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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, 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.

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

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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^{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, 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.

Key Words Approximately Unbiased test, hybridisation, Incomplete Lineage Sorting, Incongruence Length Difference test, Next Generation Sequencing, symbiont

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) increasing attention is being focused on the evolutionary potential within this family.

Coral symbionts have been thought to be nearly exclusively asexual (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 cases, 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 and colleagues (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 and colleagues (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 so this topic warrants further study. The current study aimed to gather further defendable 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 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^{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); methods for acquisition of sequence data can be found within that study. Briefly, a wide range of 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). In addition, three sites were sampled on the northern coast of Timor: HEW (Hera West); LIE (Lamsana Inlet East); and LIW (Lamsana

Inlet West). Symbiont DNA was extracted, and the three gene regions of interest were amplified. The *cob* and psbA^{ncr} were sequenced with traditional Sanger sequencing, while the ITS2 region underwent Next Generation Sequencing (NGS). As the incongruence tests utilised (see below) require a single sequence *per* sample, the most dominant ITS2 sequence from the NGS in each sample was extracted (an 'ASV' in Brian *et al.* 2019). 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^{ncr} sequences. This left between 18 and 28 samples *per* site ($\bar{x}$ = 22.6), with a total of 158 samples used.

Incongruence tests

Ideally, a statistical test would be able to test the null hypothesis 'Dataset X and Dataset Y are congruent', against an alternate hypothesis 'Dataset X and Dataset Y are incongruent.' No such test exists for phylogenetic data, so other tests with slightly different hypotheses have been frequently employed as an approximation. Two of these tests are 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 congruent, 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 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 in-built 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 (*cob*, psbA^{ncr}, 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^{ncr} regions, respectively. In total, 27 separate datasets were assembled. *Durusdinium glynnii* (D1) was used as the outgroup for these analyses (GenBank Accession Numbers: KY131780 (*cob*); JN558075 (ITS2); MH329571 (psbA^{ncr})). 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^{ncr} 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 $\alpha = 0.05$.

Approximately Unbiased (AU) tests

Maximum likelihood trees were generated for all gene regions by individual site (all possible combinations of {*cob*, psbA^{ncr}, ITS2} and {BBR, BHB, BLS, BSP, HEW, LIE, LIW} *i.e.* 21 different trees). Trees were also made for each gene region for Atauro Island and Timor datasets (*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 `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 PAUP* defaults. *cob* datasets had 1000 bootstrap replicates, while the ITS2 and psbA^{ncr} datasets had 100 replicates. In addition, for the psbA^{ncr} datasets, the number of addition sequence replicates was set to 2 (*versus* the default of 10). The exception is the Atauro Island and Timor datasets, which had 1000 replicates using the `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 `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 used as the base, and all three trees (from the *cob* BBR, ITS2 BBR and psbA^{ncr} BBR alignments) were compared with the AU test. This was then repeated using the ITS2 BBR and psbA^{ncr} BBR alignments as bases to compare the same three trees. 10000 RELL 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 ($\alpha = 0.0085$).

Post hoc *analyses*

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^{ncr}; ITS2 *vs.* psbA^{ncr}). 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.

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.

In an effort to verify the conclusions subsequently drawn, the *actin* gene (symbiont nuclear DNA) was sequenced for putative hybrid samples and closely related samples. 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 H_2O 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

297 correct length (~900 bp) were excised with a pipette tip and reamplified using 20 cycles of the
298 above conditions. Prior to sequencing, the samples were purified with MagNA PCR clean-up
299 solution (0.1% carboxyl-modified Sera-Mag Magnetic Speed-Beads (Fisher Scientific), 18% w/v
300 PEG-8000, 1 M NaCl, 10 mM Tris–HCl, 1 mM EDTA, 0.05% Tween 20, pH 8.0; Rohland and
301 Reich 2012).
302

303 **Results**
304

305 *Incongruence Length Difference tests*
306

307 The Timor sites showed no evidence of incongruence among the *cob*, psbA^{ncr} and ITS2 gene
308 regions, either when considered all together or as separate sites (ILD test, p = 1 for all). This p-
309 value is not concerning; it simply indicates that among the replicates, the partitions were never
310 more parsimonious than random partitions. The Atauro dataset as a whole did not show statistically
311 substantiated evidence of incongruence, though it approached significance (p = 0.0874). In this
312 case, it is valid to use the term 'approaching significance', as the test statistic is directly correlated
313 to the number of replicates for which the original partitions were found to vary from random data
314 (Planet 2006). Looking at each Atauro site individually, BBR and BLS were strongly congruent
315 (p = 0.99), while BHB displayed an equivocal result (p = 0.129) and BSP was strongly incongruent
316 between partitions (p = 0.001). However, these tests on three partitions could not identify where
317 potential incongruences were located.
318

319 *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^{ncr} or ITS2 trees were chosen as the best explanation of the *cob* dataset. The test always found incongruence when using the psbA^{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 showed no incongruence for either the *cob* or ITS2 gene regions; all three trees (*cob*, ITS2, psbA^{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^{ncr} tree) made from all Atauro samples was incongruent with the *cob* dataset, and both the *cob* and psbA^{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^{ncr}) and nuclear (ITS2) gene regions. In all cases, the AU test was unable to reject congruence between the *cob* and psbA^{ncr} regions. However, it did reject congruence between the ITS2 and psbA^{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 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 congruent when considered together. Site LIW is strongly congruent at all regions. This shows that these two-way tests are functioning as expected. BLS is also strongly congruent; 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

366 divergent psbA^{ncr} sequence, and does not show a reticulate pattern (Fig. S1). For the organellar
367 gene regions, BHB146 belongs to the *Cladocopium* C1 radiation (symbiont types C42a and C1v
368 respectively, see Brian *et al.* 2019). For the ITS2 region, it is identified as type C1#, which groups
369 more closely with the *Cladocopium* C3 radiation.
370
371 The BSP tanglegrams (Fig. 4) also support the statistical analyses, with six potentially incongruent
372 samples identified. After inspection of the raw sequence data, four of these were disregarded
373 (BSP211, BSP320, BSP372, BSP387), as they were more likely caused by parsimony-
374 uninformative mutations in a single sequence (Fig. S2). However, two samples were verified as
375 incongruent (BSP343 and BSP364, Figs. 5 and 6). BSP364 belongs to two different previously
376 defined subclades: a variant of *Cladocopium* type C40 for psbA^{ncr}, and type C3z for ITS2. BSP343
377 also shows clear incongruence between the organellar and nuclear genes regions. The psbA^{ncr} is a
378 variant of *Cladocopium* type C40, which groups it most closely with the psbA^{ncr} C3z clade (Fig.
379 4b), while the ITS2 region features type C3u, which places it as distinct from both the C3z and
380 C40 groups.
381
382 Pairwise tanglegrams and hybridisation analyses were also executed for the whole Atauro Island
383 dataset (sites BBR, BHB, BLS, BSP). Despite the inclusion of two more sites, the analyses showed
384 that incongruence was caused by exactly the same samples as found by the individual site analyses,
385 affirming BHB and BSP as sites with incongruent samples. Further, no other sites contributed any
386 incongruent samples. The overall results are presented in Table 3, which demonstrates that ITS2
387 comparisons displayed the most incongruence, while any incongruences between *cob* and psbA^{ncr}
388 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).

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^{ncr} region. The psbA^{ncr} region is highly variable, 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^{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

412 reject congruence between the *cob* and psbA^{ncr} regions for sites BSP and BHB despite the tree
413 hybridisation analyses finding potential incongruence (Fig. 2c, 4c), and so appears to be reasonably
414 conservative in this case. The results of the AU and ILD tests are also compelling because they are
415 differential: they show consistently different patterns between datasets and are therefore likely
416 responding to genuine phylogenetic signals. This was confirmed by looking at the raw sequence
417 data, and shows the efficacy of the approach taken here. With such a wide range of samples,
418 initially searching for incongruences in sequence data would be functionally impossible, as it
419 would require comparing all possible combinations of sequences (in this study, this would require
420 2.95×10^{282} comparisons). However, the stepwise use of analyses allowed the initial
421 identification of which sites may host incongruent samples, and then visualisation on phylogenetic
422 trees allowed simple alignments of appropriate samples to be generated, where incongruence could
423 clearly be refuted or confirmed. In addition, given the issues with tests in isolation, the multiplicity
424 of analyses used generates a far more convincing picture of reticulate evolution.
425
426 *Hybridisation in* Cladocopium*?*
427
428 Incongruence was comprehensively established for the samples BHB146, BSP343 and BSP364.
429 However, this does not necessarily translate to hybridisation, as there are a range of analytical or
430 biological factors that can cause incongruence in phylogenetic data. For example, one
431 hypothesised to be quite common but insidious in its undetectable nature is heterotachy, shifts in
432 site-specific evolutionary rates through time (Som 2014). While there is no particular way to
433 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 (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

457 Zhong 2000; Joly *et al.* 2009). Therefore, ILS as a cause of the observed incongruence cannot be
458 statistically refuted. However, there is good evidence that the patterns observed here are more
459 likely to be caused by symbiont hybridisation.
460
461 First, the pattern of incongruence observed, with organellar cytoplasmic genes being different to
462 nuclear genes, accords with a large body of prior theory on hybridisation. Nuclear genes are largely
463 inherited biparentally, and the ITS2 region is no exception (Baldwin *et al.* 1995; Rybalka *et al.*
464 2013). However, the cytoplasm tends to be inherited maternally (Rieseberg *et al.* 1996). This
465 difference is largely due to gametogenesis and fertilisation, where the male gamete typically only
466 contains nuclear information, while the female gamete (egg) contains the cytoplasm that will be
467 passed on to the zygote. Therefore, if an organism encounters a population of another species and
468 produces viable hybrids, theory predicts that over time, repeated backcrossing with the more
469 common species (introgression) will produce hybrids with divergent organellar and nuclear
470 signals. While the nature of the sexual life cycle has yet to be fully elucidated in the
471 Symbiodiniaceae, previous evidence has shown that other unicellular dinoflagellates produce
472 gametes (Brawley and Johnson 1992). In addition, the presence of 'plus' and 'minus' mating types,
473 analogous to gender, has been shown in the dinoflagellate *Alexandrium tamarense* (Brosnahan
474 2011). Therefore, it is reasonable to assume that Symbiodiniaceae also produce distinct gametes
475 (as opposed to conducting sex *via* fusion, for example), making this mechanism eminently
476 plausible. The documentation of functional meiotic genes in Symbiodiniaceae (Chi *et al.* 2014,
477 Levin *et al.* 2016) supports this assertion. Such a pattern of discordance between cytoplasmic and
478 nuclear genes caused by hybridisation has been recorded for taxa as diverse as plants (Rieseberg
479 *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 gene 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. Further, the psbA^{ncr} region can also be intragenomically variable (LaJeunesse and Thornhill 2011); it may be expected then that this would also cause occasional incongruences with the *cob* gene, something that was not observed.

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^{ncr} region, and C3z for the ITS2 region. Significantly, there were also samples recovered which were type C40 for both the psbA^{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^{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. 7. Only the psbA^{ncr} and ITS2 genes are presented, as the *cob* gene was invariant in this case.

Fig. 7b 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 (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. 7a). 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 an analysis of the background symbiont populations, identified *via* NGS. At site BSP, there were eight additional samples with C3z as the dominant ITS2 sequence (that were also C3z for psbA^{ncr} and *cob* regions), and thirteen with type C40 dominant (also with congruent cytoplasmic sequences). Of those eight C3z samples, seven had no C40 sequences in their genomes, while one had C40 traces at a frequency of 0.61%. Similarly, there were almost no traces of C3z sequences in the thirteen C40 samples (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#) did not occur frequently enough to conduct a similar analysis, 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 reasonably 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 that *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,

548 both these sites have been shown to be rich in Symbiodiniaceae diversity, when compared with
549 the Timor sites (Brian *et al.* 2019). This suggests that putative hybridisation may be limited to
550 high-quality sites that maintain high levels of symbiont diversity.
551
552 *Previous tests of incongruence*
553
554 No previous study on Symbiodiniaceae seriously considers symbiont hybridisation, except that of
555 Wilkinson and colleagues (2015), which also finds evidence for its existence. However, aside from
556 the potential examples of hybridisation mentioned in the Introduction of this study (LaJeunesse *et*
557 *al.* 2003, 2004; LaJeunesse 2005), three other studies bear mention. Sampayo *et al.* (2009) also
558 focused on the basis that hybridisation can cause incongruence between genes from different
559 organelles, and built trees from mitochondrial, chloroplast and rDNA nuclear gene regions to test
560 this. Based on visual inspection of these trees, they concluded that different symbiont lineages
561 (types) within *Cladocopium* are reproductively isolated. Interestingly, they did also use the ILD
562 test to formally test incongruence, which returned a p-value of 0.01, though this result was not
563 explored further. Pochon and colleagues (2014) assessed six genes from three different organelles
564 (mitochondrion, nucleus and chloroplast). In all cases, they found evidence of incongruence
565 between pairwise comparisons of genes, using the AU test. While they go on to discuss the
566 implications for concatenation in some detail, the cause of these incongruences was likewise not
567 explored further. Another study from Pochon *et al.* (2006) found the surprising result of
568 incongruence between whole genera rendered from nr28S and cp23S data, using the Shimodaira-
569 Hasegawa test. However, when they removed all but two members of each clade, the test then
570 showed congruence between datasets. This indicated incongruence was being caused by the

accumulation of within-clade mismatches between the nucleus and chloroplasts, something which is also broadly agreeable with a hypothesis of hybridisation. 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 mechanism for adaptive change in *Cladocopium*, and potentially for Symbiodiniaceae in general. 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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Data Accessibility

All novel sequences obtained during sequencing have been deposited in GenBank. The original data from Brian *et al.* 2019 have the following accession numbers: *cob* gene MH236749 – MH236764; ITS2 region MH236765 – MH236784; psbA^{ncr} region MH329431 – MH329571. The novel *actin* gene sequences have been deposited with accession numbers MK520897 – MK520906. Datasets used in the ILD and AU tests, in addition to annotated exemplar code to run both analyses, are available online at https://github.com/brianjosh/Cladocopium_alignments.

Figure 1(on next page)

Stepwise analyses performed *per* site to identify incongruences in *Cladocopium*.

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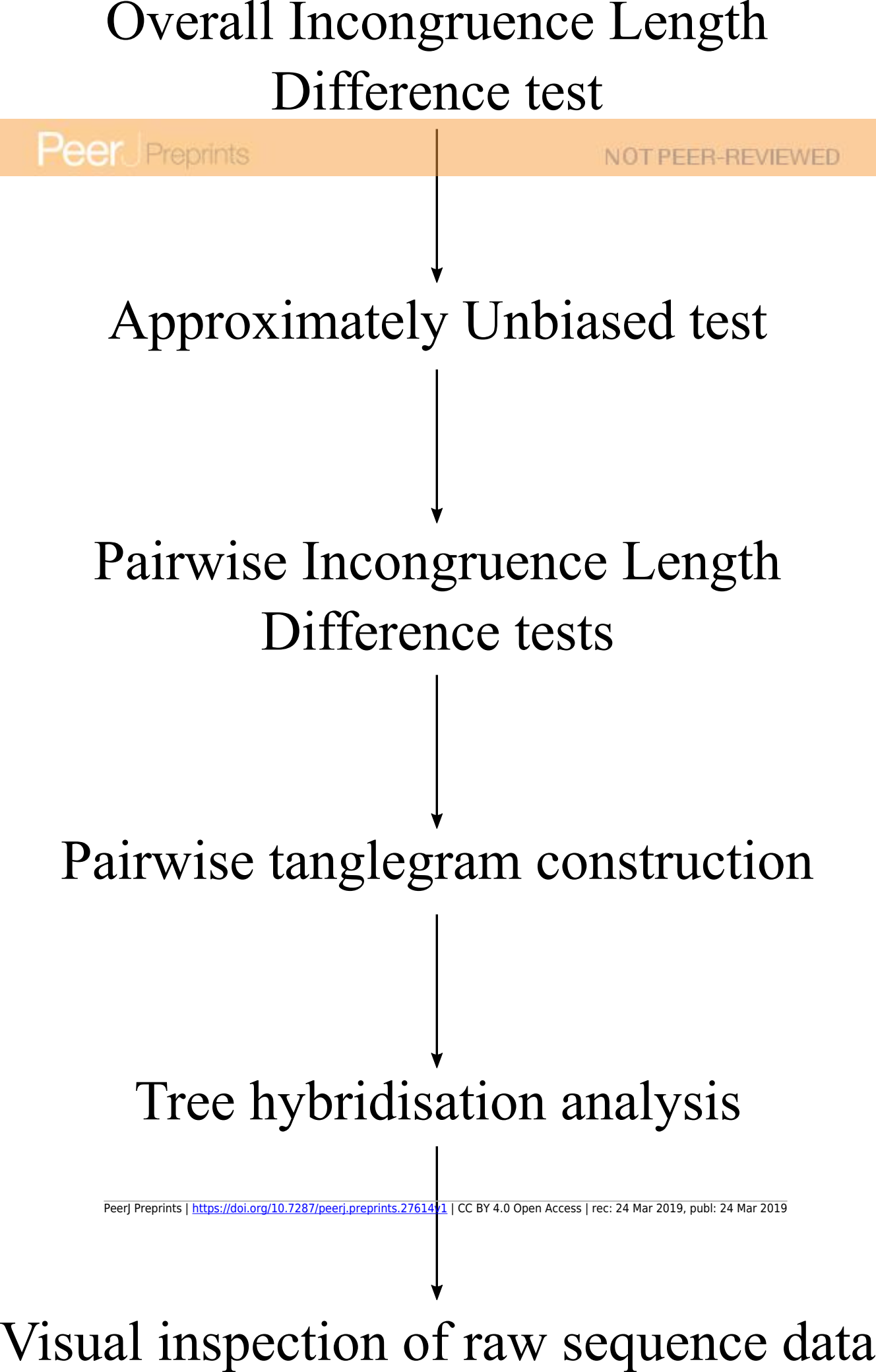

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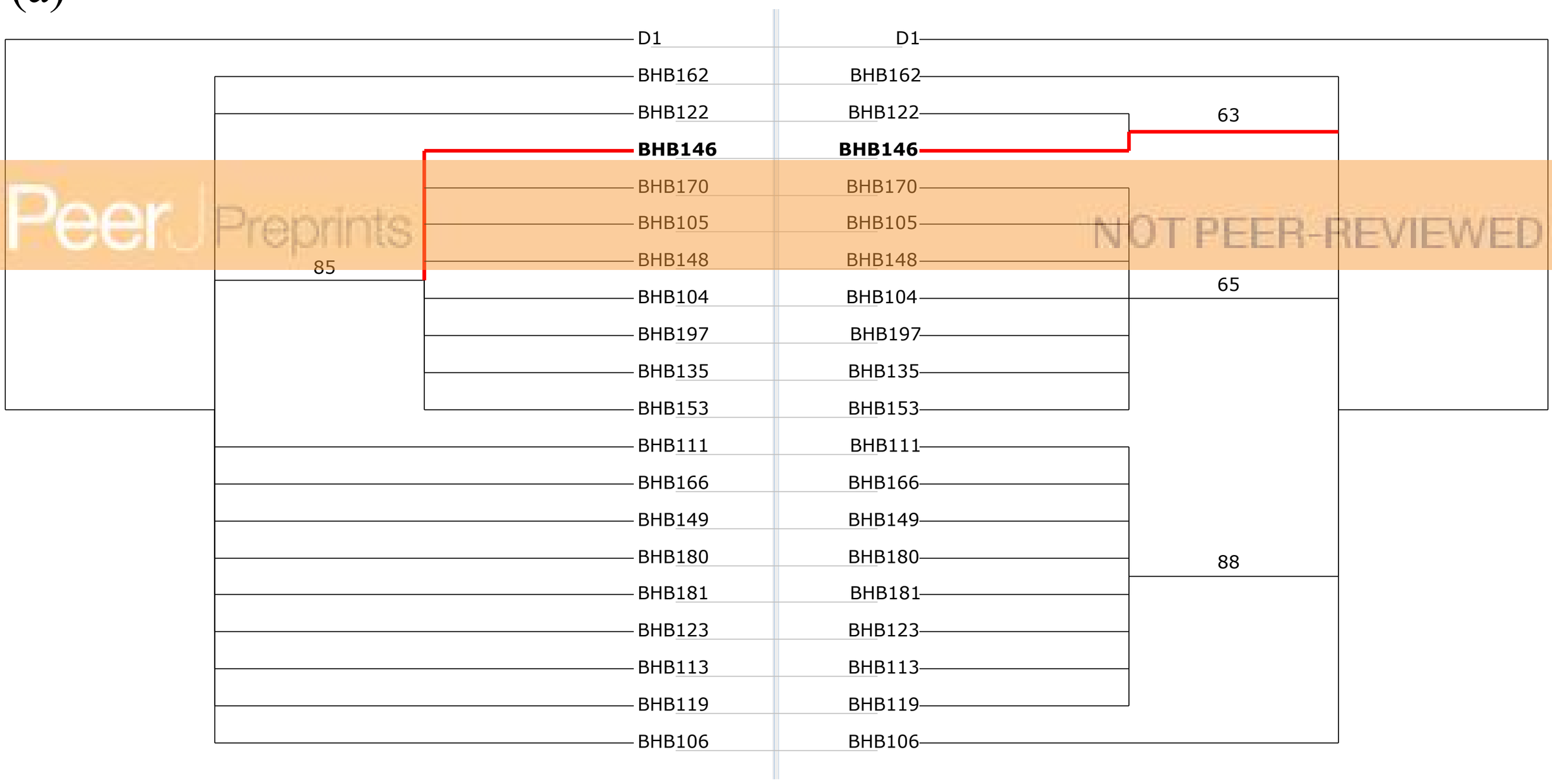
Figure 2(on next page)

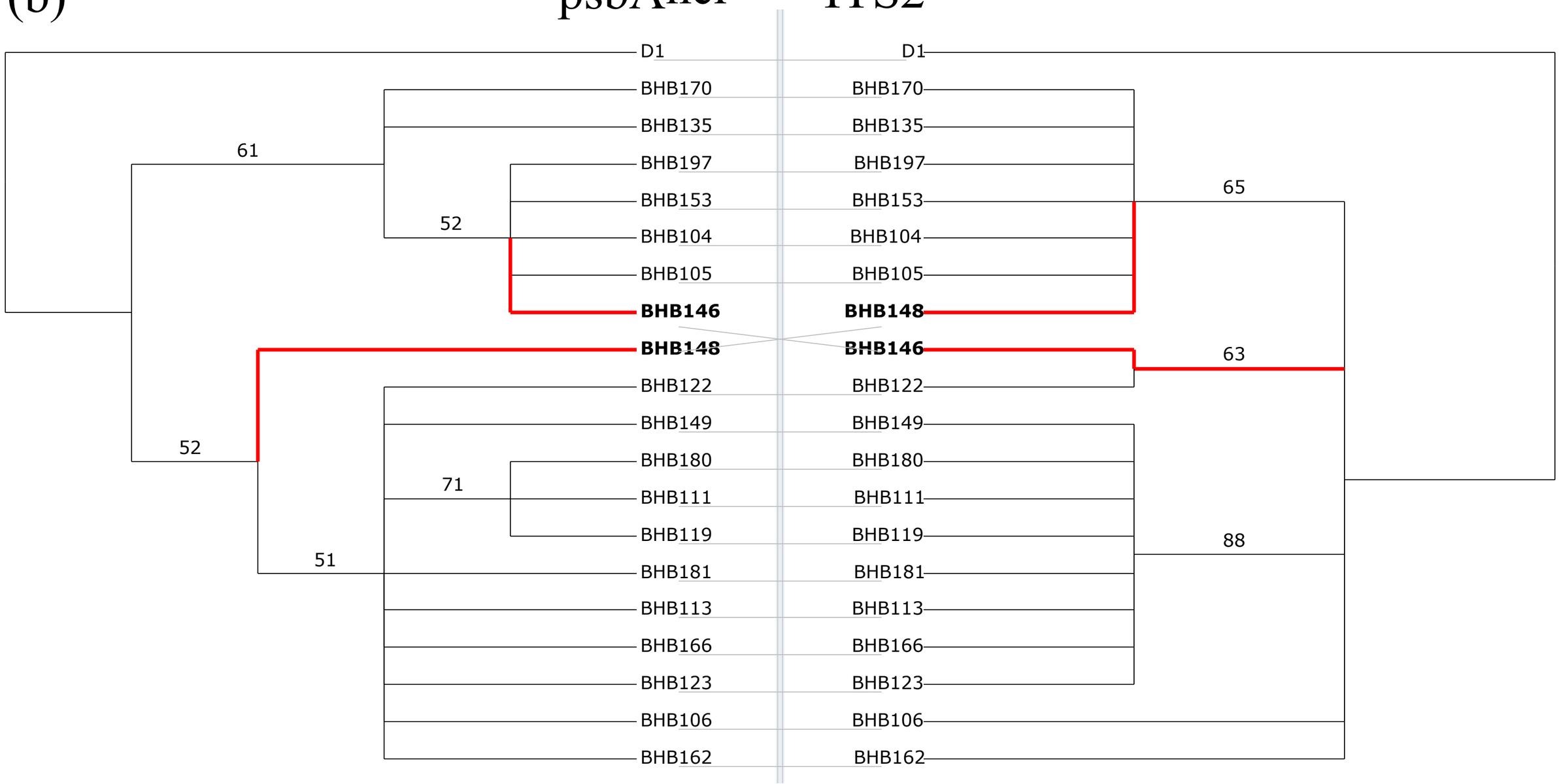
Pairwise tanglegrams for site BHB.

Red branches with bolded taxa labels indicate incongruent samples, as identified by the tree hybridisation analysis (Huson and Linz 2016). Branch labels are ML bootstrap values (1000 replicates for *cob*, 100 for ITS2 and psbA^{ncr}). (a) *cob vs.* ITS2: found incongruent by ILD and AU tests. (b) psbA^{ncr} *vs.* ITS2: found incongruent by ILD and AU tests. (c) *cob vs.* psbA^{ncr}: found congruent by ILD and AU tests.

(a)



(b)



(c)

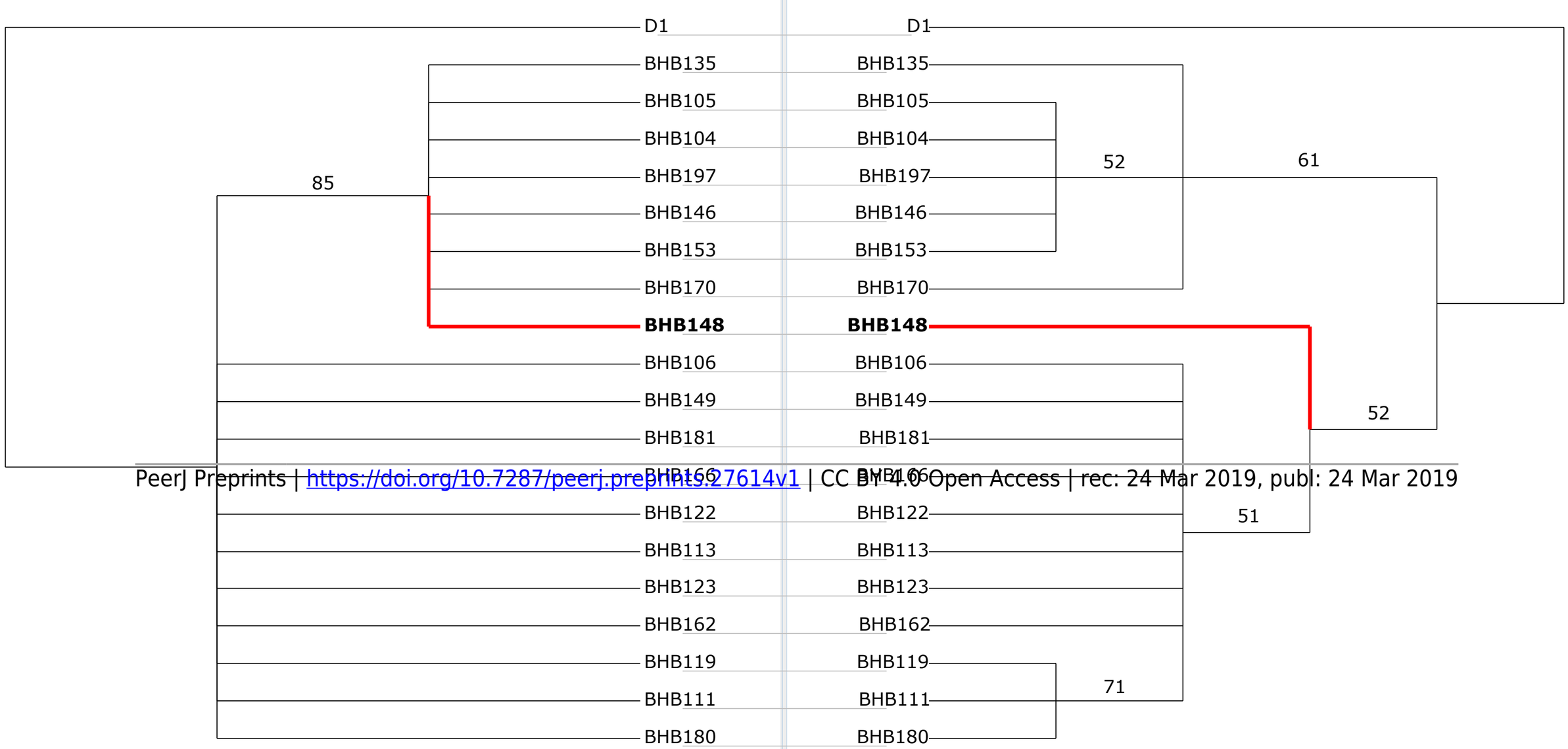


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Figure 3(on next page)

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#).

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(a) *cob*

```
                310       315                455
BHB104  GGGAGTAC  ...  TTCTT
BHB105  GGGAGTAC  ...  TTCTT
BHB122  GGGGGTAC  ...  TTGTT
BHB146  GGGAGTAC  ...  TTCTT
BHB149  GGGGGTAC  ...  TTGTT
```

(b) ITS2

```
                    225       230       235
BHB104  AGG--TTTCTACCTTCGTG
BHB105  AGG--TTTCTACCTTCGTG
BHB122  AAG--TTTCTACCTTCGCG
BHB146  AAG--TTTCTACCTTCGCG
BHB149  AAG--TTTCTACCTTCGTG
```

(c) psbA^{ncr}

```
                270       275       280       285
BHB104  CCCTTCGGG-GTGCACAT
BHB105  CCCTTCGGG-GTGCACAT
BHB122  CCCGTAGGG-GTACCCAT
BHB146  CCCTTCGGG-GTGCACAT
BHB149  CCCGTAGGG-GTACCCAT
```

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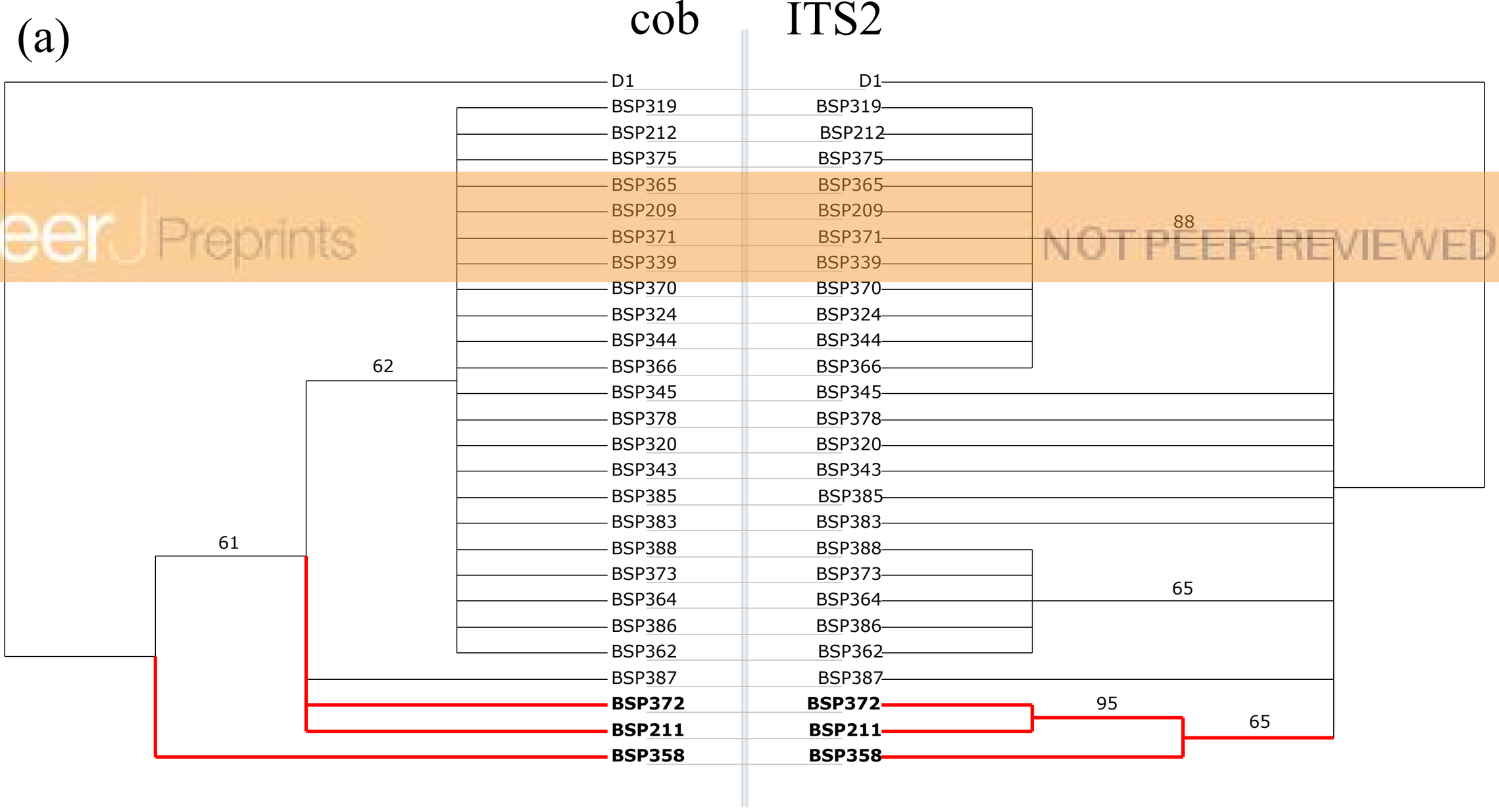
Figure 4(on next page)

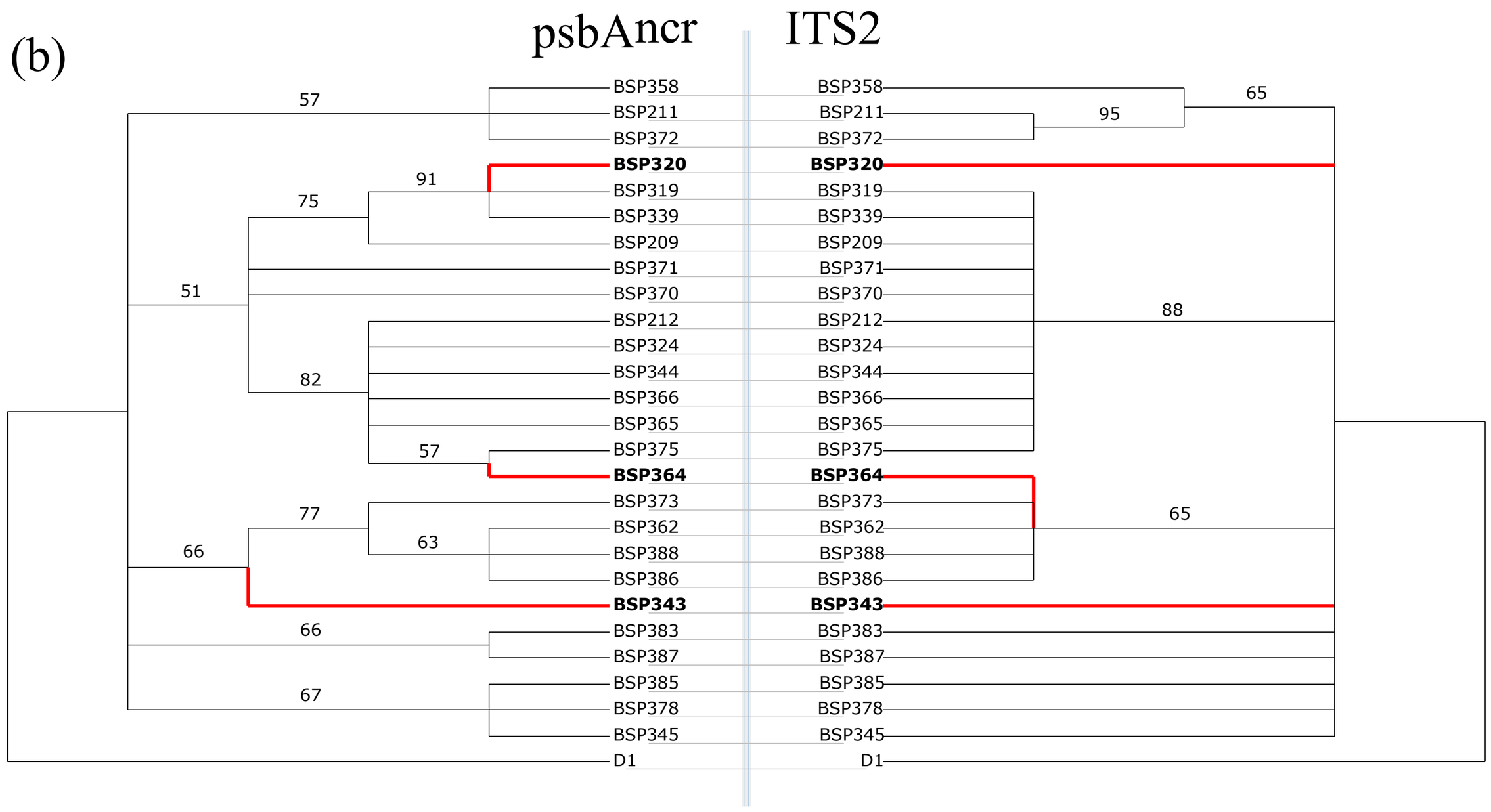
Pairwise tanglegrams for site BSP.

Red branches with bolded taxa labels indicate incongruent samples, as identified by the tree hybridisation analysis (Huson and Linz 2016). Branch labels are ML bootstrap values (1000 replicates for *cob*, 100 for ITS2 and psbA^{ncr}). (a) *cob vs.* ITS2: found incongruent by ILD and AU tests. (b) psbA^{ncr} *vs.* ITS2: found incongruent by ILD and AU tests. (c) *cob vs.* psbA^{ncr}: found congruent by ILD and AU tests.

(a)



(b)



(c)

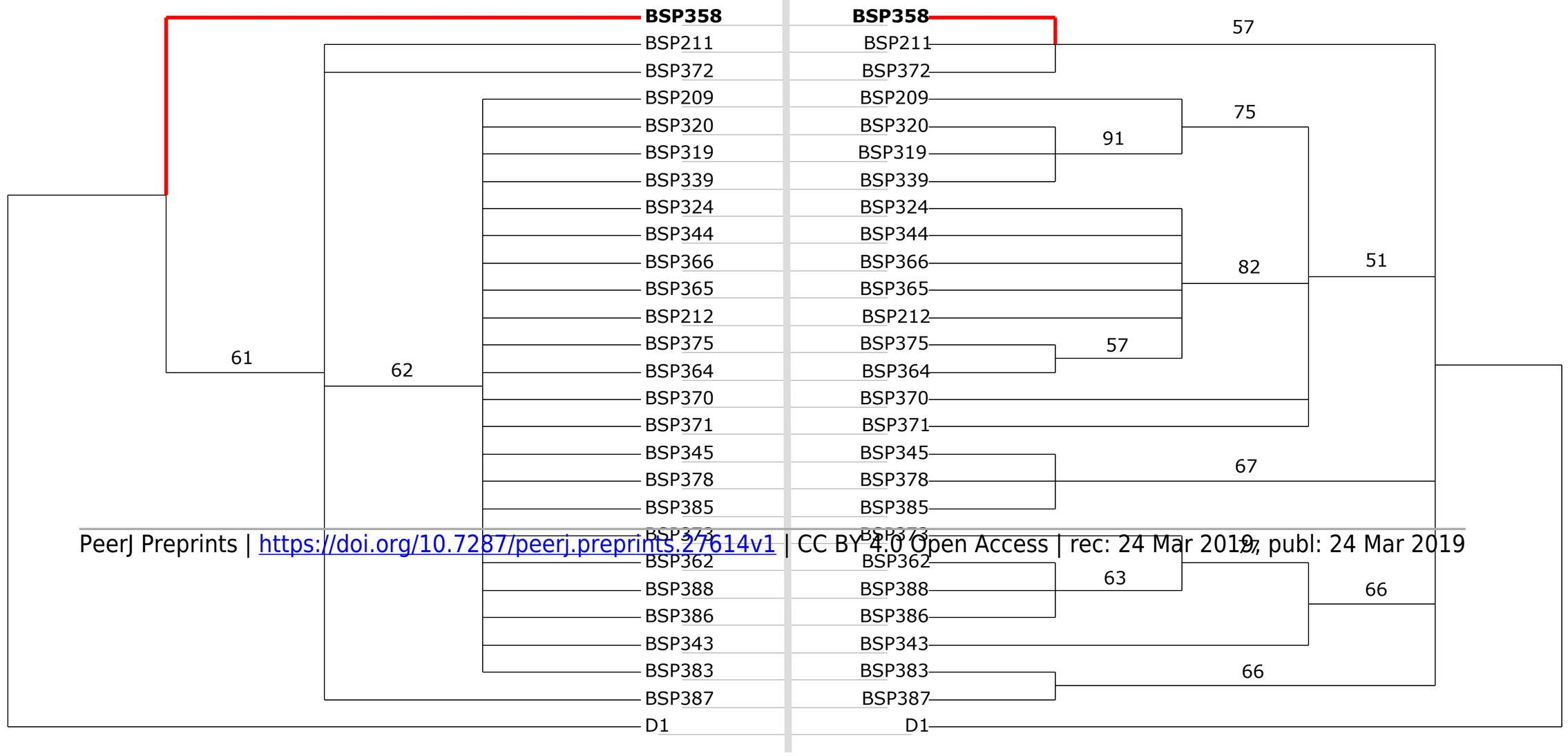


Figure 5(on next page)

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 (not presented) identify it as *Cladocopium* type C3u. (b) In the organellar psbA^{ncr} region, BSP343 groups with BSP386 and BSP388, as a variant of *Cladocopium* type C40. The *cob* gene was invariant in this case.

(a) ITS2

```
                205       210                 290
BSP343  TG-CGCGC  ...  CCGCT
BSP383  TG-CGCGC  ...  CCGCT
BSP386  TG-TGCGC  ...  CTGCT
BSP387  TG-CGCGC  ...  CCGCT
BSP388  TG-TGCGC  ...  CTGCT
```

(b) psbA^{ncr}

```
                425       430       435
BSP343  ATGCC-CCACA-GGGGCAT
BSP383  ACACC-CCGGA-GGGGTGT
BSP386  ATGCC-CCACA-GGGGCAT
BSP387  ACACC-CCGGA-GGGGTGT
BSP388  ATGCC-CCACA-GGGGCAT
```

Figure 6(on next page)

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^{ncr} region, BSP364 groups with BSP344 and BSP366, as a variant of *Cladocopium* type C40. The *cob* gene was invariant in this case.

(a) ITS2

```
                255       260       265
BSP344  TGCTTGCGACCGCTGG
BSP362  TGCTTGCAACTGCTGG
BSP364  TGCTTGCAACTGCTGG
BSP366  TGCTTGCGACCGCTGG
BSP373  TGCTTGCAACTGCTGG
```

(b) psbA^{ncr}

```
                310       315       320
BSP344  ATG-AAAAGAAAAAAGA
BSP362  ACGAAAAAGAAAAATA
BSP364  ATG-AAAAGAAAAAAGA
BSP366  ATG-AAAAGAAAAAAGA
BSP373  ACGAAAAAGAAAAATA
```

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Figure 7 (on next page)

Predictions under Incomplete Lineage Sorting.

(a) General pattern expected for ILS. A single ancestral population with polymorphism in both the psbA^{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.

(a)

(b)

Speciation Event

C40

C3z

psbAncr

C40

C3z

ITS2

C40

C3z

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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 α = 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 α = 0.0085); p-values likely due to type I error are designated by º (see Discussion).

Dataset	Gene region used for test	Best tree	Tree to compare with best tree	AU p-value
BBR	*cob*	psbA^{ncr}	ITS2	<0.0001º
			cob	0.4417
	ITS2	ITS2	*cob*	0.4056
			psbA^{ncr}	0.7712
	psbA^{ncr}	psbA^{ncr}	*cob*	<0.0001º
			ITS2	<0.0001º
BHB	*cob*	*cob*	ITS2	<0.0001*
			psbA^{ncr}	0.5631
	ITS2	ITS2	*cob*	<0.0001*
			psbA^{ncr}	<0.0001*
	psbA^{ncr}	psbA^{ncr}	*cob*	<0.0001º
			ITS2	<0.0001º
BLS	*cob*	*cob*	ITS2	<0.0001*
			psbA^{ncr}	0.3456
	ITS2	ITS2	*cob*	0.4163
			psbA^{ncr}	0.1806
	psbA^{ncr}	psbA^{ncr}	*cob*	<0.0001º
			ITS2	<0.0001º
BSP	*cob*	*cob*	ITS2	0.0493
			psbA^{ncr}	<0.0001*
	ITS2	ITS2	*cob*	<0.0001*
			psbA^{ncr}	<0.0001*
	psbA^{ncr}	psbA^{ncr}	*cob*	<0.0001º
			ITS2	<0.0001º
HEW	*cob*	psbA^{ncr}	ITS2	0.1562
			psbA^{ncr}	0.1562
	ITS2	ITS2	*cob*	0.5465
			psbA^{ncr}	0.5465
	psbA^{ncr}	psbA^{ncr}	*cob*	<0.0001º
			ITS2	<0.0001º
LIE	*cob*	ITS2	*cob*	0.0183
			psbA^{ncr}	0.2336
	ITS2	ITS2	*cob*	0.0870
			psbA^{ncr}	0.4727
	psbA^{ncr}	psbA^{ncr}	*cob*	<0.0001º
			ITS2	<0.0001º
LIW	*cob*	*cob*	ITS2	0.0409
			psbA^{ncr}	0.0811
	ITS2	ITS2	*cob*	0.2490
			psbA^{ncr}	0.6638
	psbA^{ncr}	psbA^{ncr}	*cob*	<0.0001º
			ITS2	<0.0001º

Atauro	*cob*	*cob*	ITS2	<0.0001*
			psbA^{ncr}	0.0125
	ITS2	ITS2	*cob*	<0.0001*
			psbA^{ncr}	<0.0001*
	psbA^{ncr}	psbA^{ncr}	*cob*	<0.0001º
			ITS2	<0.0001º
Timor	*cob*	*cob*	ITS2	0.5604
			psbA^{ncr}	0.0196
	ITS2	ITS2	*cob*	<0.0001*
			psbA^{ncr}	0.0935
	psbA^{ncr}	psbA	*cob*	<0.0001º
			ITS2	<0.0001º

5

Table 2(on next page)

Results of pairwise Incongruence Length Difference tests.

Conclusions were drawn at α=0.05. Statistical significance is designated by *.

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Table 2: Results of pairwise Incongruence Length Difference tests. Conclusions were drawn at α=0.05. Statistical significance is designated by *.

Dataset	Partitions tested	p-value
BHB	*cob* vs. ITS2	0.006*
	cob vs. psbA^{ncr}	0.847
	ITS2 vs. psbA^{ncr}	0.021*
BSP	*cob* vs. ITS2	0.011*
	cob vs. psbA^{ncr}	0.223
	ITS2 vs. psbA^{ncr}	0.001*
Atauro	*cob* vs. ITS2	0.01*
	cob vs. psbA^{ncr}	1
	ITS2 vs. psbA^{ncr}	0.01*
BLS	*cob* vs. ITS2	1
	cob vs. psbA^{ncr}	1
	ITS2 vs. psbA^{ncr}	0.778
LIW	*cob* vs. ITS2	1
	cob vs. psbA^{ncr}	1
	ITS2 vs. psbA^{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^{ncr} *vs*. ITS2	**BHB146,** BHB148
	cob vs. psbA^{ncr}	BHB148
BSP	*cob vs.* ITS2	BSP211, BSP358, BSP372
	psbA^{ncr} *vs*. ITS2	BSP320, **BSP343**, **BSP364**
	cob vs. psbA^{ncr}	BSP358
Atauro	*cob vs.* ITS2	**BHB146,** BSP372, BSP387
	psbA^{ncr} *vs*. ITS2	**BHB146,** BHB148, **BSP343, BSP364,** BSP372
	cob vs. psbA^{ncr}	BHB148, BSP372, BSP387