be quite common in the ITS2 region, thanks to its multiple-copy nature (Thornhill et al. 2007). Through this mechanism, ancestral polymorphisms may persist at low levels in the genome. Therefore, it is possible that the divergent sequences recovered actually represent a single symbiont population, which has multiple ancestral polymorphisms present via ILS (i.e. intragenomic variation). Through stochastic DNA processes such as unequal crossing over, slipped-strand mispairing and transposition, these intragenomic variants may be eliminated or promoted in the multiple-copy array (i.e. concerted evolution, see Nei and Rooney 2005). Hence, in the samples from a single reproductively isolated population, one ancestral polymorphism may be dominant in the ITS2 region of some, while a different ancestral polymorphism may be dominant in others. This would cause the patterns observed in this study, with the ITS2 region being occasionally incongruent with the organellar regions.

Ideally, a statistical test would be carried out to differentiate between hybridisation and ILS, and such tests do exist. However, they require inputs of information which are not currently available for *Cladocopium*, such as: (a) An understanding of the effective population size $N_e$ (Pelser et al. 2010); (b) a large number of genes, at least some of which must be adjacent (Pollard et al. 2006; Meng and Kubatko 2009); or (c) strictly bifurcating trees and clearly defined species (Sang and
Zhong 2000; Joly et al. 2009). Therefore, ILS as a cause of the observed incongruence cannot be statistically refuted. However, there is good evidence that the patterns observed here are more likely to be caused by symbiont hybridisation.

First, the pattern of incongruence observed, with organellar cytoplasmic genes being different to nuclear genes, accords with a large body of prior theory on hybridisation. Nuclear genes are largely inherited biparentally, and the ITS2 region is no exception (Baldwin et al. 1995; Rybalka et al. 2013). However, the cytoplasm tends to be inherited maternally (Rieseberg et al. 1996). This difference is largely due to gametogenesis and fertilisation, where the male gamete typically only contains nuclear information, while the female gamete (egg) contains the cytoplasm that will be passed on to the zygote. Therefore, if an organism encounters a population of another species and produces viable hybrids, theory predicts that over time, repeated backcrossing with the more common species (introgression) will produce hybrids with divergent organellar and nuclear signals. While the nature of the sexual life cycle has yet to be fully elucidated in the Symbiodiniaceae, previous evidence has shown that other unicellular dinoflagellates produce gametes (Brawley and Johnson 1992). In addition, the presence of ‘plus’ and ‘minus’ mating types, analogous to gender, has been shown in the dinoflagellate *Alexandrium tamarense* (Brosnahan 2011). Therefore, it is reasonable to assume that Symbiodiniaceae also produce distinct gametes (as opposed to conducting sex via fusion, for example), making this mechanism eminently plausible. The documentation of functional meiotic genes in Symbiodiniaceae (Chi et al. 2014, Levin et al. 2016) supports this assertion. Such a pattern of discordance between cytoplasmic and nuclear genes caused by hybridisation has been recorded for taxa as diverse as plants (Rieseberg et al. 1996; Pelser et al. 2010; Sun et al. 2015), beetles (Sota and Vogler 2001) and indeed corals.
van Oppen et al. 2001). In general, hybridisation is predicted to cause incongruence between nuclear and cytoplasmic markers in both multicellular and unicellular taxa (Bull et al. 1993). Other factors due to hybridisation, such as semigamy or differential fitness of nuclear-cytoplasmic combinations, can also cause incongruence between nuclear and cytoplasmic gene trees (Rieseberg et al. 1996). Therefore, the fact that this was the pattern observed in this study is strong circumstantial evidence that hybridisation is the explanation.

In addition, hybridisation is made more likely in comparison to ILS by the fact that all of the incongruent ITS2 sequences were previously defined types (i.e. not unique sequences), that were also present in non-incongruent relationships in the analyses. For example, BSP364 had a generic Cladocopium type C3 sequence for the cob gene, was a C40 type for the psbA\textsuperscript{ncr} region, and C3z for the ITS2 region. Significantly, there were also samples recovered which were type C40 for both the psbA\textsuperscript{ncr} and ITS2 regions (samples BSP319-BSP375, Fig. 4b), and samples which were type C3z for both regions (samples BSP373-BSP386, Fig. 4b). This confirms that they are clearly separate types, supported by the fact that they differ by four base pairs in the ITS2 sequence and 64 base pairs in the psbA\textsuperscript{ncr} region (including a 49 base pair deletion in the C40 sequences), indicating that this is not just a non-diagnostic polymorphism (Wilkinson et al. 2015). The implications for this being caused by ILS are given in Fig. 8. Only the psbA\textsuperscript{ncr} and ITS2 genes are presented, as the cob gene was invariant in this case.

Fig. 8b graphically represents the process that would be required for the observed patterns to be due to ILS. Given that symbiont sex is now strongly supported (though in low frequency; Thornhill et al. 2017), it seems unlikely that a divergent ancestral polymorphism could be maintained as the
dominant sequence in some samples within type C40, as it would be expected that repeated
recombination would eventually remove C3z traces from the C40 genome, or vice versa (Fig. 8a).
It is more parsimonious that a hybridisation event has occurred between symbiont types C40 and
C3z, with backcrossing leading to incongruence between organellar and nuclear genes. This is
strongly supported by the analysis of the background symbiont populations (Fig. 7). The results
show that there is little evidence of C40 and C3z sequences being shared within samples. The C3z
population had almost no C40 sequences present at all, with just one sample having an extremely
low background abundance of C40 (Fig. 7b). C3z sequences were slightly more common in C40
samples (Fig. 7d). However, the low proportion of background sequences in this population (Fig.
7c) meant that overall the presence of C3z in the C40 population was negligible (mean = 1.61%,
median = 0). This reveals essentially pure populations of C40 and C3z at site BSP, something
which strongly favours hybridisation versus ILS as causing the mixed pattern in BSP364
(Wilkinson et al. 2015). While the other two putative hybrid ITS2 types (C3u, C1#) do not have
large populations to compare, the same basic pattern was also observed for BSP343, which was
identified as Cladocopium type C40 for the organelle regions, and type C3u for ITS2. If this was
to be caused by ILS, then both variants would be expected to occur in the ITS2 region, (with one
at low frequency), but the NGS data revealed no trace of ITS2 type C40 in that sample. Further,
the divergences observed (i.e. C40/C3u, C40/C3z, C1/C1#) all coalesce at the ‘ancestral’ types C1
or C3, rather than one representing an intermediate evolutionary step to the other. Therefore, ILS
would also predict these ancestral sequences to be in the ITS2 genome in low frequencies.
However, this was only observed in BSP343 (as the fourth most common sequence); neither
BHB146 nor BSP364 showed any evidence of these ancestral sequences. While it is acknowledged
that hybridisation and ILS are not mutually exclusive and the incongruences observed could be
caused by a combination of both, the weight of evidence suggests that these results are more likely a result of interspecific hybridisation between distinct symbiont types.

Potentially, the two competing hypotheses could be distinguished by sequencing another nuclear gene, less susceptible to intragenomic variation, for both putative hybrid samples and closely related sequences. If the patterns were due to hybridisation, it would be expected that the additional nuclear gene would support the ITS2 identity, and cluster the sample with the same group as presented in the ITS2 trees (Figs. 2, 4). In contrast, if the incongruence was caused by ILS, the additional marker would cluster the putative hybrid with the same samples as the organellar gene regions. This was attempted using the *actin* gene. Unfortunately, low resolution (and difficulties in amplification leading to short usable sequences) meant that neither scenario was supported, as the sequences were not variable enough to recover the groups observed in Figures 2 and 4. The other currently-available Symbiodiniaceae nuclear gene markers either suffer from the same issue of significant intragenomic variation (ITS1), or are lower-resolution than *actin* (SSU, LSU, 5.8S, elf2), and therefore the patterns observed cannot currently be independently verified. The further development of highly-variable, reliably amplifiable nuclear gene markers should be a priority for Symbiodiniaceae systematics. However, ILS (and indeed all analytical factors), are random or would be expected to affect all sites. The results obtained, however, are anything but random, with two sites consistently being recovered as incongruent in contrast to all others, despite those incongruences coming from a range of host species that were present at all sites. In addition, both these sites have been shown to be rich in Symbiodiniaceae diversity, when compared with the Timor sites (Brian et al. 2019). This suggests that putative hybridisation may be limited to high-quality sites that maintain high levels of symbiont diversity.
Intragenomic variation within the ITS2 region could lead to the incongruences observed via ILS, though the discussion above suggests that hybridisation should be favoured as an explanation. However, the psbA\textsuperscript{ncr} region can also be intragenomically variable (LaJeunesse and Thornhill 2011), with intragenomic sequence ratios that may fluctuate within a single species. Combined with the intragenomic variation of the ITS2 region, this generates the potential for a wide range of ITS2-psbA\textsuperscript{ncr} combinations within a single genome. For example, one member of a population may have a 9:1 ratio of variant A:variant B within its multiple-copy ITS2 sequences, and a 9:1 ratio of variant A:variant B in its multiple-copy psbA\textsuperscript{ncr} sequences. A second member of the same population could plausibly have an 8:2 ratio of variant A:variant B within its ITS2 sequences, and a 4:6 ratio of variant A:variant B in its psbA\textsuperscript{ncr} sequences. Therefore, there is a possible difference between the most common sequences in total, and the most common associations between ITS2 and psbA\textsuperscript{ncr} ratio types. If only the most common sequences are studied, there is the potential for some natural associations (\textit{i.e.} not caused by hybridisation) to appear as incongruences. The present study attempts to draw conclusions based on common associations between nuclear and organellar genes, but is only able to utilise common sequences. This is explicit for the psbA\textsuperscript{ncr} region (as Sanger sequencing only amplifies the most common overall sequence), and implicit for the ITS2 region (as the nature of the tests necessitated the selection of the most common overall sequence from NGS data). Within a single genome, a solution would be to sequence multiple markers from the same DNA strand through long-read sequencing, which would preserve the ratio of intragenomic variants. However, this does not work for markers across multiple organellar and nuclear genomes, and currently there is no other acceptable solution to this problem for
Symbiodiniaceae. While perhaps unlikely, this issue could potentially explain the patterns seen, and should be acknowledged.

**Previous tests of incongruence**

No previous study on Symbiodiniaceae seriously considers symbiont hybridisation, except that of Wilkinson *et al.* (2015), which also finds evidence for its existence. However, aside from the potential examples of hybridisation mentioned in the Introduction of this study (LaJeunesse *et al.* 2003, 2004; LaJeunesse 2005), three other studies bear mention. Sampayo *et al.* (2009) also focused on the basis that hybridisation can cause incongruence between genes from different organelles, and built trees from mitochondrial, chloroplast and rDNA nuclear gene regions to test this. Based on visual inspection of these trees, they concluded that different symbiont lineages (types) within *Cladocopium* are reproductively isolated. Interestingly, they did also use the ILD test to formally test incongruence, which returned a p-value of 0.01, though this result was not explored further. Pochon *et al.* (2014) assessed six genes from three different organelles (mitochondrion, nucleus and chloroplast). In all cases, they found evidence of incongruence between pairwise comparisons of genes, using the AU test. While they go on to discuss the implications for concatenation in some detail, the cause of these incongruences was likewise not explored further. Another study from Pochon *et al.* (2006) found the surprising result of incongruence between whole genera rendered from nr28S and cp23S data, using the Shimodaira-Hasegawa test. However, when they removed all but two members of each clade, the test then showed congruence between datasets. This indicated incongruence was being caused by the accumulation of occasional within-clade mismatches between the nucleus and chloroplasts, something which is also broadly agreeable with a hypothesis of hybridisation in low frequency.
These studies certainly do not provide conclusive evidence of hybridisation. However, it is reasonably striking that four studies conduct an explicit statistical test of incongruence within Symbiodiniaceae (Pochon *et al.* 2006, 2014; Sampayo *et al.* 2009; this study), and all four find evidence for its existence. At the very least, these add to the body of evidence that the family Symbiodiniaceae has not evolved in a simple linear fashion, and justifies a more careful consideration of patterns of incongruence within this family.

**Conclusions**

This study cannot be considered unequivocal proof of *Cladocopium* hybridisation. However, the unambiguous evidence for incongruence between nuclear and organellar gene regions shows the value of the stepwise approach taken here, and conforms to the hypothesis of hybridisation between divergent taxa. While incomplete lineage sorting remains a possibility, it is a less intuitive explanation, especially in the light of incongruent samples having clearly distinct, predefined types which were recovered in non-incongruent samples, and the failures of background populations to consistently align to its predictions. Therefore, hybridisation appears to be a credible, if infrequent, mechanism for adaptive change in *Cladocopium*, and potentially for Symbiodiniaceae in general, though multiple sources of intragenomic variation remain analytically problematic. Ascertaining the frequency and extent of this may be vital to predicting the fate of coral reefs in an environmentally unpredictable future.

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References

Baillie BK, Belda-Baillie CA, Silvestre V, Sison M, Gomez AV, Gomez ED, Monje V (2000) Genetic variation in Symbiodinium isolates from giant clams based on random-amplified-polymorphic DNA (RAPD) patterns. Marine Biology, 136, 829-836.

Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS, Donoghue MJ (1995) The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Annals of the Missouri Botanical Garden, 247-277.

Barker FK, Lutzoni FM (2002) The utility of the incongruence length difference test. Systematic Biology, 51, 625-637.

Baskett ML, Gaines SD, Nisbet RM (2009) Symbiont diversity may help coral reefs survive moderate climate change. Ecological Applications, 19, 3-17.

Baums IB, Devlin-Durante MK, LaJeunesse TC (2014) New insights into the dynamics between reef corals and their associated dinoflagellate endosymbionts from population genetic studies. Molecular Ecology, 23, 4203-4215.

Brawley SH, Johnson LE (1992) Gametogenesis, gametes and zygotes: an ecological perspective on sexual reproduction in the algae. British Phycological Journal, 27, 233-252.

Brian JI, Davy SK, Wilkinson SP (2019) Elevated Symbiodiniaceae richness at Atauro Island (Timor-Leste): a highly biodiverse reef system. Coral Reefs, 38, 123-136

Brosnahan, ML (2011) Life cycle studies of the red tide dinoflagellate species complex Alexandrium tamarense. Doctoral dissertation, Massachusetts Institute of Technology.

Bull JJ, Huelsenbeck JP, Cunningham CW, Swofford DL, Waddell PJ (1993) Partitioning and combining data in phylogenetic analysis. Systematic Biology, 42, 384-397.
Charlton ND, Craven KD, Afkhami ME, Hall BA, Ghimire SR, Young CA (2014) Interspecific hybridization and bioactive alkaloid variation increases diversity in endophytic Epichloë species of Bromus laeves. FEMS Microbiology Ecology, 90, 276-289.

Chesnick JM, Cox ER (1987) Synchronized sexuality of an algal symbiont and its dinoflagellate host, Peridinium balticum (Levander) Lemmermann. Biosystems, 21, 69-78.

Chi J, Parrow MW, Dunthorn M (2014) Cryptic sex in Symbiodinium (Alveolata, Dinoflagellata) is supported by an inventory of meiotic genes. Journal of Eukaryotic Microbiology, 61, 322-327.

Coffroth MA, Lewis CF, Santos SR, Weaver JL (2006) Environmental populations of symbiotic dinoflagellates in the genus Symbiodinium can initiate symbioses with reef cnidarians. Current Biology, 16, R985-R987.

Combosch DJ, Vollmer SV (2015) Trans-Pacific RAD-Seq population genomics confirms introgressive hybridization in Eastern Pacific Pocillopora corals. Molecular Phylogenetics and Evolution, 88, 154-162.

Davy SK, Allemand D, Weis VM (2012) Cell biology of cnidarian-dinoflagellate symbiosis. Microbiology and Molecular Biology Reviews, 76, 229-261.

Degnan JH, Rosenberg NA (2009) Gene tree discordance, phylogenetic inference, and the multispecies coalescent. Trends in Ecology and Evolution, 24, 332-340.

Dittrich-Reed DR, Fitzpatrick BM (2013) Transgressive hybrids as hopeful monsters. Evolutionary Biology, 40, 310-315.

Edvardsen B, Shalchian-Tabrizi K, Jakobsen KS, Medlin LK, Dahl E, Brubak S, Paasche E (2003) Genetic variability and molecular phylogeny of Dinophysis species (Dinophyceae) from Norwegian waters inferred from single cell analyses of rDNA. Journal of Phycology, 39, 395-408.

Ellstrand NC, Hoffman CA (1990) Hybridization as an avenue of escape for engineered genes. Bioscience, 40, 438-442.

Farris JS, Källersjö M, Kluge AG, Bult C (1994) Testing significance of incongruence. Cladistics, 10, 315-319.
Franklin EC, Stat M, Pochon X, Putnam HM, Gates RD (2012) GeoSymbio: a hybrid, cloud-based web application of global geospatial bioinformatics and ecoinformatics for Symbiodinium–host symbioses. *Molecular Ecology Resources, 12*, 369-373.

Folk RA, Mandel JR, Freudenstein JV (2017) Ancestral gene flow and parallel organellar genome capture result in extreme phylogenomic discord in a lineage of angiosperms. *Systematic Biology, 66*, 320-337.

Geneious version 8.0.5 created by Biomatters. Available from http://www.geneious.com

Goldman N, Anderson JP, Rodrigo AG (2000) Likelihood-based tests of topologies in phylogenetics. *Systematic Biology, 49*, 652-670.

Govindarajulu R, Parks M, Tennessen JA, Liston A, Ashman TL (2015) Comparison of nuclear, plastid, and mitochondrial phylogenies and the origin of wild octoploid strawberry species. *American Journal of Botany, 102*, 544-554.

Gribble KE, Anderson DM (2007) High intraindividual, intraspecific, and interspecific variability in large-subunit ribosomal DNA in the heterotrophic dinoflagellates *Protoperidinium, Diplopsalis*, and *Preperidinium* (Dinophyceae). *Phycologia, 46*, 315-324.

Hart MC, Green DH, Bresnan E, Bolch CJ (2007) Large subunit ribosomal RNA gene variation and sequence heterogeneity of *Dinophysis* (Dinophyceae) species from Scottish coastal waters. *Harmful Algae, 6*, 271-287.

Heitman J (2010) Evolution of eukaryotic microbial pathogens via covert sexual reproduction. *Cell Host & Microbe, 8*, 86-99.

Hughes TP, Kerry JT, Álvarez-Noriega M, Álvarez-Romero JG, Anderson KD, Baird AH, ..., Bridge TC (2017) Global warming and recurrent mass bleaching of corals. *Nature, 543*, 373.

Huson DH, Scornavacca C (2012). Dendroscope 3: an interactive tool for rooted phylogenetic trees and networks. *Systematic biology, 61*, 1061-1067.

Huson DH, Linz S (2016) Autumn Algorithm–Computation of Hybridization Networks for Realistic Phylogenetic Trees. *IEEE/ACM transactions on computational biology and bioinformatics.*
Joly S, McLenachan PA, Lockhart PJ (2009) A statistical approach for distinguishing hybridization and incomplete lineage sorting. *The American Naturalist*, **174**, E54-E70.

Kemp DW, Thornhill DJ, Rotjan RD, Iglesias-Prieto R, Fitt WK, Schmidt GW (2015) Spatially distinct and regionally endemic *Symbiodinium* assemblages in the threatened Caribbean reef-building coral *Orcibella faveolata*. *Coral Reefs*, **34**, 535-547.

Kirtman B, Power SB, Adedoyin AJ, Boer GJ, Bojariu R, Camilloni I, ..., Prather M (2013) Near-term climate change: projections and predictability. In: *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* (eds. Stocker TF, Qin D, ..., Plattner GK). Cambridge University Press, Cambridge.

Kishino H, Miyata T, Hasegawa M (1990) Maximum likelihood inference of protein phylogeny and the origin of chloroplasts. *Journal of Molecular Evolution*, **31**, 151-160.

LaJeunesse TC (2001) Investigating the biodiversity, ecology, and phylogeny of endosymbiotic dinoflagellates in the genus *Symbiodinium* using the internal transcribed spacer region: in search of a "species" level marker. *Journal of Phycology*, **37**, 866-880.

LaJeunesse TC (2005) “Species” radiations of symbiotic dinoflagellates in the Atlantic and Indo-Pacific since the Miocene-Pliocene transition. *Molecular Biology and Evolution*, **22**, 570-581.

LaJeunesse TC, Thornhill DJ (2011) Improved resolution of reef-coral endosymbiont (*Symbiodinium*) species diversity, ecology, and evolution through psbA non-coding region genotyping. *PLoS One*, **6**, e29013.

LaJeunesse TC, Loh WK, van Woesik R, Hoegh-Guldberg O, Schmidt GW, Fitt WK (2003) Low symbiont diversity in southern Great Barrier Reef corals, relative to those of the Caribbean. *Limnology and Oceanography*, **48**, 2046-2054.

LaJeunesse TC, Wham DC, Pettay DT, Parkinson JE, Keshavmurthy S, Chen CA (2014) Ecologically differentiated stress-tolerant endosymbions in the dinoflagellate genus *Symbiodinium* (Dinophyceae) Clade D are different species. *Phycologia*, **53**, 305-319.

LaJeunesse TC, Parkinson JE, Gabrielson PW, Jeong HJ, Reimer JD, Voolstra CR, Santos SR (2018) Systematic revision of Symbiodiniaceae highlights the antiquity and diversity of coral endosymbionts. *Current Biology*, **28**, 2570-2580.
LaJeunesse TC, Bhagooli R, Hidaka M, DeVantier L, Done T, Schmidt GW, ..., Hoegh-Guldberg O (2004) Closely related *Symbiodinium* spp. differ in relative dominance in coral reef host communities across environmental, latitudinal and biogeographic gradients. *Marine Ecology Progress Series, 284*, 147-161.

Law R, Lewis DH (1983) Biotic environments and the maintenance of sex–some evidence from mutualistic symbioses. *Biological Journal of the Linnean Society, 20*, 249-276.

Levin RA, Beltran VH, Hill R, Kjelleberg S, McDougald D, Steinberg PD, van Oppen MJ (2016) Sex, scavengers, and chaperones: transcriptome secrets of divergent *Symbiodinium* thermal tolerances. *Molecular Biology and Evolution, 33*, 2201-2215.

Meng C, Kubatko LS (2009) Detecting hybrid speciation in the presence of incomplete lineage sorting using gene tree incongruence: a model. *Theoretical Population Biology, 75*, 35-45.

Moon CD, Craven KD, Leuchtmann A, Clement SL, Schardl CL (2004) Prevalence of interspecific hybrids amongst asexual fungal endophytes of grasses. *Molecular Ecology, 13*, 1455-1467.

Moore RB, Ferguson KM, Loh WK, Hoegh-Guldberg O, Carter DA (2003) Highly organized structure in the non-coding region of the psbA minicircle from clade C *Symbiodinium*. *International Journal of Systematics and Evolutionary Microbiology, 53*, 1725-1734.

Moran EV, Alexander JM (2014) Evolutionary responses to global change: lessons from invasive species. *Ecology Letters, 17*, 637-649.

Nei M, Rooney AP (2005) Concerted and birth-and-death evolution of multigene families. *Annual Review of Genetics, 39*, 121-152.

Nitschke, M. R., Davy, S. K., & Ward, S. (2016). Horizontal transmission of *Symbiodinium* cells between adult and juvenile corals is aided by benthic sediment. *Coral Reefs, 35*, 335-344.

Pelser PB, Kennedy AH, Tepe EJ, Shidler JB, Nordenstam B, Kadereit JW, Watson LE (2010) Patterns and causes of incongruence between plastid and nuclear Senecioneae (Asteraceae) phylogenies. *American Journal of Botany, 97*, 856-873.

Pettay DT, Wham DC, Pinzon JH, LaJeunesse TC (2011) Genotypic diversity and spatial–temporal distribution of *Symbiodinium* clones in an abundant reef coral. *Molecular Ecology, 20*, 5197-5212.
855  Planet PJ (2006) Tree disagreement: measuring and testing incongruence in phylogenies. *Journal of Biomedical Informatics*, **39**, 86-102.
856  
857  Pochon X, Putnam HM, Gates RD (2014) Multi-gene analysis of *Symbiodinium* dinoflagellates: a perspective on rarity, symbiosis, and evolution. *PeerJ*, **2**, e394.
858  
859  Pochon X, Pawlowski J, Zaninetti L, Rowan R (2001) High genetic diversity and relative specificity among *Symbiodinium*-like endosymbiotic dinoflagellates in soritid foraminiferans. *Marine Biology*, **139**, 1069-1078.
860  
861  Pochon X, Montoya-Burgos JI, Stadelmann B, Pawlowski J (2006) Molecular phylogeny, evolutionary rates, and divergence timing of the symbiotic dinoflagellate genus *Symbiodinium*. *Molecular Phylogenetics and Evolution*, **38**, 20-30.
862  
863  Pochon X, Putnam HM, Burki F, Gates RD (2012) Identifying and characterizing alternative molecular markers for the symbiotic and free-living dinoflagellate genus *Symbiodinium*. *PLoS One*, **7**, e29816.
864  
865  Pollard DA, Iyer VN, Moses AM, Eisen MB (2006) Widespread discordance of gene trees with species tree in *Drosophila*: evidence for incomplete lineage sorting. *PLoS Genetics*, **2**, e173.
866  
867  Rhymer JM, Simberloff D (1996) Extinction by hybridization and introgression. *Annual Review of Ecology and Systematics*, **27**, 83-109.
868  
869  Rieseberg LH, Whitton J, Linder CR (1996) Molecular marker incongruence in plant hybrid zones and phylogenetic trees. *Acta Botanica Neerlandica*, **45**, 243-262.
870  
871  Robinson DA, Monk AB, Cooper JE, Feil EJ, Enright MC (2005) Evolutionary genetics of the accessory gene regulator (agr) locus in *Staphylococcus aureus*. *Journal of Bacteriology*, **187**, 8312-8321.
872  
873  Rodriguez RJ, White JF, Arnold AE, Redman RS (2009) Fungal endophytes: diversity and functional roles. *New Phytologist*, **182**, 314-330.
874  
875  Rohland N, Reich D (2012) Cost-effective, high-throughput DNA sequencing libraries for multiplexed target capture. *Genome Res*, **22**, 939-946.
876  
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893
Rybalka N, Wolf M, Andersen RA, Friedl T (2013) Congruence of chloroplast-and nuclear-encoded DNA sequence variations used to assess species boundaries in the soil microalga Heterococcus (Stramenopiles, Xanthophyceae). BMC Evolutionary Biology, 13, 39.

Sampayo EM, Dove S, LaJeunesse TC (2009) Cohesive molecular genetic data delineate species diversity in the dinoflagellate genus Symbiodinium. Molecular Ecology, 18, 500-519.

Sang T, Zhong Y (2000) Testing hybridization hypotheses based on incongruent gene trees. Systematic Biology, 49, 422-434.

Santos SR, Coffroth MA (2003) Molecular genetic evidence that dinoflagellates belonging to the genus Symbiodinium Freudenthal are haploid. The Biological Bulletin, 204, 10-20.

Santos SR, Taylor DJ, Coffroth MA (2001) Genetic comparisons of freshly isolated versus cultured symbiotic dinoflagellates: implications for extrapolating to the intact symbiosis. Journal of Phycology, 37, 900-912.

Santos SR, Shearer TL, Hannes AR, Coffroth MA (2004) Fine-scale diversity and specificity in the most prevalent lineage of symbiotic dinoflagellates (Symbiodinium, Dinophyceae) of the Caribbean. Molecular Ecology, 13, 459-469.

Schardl CL, Leuchtmann A, Tsai HF, Collett MA, Watt DM, Scott DB (1994) Origin of a fungal symbiont of perennial ryegrass by interspecific hybridization of a mutualist with the ryegrass choke pathogen, Epichloe typhina. Genetics, 136, 1307-1317.

Shimodaira H (2002) An approximately unbiased test of phylogenetic tree selection. Systematic Biology, 51, 492-508.

Shimodaira H, Hasegawa M (1999) Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Molecular Biology and Evolution, 16, 1114-1116.

Shymanovich T, Charlton ND, Musso AM, Scheerer J, Cech NB, Faeth SH, Young CA (2017) Interspecific and intraspecific hybrid Epichloë species symbiotic with the North American native grass Poa alsodes. Mycologia, 109, 459-474.

Som A (2014) Causes, consequences and solutions of phylogenetic incongruence. Briefings in Bioinformatics, 16, 536-548.

Sota T, Vogler AP (2001) Incongruence of mitochondrial and nuclear gene trees in the carabid beetles Ohomopterus. Systematic Biology, 50, 39-59.
Stat M, Pochon X, Cowie RO, Gates RD (2009) Specificity in communities of Symbiodinium in corals from Johnston Atoll. *Marine Ecology Progress Series*, **386**, 83-96.

Strimmer K, Rambaut A (2002) Inferring confidence sets of possibly misspecified gene trees. *Proceedings of the Royal Society of London B: Biological Sciences*, **269**, 137-142.

Sun M, Soltis DE, Soltis PS, Zhu X, Burleigh JG, Chen Z (2015) Deep phylogenetic incongruence in the angiosperm clade Rosidae. *Molecular Phylogenetics and Evolution*, **83**, 156-166.

Swofford DL (2002) PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.0a161. Sinauer Associates, Sunderland, Massachusetts

Thornhill DJ, LaJeunesse TC, Santos SR (2007) Measuring rDNA diversity in eukaryotic microbial systems: how intragenomic variation, pseudogenes, and PCR artifacts confound biodiversity estimates. *Molecular Ecology*, **16**, 5326-5340.

Thornhill DJ, Lewis AM, Wham DC, LaJeunesse TC (2014) Host-specialist lineages dominate the adaptive radiation of reef coral endosymbionts. *Evolution*, **68**, 352-367.

Thornhill DJ, Howells EJ, Wham DC, Steury TD, Santos SR (2017) Population genetics of reef coral endosymbionts (*Symbiodinium*, Dinophyceae). *Molecular Ecology*, **26**, 2640-2659.

Trench RK (1997) Diversity of symbiotic dinoflagellates and the evolution of microalgal-invertebrate symbioses. In *Proceedings of the 8th International Coral Reef Symposium*, **2**, 1275-1286.

Tsai HF, Liu JS, Staben C, Christensen MJ, Latch GC, Siegel MR, Schardl CL (1994) Evolutionary diversification of fungal endophytes of tall fescue grass by hybridization with *Epichloë* species. *Proceedings of the National Academy of Sciences*, **91**, 2542-2546.

van Oppen MJH, McDonald BJ, Willis B, Miller DJ (2001) The evolutionary history of the coral genus *Acropora* (Scleractinia, Cnidaria) based on a mitochondrial and a nuclear marker: reticulation, incomplete lineage sorting, or morphological convergence?. *Molecular Biology and Evolution*, **18**, 1315-1329.

van Oppen MJH, Oliver JK, Putnam HM, Gates RD (2015) Building coral reef resilience through assisted evolution. *Proceedings of the National Academy of Sciences*, **112**, 2307-2313.
Wilkinson SP, Fisher PL, van Oppen MJH, Davy SK (2015) Intra-genomic variation in symbiotic dinoflagellates: recent divergence or recombination between lineages?, *BMC Evolutionary Biology*, **15**, 46.

Willis BL, van Oppen MJH, Miller DJ, Vollmer SV, Ayre DJ (2006) The role of hybridization in the evolution of reef corals. *Annual Review of Ecology, Evolution, and Systematics*, **37**, 489-517.

Zhang H, Bhattacharya D, Lin S (2005) Phylogeny of dinoflagellates based on mitochondrial cytochrome B and nuclear small subunit rDNA sequence comparisons. *Journal of Phycology*, **41**, 411-420.
Figure 1 (on next page)

Stepwise analyses performed per site to identify incongruences in *Cladocopium*. 
Overall Incongruence Length Difference test

Approximately Unbiased test

Pairwise Incongruence Length Difference tests

Pairwise tanglegram construction

Tree hybridisation analysis

Visual inspection of raw sequence data
**Figure 2**(on next page)

Pairwise tanglegrams for site BHB.

Red branches with bolded taxa labels indicate incongruent samples, as identified by the tree hybridisation analysis (executed in Dendroscope 3.0 [Huson and Linz 2016]). Branch labels are ML bootstrap values (1000 replicates for cob, 100 for ITS2 and psbAncr). (a) cob vs. ITS2: found incongruent by ILD and AU tests. (b) psbAncr vs. ITS2: found incongruent by ILD and AU tests. (c) cob vs. psbAncr: found congruent by ILD and AU tests.
Figure 3 (on next page)

Short selections of raw sequence data for incongruent sample BHB146 and related sequences (polymorphisms in bold).

In organellar gene regions (a) and (c), BHB146 groups with samples BHB104 and BHB105 (Cladocopium type C42a, C1v). In the nuclear gene region (b), BHB groups with BHB122 and BHB149 (Cladocopium type C1#).
(a) *cob*

| Sample  | Sequence          |
|---------|-------------------|
| BHB104  | GGGGAAGTAC ... CTT |
| BHB105  | GGGGAAGTAC ... CTT |
| BHB122  | GGGGGGATAC ... TTGTT |
| BHB146  | GGGGAAGTAC ... CTT |
| BHB149  | GGGGGGATAC ... TTGTT |

(b) *ITS2*

| Sample  | Sequence                      |
|---------|-------------------------------|
| BHB104  | AGG---TTTCTACCTTTCGTG         |
| BHB105  | AGG---TTTCTACCTTTCGTG         |
| BHB122  | AGG---TTTCTACCTTTCCGG         |
| BHB146  | AGG---TTTCTACCTTTCCGG         |
| BHB149  | AGG---TTTCTACCTTTCCGG         |

(c) *psbA*^{ncr}

| Sample  | Sequence                                      |
|---------|-----------------------------------------------|
| BHB104  | CCCCTCGGG-GTGCAACAT                          |
| BHB105  | CCCCTCGGG-GTGCAACAT                          |
| BHB122  | CCCGTAGGG-GTACCCCAT                          |
| BHB146  | CCCCTCGGG-GTACCCCAT                          |
| BHB149  | CCCGTAGGG-GTACCCCAT                          |
Pairwise tanglegrams for site BSP.

Red branches with bolded taxa labels indicate incongruent samples, as identified by the tree hybridisation analyses (executed in Dendroscope 3.0 [Huson and Linz 2016]). Branch labels are ML bootstrap values (1000 replicates for cob, 100 for ITS2 and psbAncr). (a) cob vs. ITS2: found incongruent by ILD and AU tests. (b) psbAncr vs. ITS2: found incongruent by ILD and AU tests. (c) cob vs. psbAncr: found congruent by ILD and AU tests.
Short selections of raw sequence data for incongruent sample BSP343 and related sequences (polymorphisms in bold).

(a) In the nuclear ITS2 region, BSP343 groups with samples BSP383 and BSP387; point mutations at base pairs 23 and 238 (available in Data Availability) identify it as *Cladocopium* type C3u. (b) In the organellar psbAncr region, BSP343 groups with BSP386 and BSP388, as a variant of *Cladocopium* type C40. The cob gene was invariant in this case.
(a) ITS2

\[
\begin{array}{c|c|c|c|c}
\text{BSP343} & \text{TG-CGCGC} & \ldots & \text{CGCT} \\
\text{BSP383} & \text{TG-CGCGC} & \ldots & \text{CGCT} \\
\text{BSP386} & \text{TG-TGCAGC} & \ldots & \text{CTGCT} \\
\text{BSP387} & \text{TG-CGCGC} & \ldots & \text{CGCT} \\
\text{BSP388} & \text{TG-TGCAGC} & \ldots & \text{CTGCT} \\
\end{array}
\]

(b) psbA\text{ncr}

\[
\begin{array}{c|c|c|c|c}
\text{BSP343} & \text{ATGCC-CCACA-GGGGGCAT} \\
\text{BSP383} & \text{ACACC-CCGGA-GGGGTGT} \\
\text{BSP386} & \text{ATGCC-CCACA-GGGGGCAT} \\
\text{BSP387} & \text{ACACC-CCGGA-GGGGTGT} \\
\text{BSP388} & \text{ATGCC-CCACA-GGGGGCAT} \\
\end{array}
\]
Short selections of raw sequence data for incongruent sample BSP364 and related sequences (polymorphisms in bold).

(a) In the nuclear ITS2 region, BSP364 groups with samples BSP362 and BSP373 (*Cladocopium* type C3z). (b) In the organellar *psbA* region, BSP364 groups with BSP344 and BSP366, as a variant of *Cladocopium* type C40. The *cob* gene was invariant in this case.
(a) ITS2
BSP344   TGCTTTCGACCGTGG
BSP362   TGCTTTCGACCTGG
BSP364   TGCTTTCGACCTGG
BSP366   TGCTTTCGACCGTGG
BSP373   TGCTTTCGACCTGG

(b) psbA\text{ncr}
BSP344   ATG-AAAAAGAAAAAAGA
BSP362   ACGAAAAAGAAAAATAGA
BSP364   ATG-AAAAAGAAAAAAGA
BSP366   ATG-AAAAAGAAAAAAGA
BSP373   ACGAAAAAGAAAAATAGA
Figure 7 (on next page)

Average proportions of different background sequence populations for samples identified as C3z (8 samples) and C40 (13 samples) from site BSP.

(a) For C3z population, proportion of sequences that were C3z vs. non-C3z sequences (i.e. background sequences), averaged over all 8 samples. (b) Average proportion of background sequences for C3z samples, identified using the database of Franklin et al. 2012 and GenBank BLAST search. Sequences followed by stars indicate novel sequences and are named according to their most closely related sequence in the databases. Parentheses indicate the total number of samples (out of 8) that the background sequence appeared in. Rare sequences (<3% average individual abundance) are clustered together. The red box indicates the background sequence with the organellar identity of the putative hybrid (BSP364). (c) For C40 population, proportion of sequences that were C40 vs. non-C40 sequences (i.e. background sequences), averaged over all 13 samples. (d) Average proportion of background sequences for C40 samples. Parentheses indicate the total number of samples (out of 13) that the background sequence appeared in. The red box indicates the background sequence with the nuclear identity of the putative hybrid. Other details as per (b).
Background Sequences

(a) C3z (n=8)

(b) C3 (7)

(c) C40 (n=13)

(d) C3 (12)

Low-abundance sequences

C3z** (7)
C3z* (7)
C3d/C21* (1)
C3d/C21 (1)
C3.10 (7)
C3* (4)
C3 (12)
C115 (12)
C40 (1)

Manuscript to be reviewed
Predictions under Incomplete Lineage Sorting.

(a) General pattern expected for ILS. A single ancestral population with polymorphism in both the psbA\textsuperscript{ncr} and ITS2 regions is present before a speciation event. After speciation, the ITS2 polymorphism fails to segregate, while through stochastic processes the C40 polymorphism is eliminated and leads to incongruence between nuclear and chloroplast genes. (b) The process of ILS that would be required for this example. The ITS2 region fails to segregate after speciation; despite the extensive presence of C40 alleles, a small subpopulation of symbionts with dominant C3z alleles is maintained (weak dashed blue line) in the C40 population and both are recovered in present-day sampling, at the same site, as pure C3z populations.
Speciation Event

(a) (b)
Table 1 (on next page)

Results of the Approximately Unbiased (AU) tests.

P-values presented are whether a candidate tree is statistically differentiable from the best tree. Statistical significance is designated by * (conclusions drawn at Bonferroni-corrected $\alpha = 0.0085$); p-values likely due to type I error are designated by º (see Discussion).
Table 1: Results of the Approximately Unbiased (AU) tests. P-values presented are whether a candidate tree is statistically differentiable from the best tree. Statistical significance is designated by * (conclusions drawn at Bonferroni-corrected $\alpha = 0.0085$); p-values likely due to type I error are designated by ° (see Discussion).

| Dataset | Gene region used for test | Best tree | Tree to compare with best tree | AU p-value |
|---------|---------------------------|-----------|-------------------------------|------------|
| BBR     | cob                       | psbA<sub>ncr</sub> | ITS2 | <0.0001° |
|         |                           |           | cob                           | 0.4417     |
| ITS2    | ITS2                      | psbA<sub>ncr</sub> | cob | 0.4056 |
|         |                           |           | psbA<sub>ncr</sub> | 0.7712     |
| psbA<sub>ncr</sub> | psbA<sub>ncr</sub> | cob            | <0.0001° |
|         |                           |           | ITS2 | <0.0001° |
| BHB     | cob                       | cob       | ITS2 | <0.0001° |
|         |                           |           | psbA<sub>ncr</sub> | 0.5631     |
| ITS2    | ITS2                      | cob       | psbA<sub>ncr</sub> | <0.0001° |
|         |                           |           | psbA<sub>ncr</sub> | <0.0001° |
| psbA<sub>ncr</sub> | psbA<sub>ncr</sub> | cob | <0.0001° |
|         |                           |           | ITS2 | <0.0001° |
| BLS     | cob                       | cob       | ITS2 | <0.0001° |
|         |                           |           | psbA<sub>ncr</sub> | 0.3456     |
| ITS2    | ITS2                      | cob       | psbA<sub>ncr</sub> | 0.4163     |
|         |                           |           | psbA<sub>ncr</sub> | 0.1806     |
| psbA<sub>ncr</sub> | psbA<sub>ncr</sub> | cob | <0.0001° |
|         |                           |           | ITS2 | <0.0001° |
| BSP     | cob                       | cob       | ITS2 | 0.0493 |
|         |                           |           | psbA<sub>ncr</sub> | <0.0001° |
| ITS2    | ITS2                      | cob       | psbA<sub>ncr</sub> | <0.0001° |
|         |                           |           | psbA<sub>ncr</sub> | <0.0001° |
| psbA<sub>ncr</sub> | psbA<sub>ncr</sub> | cob | <0.0001° |
|         |                           |           | ITS2 | <0.0001° |
| HEW     | cob                       | psbA<sub>ncr</sub> | ITS2 | 0.1562 |
|         |                           |           | psbA<sub>ncr</sub> | 0.1562     |
| ITS2    | ITS2                      | cob       | psbA<sub>ncr</sub> | 0.5465     |
|         |                           |           | psbA<sub>ncr</sub> | 0.5465     |
| psbA<sub>ncr</sub> | psbA<sub>ncr</sub> | cob | <0.0001° |
|         |                           |           | ITS2 | <0.0001° |
| LIE     | cob                       | ITS2      | cob | 0.0183 |
|         |                           |           | psbA<sub>ncr</sub> | 0.2336     |
| ITS2    | ITS2                      | cob       | psbA<sub>ncr</sub> | 0.4727     |
|         |                           |           | psbA<sub>ncr</sub> | <0.0001° |
| psbA<sub>ncr</sub> | psbA<sub>ncr</sub> | cob | <0.0001° |
|         |                           |           | ITS2 | <0.0001° |
| LIW     | cob                       | cob       | ITS2 | 0.0409 |
|         |                           |           | psbA<sub>ncr</sub> | 0.0811     |
| ITS2    | ITS2                      | cob       | psbA<sub>ncr</sub> | 0.2490     |
|         |                           |           | psbA<sub>ncr</sub> | 0.6638     |
| psbA<sub>ncr</sub> | psbA<sub>ncr</sub> | cob | <0.0001° |
|         |                           |           | ITS2 | <0.0001° |
|     | cob | cob | ITS2   | psbA<sup>ncr</sup> | 0.0125 |
|-----|-----|-----|--------|---------------------|--------|
| Atauro |     |     |        | cob                 | <0.0001* |
|       | ITS2 | ITS2|        | psbA<sup>ncr</sup> | <0.0001* |
|       | psbA<sub>ncr</sub> | psbA<sub>ncr</sub> |        | cob                 | <0.0001° |

|     | cob | cob | ITS2   | psbA<sup>ncr</sup> | 0.5604 |
|-----|-----|-----|--------|---------------------|--------|
| Timor |     |     |        | cob                 | <0.0001* |
|       | ITS2 | ITS2|        | psbA<sup>ncr</sup> | 0.0935 |
|       | psbA<sub>ncr</sub> | psbA   |        | cob                 | <0.0001° |
|       |     |     |        | ITS2                 | <0.0001° |
Table 2 (on next page)

Results of pairwise Incongruence Length Difference tests.

Conclusions were drawn at a Bonferroni-corrected $\alpha=0.017$. Statistical significance is designated by *. 

Table 2: Results of pairwise Incongruence Length Difference tests. Conclusions were drawn at a Bonferroni-corrected $\alpha=0.017$. Statistical significance is designated by *.

| Dataset | Partitions tested | p-value |
|---------|-------------------|---------|
| BHB     | cob vs. ITS2      | 0.006*  |
|         | cob vs. psbA\textsuperscript{ncr} | 0.847   |
|         | ITS2 vs. psbA\textsuperscript{ncr} | 0.021   |
| BSP     | cob vs. ITS2      | 0.011*  |
|         | cob vs. psbA\textsuperscript{ncr} | 0.223   |
|         | ITS2 vs. psbA\textsuperscript{ncr} | 0.001*  |
| Atauro  | cob vs. ITS2      | 0.01*   |
|         | cob vs. psbA\textsuperscript{ncr} | 1       |
|         | ITS2 vs. psbA\textsuperscript{ncr} | 0.01*   |
| BLS     | cob vs. ITS2      | 1       |
|         | cob vs. psbA\textsuperscript{ncr} | 1       |
|         | ITS2 vs. psbA\textsuperscript{ncr} | 0.778   |
| LIW     | cob vs. ITS2      | 1       |
|         | cob vs. psbA\textsuperscript{ncr} | 1       |
|         | ITS2 vs. psbA\textsuperscript{ncr} | 1       |
Table 3 (on next page)

Summary of incongruent samples inferred from tanglegrams and tree hybridisation analyses.

Bolded samples are those verified to be incongruent.
Table 3: Summary of incongruent samples inferred from tanglegrams and tree hybridisation analyses. Bolded samples are those verified to be incongruent.

| Dataset | Comparison               | Incongruent Samples |
|---------|--------------------------|---------------------|
| BHB     | cob vs. ITS2             | BHB146              |
|         | psbA<sup>ncf</sup> vs. ITS2 | BHB146, BHB148     |
|         | cob vs. psbA<sup>ncf</sup> | BHB148              |
| BSP     | cob vs. ITS2             | BSP211, BSP358, BSP372 |
|         | psbA<sup>ncf</sup> vs. ITS2 | BSP320, BSP343, BSP364 |
|         | cob vs. psbA<sup>ncf</sup> | BSP358              |
| Atauro  | cob vs. ITS2             | BHB146, BSP372, BSP387 |
|         | psbA<sup>ncf</sup> vs. ITS2 | BHB146, BHB148, BSP343, BSP364, BSP372 |
|         | cob vs. psbA<sup>ncf</sup> | BHB148, BSP372, BSP387 |