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PRIMARY RESEARCH PAPER

Molecular phylogenetics of the genus *Physalia* (Cnidaria: Siphonophora) in New Zealand coastal waters reveals cryptic diversity

D. R. Pontin · R. H. Cruickshank

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Abstract *Physalia* is a genus of pelagic colonial hydrozoans often known by common names such as 'Portuguese-man-of-war' or 'bluebottle'. Siphonophore systematists generally recognise only a single species in this genus, *Physalia physalis*, however the name *Physalia utriculus* is also still in common use, which has led to considerable taxonomic confusion. With some morphological variation between global regions there is the possibility that this genus holds a substantial amount of cryptic variation. We seek to examine the genetic structure of *Physalia* present in New Zealand coastal waters. Fifty-four specimens collected from 13 locations around New Zealand and Australia were sequenced for both mitochondrial cytochrome *c* oxidase I (COI) and the first internal transcribed spacer (ITS1) of the nuclear ribosomal cistron. Sequences were analysed using maximum likelihood and split decomposition neighbour networks to determine conflict between clans (the unrooted analog of clades). Three clans were identified from both the COI and ITS sequences. The results are complex and clans are not consistent between the two genes. Nevertheless, it seems that there is substantial cryptic diversity amongst *Physalia* present in New Zealand coastal waters.

Keywords Cnidaria · Siphonophora · DNA barcoding · Integrated taxonomy · *Physalia physalis* · Neighbour networks

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D. R. Pontin · R. H. Cruickshank (✉)
Department of Ecology, Faculty of Agriculture and Life Sciences, Lincoln University, PO Box 84, Canterbury, New Zealand
e-mail: rob.cruickshank@lincoln.ac.nz

D. R. Pontin
e-mail: david.r.pontin@gmail.com

Introduction

The diversity of cnidarians has often been underestimated due to their morphological simplicity and the historical belief that oceans provide little barrier to gene flow so there are few opportunities for allopatric divergence (Palumbi, 1992; Knowlton, 2000; Dawson & Jacobs, 2001). As a consequence, there are believed to be a number of widespread cosmopolitan species in ocean ecosystems. However, with the growing use of molecular techniques, studies are now showing that species once thought to be cosmopolitan often consist of many cryptic species. For example, Dawson & Jacobs (2001) showed that *Aurelia aurita* (the moon jellyfish), commonly believed to be cosmopolitan with little or no geographic variation, can be reclassified into seven species based on molecular data. Another cosmopolitan species is *Physalia physalis* (L.) (Portuguese man-of-war), which is thought to inhabit all the

world's oceans (Lane, 1960; Yanagihara et al., 2002). There is considerable potential for the genus *Physalia* to contain hidden cryptic diversity and the last taxonomic review was over 50 years ago (Totton, 1960). The primary issue that needs to be addressed within this genus is the number and identity of species and the extent of their geographical distributions, as there has been significant debate over this. This is an important question that deserves attention as *Physalia* is the most commonly encountered of all the siphonophores (unlike all others, it lives at the surface of the ocean), and is of medical importance due to its potent sting.

According to the taxonomy of Cnidaria proposed by Collins (2002), *Physalia* is placed in the suborder Cystonectae of the order Siphonophora, which is considered the sister taxon to all other siphonophores. *Physalia* taxonomy has been revised many times from Lamark's early work (Lamark, 1801), through to a revision by Totton (1960), however it is still unclear exactly how many species are in the genus, with two species names commonly appearing in the literature: *P. physalis* and *P. utriculus*. This confusion has been exacerbated by the continued use of the name *P. utriculus* outside of the core siphonophore literature despite the consensus amongst siphonophore systematists that there is only a single species of *Physalia* (Totton, 1960; Bouillon et al., 2006). For example, Collins (2002), Mandojana (1990), Yanagihara et al. (2002) and Alam et al. (2002) all use the name *P. utriculus* in their papers. *P. physalis* as the sole recognised species has a global distribution (Totton, 1960; Pagès & Gili, 1992; Bouillon et al., 2006), whereas *P. utriculus* is usually considered to be confined to the Pacific (Yanagihara et al., 2002), where it is often referred to by the common name 'bluebottle'. The morphological characteristics used to differentiate *P. utriculus*, namely a single tentacle and small size (<8 cm long pneumatophore (float); Fenner, 1997), are also juvenile characters of *P. physalis*. Totton (1960), after examining individuals from around the world, noted that although there was variation, in his opinion this was not sufficient to indicate additional species, highlighting that morphological identification of potential species other than *P. physalis* is difficult. Molecular techniques, as the initial part of an 'integrated taxonomy' approach (Dayrat, 2005), provide a possible tool for resolving the taxonomic ambiguity of *Physalia* and determining whether there are any additional species in this genus.

An integrated taxonomic approach to determining whether there are cryptic species in the genus *Physalia* has potential problems, especially with respect to choosing which genes to analyse. To detect and determine potential species boundaries it is important to use genes that evolve rapidly enough to detect such boundaries. Le Goff-Vitry et al. (2004), Collins et al. (2005, 2008), Dunn et al. (2005), Govindarajan et al. (2005), and Moura et al. (2008) have all successfully used the mitochondrial gene, 16S rDNA, to examine hydrozoan species boundaries. An alternative approach is the use of mitochondrial cytochrome *c* oxidase I (COI) as a universal DNA barcode as proposed by Hebert et al. (2003). The choice between 16S and COI is compounded by a lack of published sequences to guide selection and validate the results. There is one published sequence for *P. physalis* for both 16S (AY935284) and (at the time of analysis) COI (AY937374) in Genbank (Dunn et al., 2005). These sequences are from the same specimen. There is also one 16S sequence for *P. utriculus* (AY512511). Because COI is capable of determining species boundaries in Hydrozoa (Huang et al., 2008; Ortman et al., 2010), and is recognised as a primary gene choice for species delimitation in a wide range of other taxa, it is appropriate to use COI to identify potential species boundaries in *Physalia*.

An issue with using mtDNA for species identification is that mtDNA diversity is not always correlated with nuclear gene diversity due to sex-based dispersal, incomplete lineage sorting, or mitochondrial introgression (Moritz, 1994). It is therefore important to use both sources of genetic information when assessing evolutionarily distinct populations (Cronin, 1993). The first internal transcribed spacer (ITS1) of the nuclear ribosomal cistron is a commonly used region for this purpose as it evolves rapidly and can differentiate between closely related species (Hills & Davis, 1986). Moreover, ITS has been used for this purpose within the Scyphozoa (Dawson, 2003), Anthozoa (Goulet & Coffroth, 2003) and Hydrozoa (Zhang et al., 2009), to good effect.

As a first step towards investigating genetic diversity within *Physalia* we investigated the population genetic structure of this genus at a local scale using DNA sequences from the mitochondrial COI and nuclear ITS1 regions to assess the amount of genetic variation amongst *Physalia* in New Zealand coastal waters.

Materials and methods

Sampling

A total of 55 specimens were collected from 13 locations around New Zealand and Australia (Fig. 1). Specimens were either collected directly from the ocean, or from the beach as they washed ashore. Excess sea water was removed from each specimen by blotting with a paper towel before being placed in 100% ethanol and stored at −20°C. The morphology of each specimen was briefly inspected to assess the size of the individual and the number of tentacles to establish if there was any macro-morphological variation.

DNA extraction and sequencing

Total genomic DNA was extracted from the gastrozooids or tentacles of each specimen using either the DNeasy® Tissue Kit (Qiagen) or the AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen) following the manufacturers' protocols. 2.5 µl of extracted DNA was amplified by polymerase chain reaction (PCR) in a total volume of 25 µl with 2.5 µl 10× PCR buffer (Qiagen), 2.5 µl 8 mM dNTPs (i.e. 2 mM of each, New England Biolabs), 1.2 µl of each 10 µM primer (Invitrogen) and 0.2 µl (1 unit) of *Taq* DNA polymerase (5 units/µl; Qiagen). The PCR reaction began with 2 min denaturation at 94°C, followed by 33 cycles of 40 s at 92°C, 40 s at 45°C and 90 s at 72°C, finishing with a 5-min extension at 72°C and cooling to 4°C. COI was amplified using the primers HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) and LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) (Folmer et al., 1994). ITS1 was amplified using the primers CAS18sF1 (5′-TACACACCGCCCGTCGCTACTA-3′) and CAS5p8sB1d (5′-ATGTGCGTTCRAAATGTCGATGTTCA-3′) (Zhang et al., 2003). Amplifications were confirmed by electrophoresis. PCR products (2–3 µl) were sequenced (10 µl total volume) using 0.5 µl BigDye™ (Applied Biosystems), 2 µl sequencing buffer and 0.8 µl of 10 µM primer. The sequencing reaction began with a 1 min denaturation at 96°C,

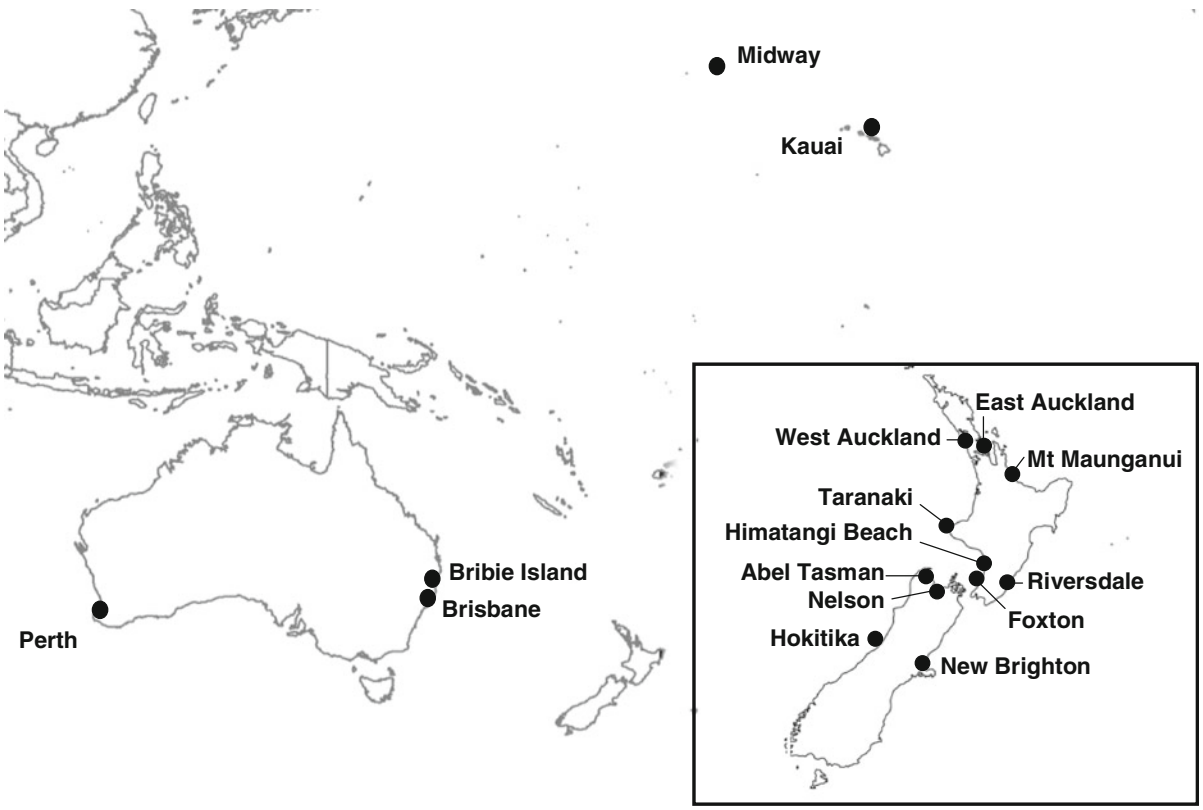

Fig. 1 Locations of *Physalia* collection sites

followed by 25 cycles of 10 s at 96°C, 5 s at 50°C and 4 min at 60°C, then cooling to 10°C. Sequencing products were purified by ethanol precipitation, air dried and run on an ABI-PRISM® 377 automated sequencer (Applied Biosystems) according to the manufacturer's instructions. Both strands were sequenced to improve accuracy. Sequences were obtained for both genes for all specimens except three (NB103, AE14 and N1) for which only COI was obtained, and one (T1) for which only ITS1 was obtained. Sequences were deposited in GenBank under the accession numbers FJ847271–FJ847322 (COI) and FJ916936–FJ916989 (ITS1). Further COI sequences were sourced from Dr Brenden Holland (University of Hawaii) from Brisbane (3), Kauai (2) and Midway (1). One COI sequence labelled *P. physalis* (Atlantic Ocean) was obtained from GenBank (AY937374).

Phylogenetic analysis

ITS1 sequences were aligned using PRANK (Loytynoja & Goldman, 2005) with default parameters. The total length of the alignment was 566 bp for COI and 456 bp for ITS1. Phylogenetic trees were constructed using maximum likelihood (ML) as implemented in the computer program PAUP*4 (Swofford, 2003), using a heuristic search with starting trees obtained by stepwise addition and branch swapping by tree bisection and reconnection (TBR). Trees were also constructed using distance methods and maximum parsimony (for parsimony analyses, gaps were treated as a fifth state) but as these were broadly congruent with the ML trees, only the likelihood trees are presented. For COI, trees were constructed using *Rhizophysa filiformis* (GenBank accession no. AY937377; Dunn et al., 2005) as an outgroup as this is in the same suborder (Cystonectae) as *Physalia*, however this placed the root of the tree on a very long branch within a group of closely related sequences, possibly due to the large distance between the ingroup and the outgroup, which we consider likely to be incorrect. No ITS1 sequences are available for other members of the Cystonectae. For these reasons all trees have been left unrooted. Parameters for ML models were determined by MODELTEST using the AIC (Posada & Crandall, 1998). One thousand bootstrap replicates were performed to estimate clan support. As the trees presented in this article are all unrooted we prefer to use the term 'clan' (sensu Wilkinson et al., 2007) rather than 'clade' to denote the unrooted analog of a monophyletic group. Split decomposition neighbour networks were calculated for both genes using SplitsTree4 (Hudson & Bryant, 2006) to assess potential conflict within the datasets. For genes where areas of conflict were identified, a sliding window was implemented across the gene. Window lengths starting at 50 base pairs (bp) and increasing by 50 bp up to 75% of the length of the sequence were assessed. For each window, 1,000 parsimony bootstrap replicates were performed using the fast stepwise addition option in PAUP*4. For each window, the bootstrap support for each node was extracted using a MATLAB 7.6.0 script, and conflicting signals were isolated.

Species delimitation

Bayesian phylogenetic analyses were run on the COI data for 20 million generations in BEAST v1.6.1 (Drummond & Rambaut, 2007) assuming a GTR+I+Γ substitution model and three different clock models; uncorrelated lognormal (Drummond et al., 2006), random local (Drummond & Suchard, 2010) and strict molecular clocks. For the uncorrelated lognormal clock, the mean value of the ucld.stdev parameter for 18,000 sampled trees (after removal of the first 10% as burnin) was 0.369 (ESS = 1,271), which is less than 1 (the threshold for statistical rejection of the hypothesis of a strict molecular clock), and the posterior frequency distribution of ucld.stdev closely abutted zero (Electronic Supplementary Material (ESM), Fig. S1). For the random local clock, the mean value of the *K* parameter (rateChangeCount = the number of discrete rate changes) for 18,000 sampled trees (after removal of the first 10% as burnin) was 0.653 (ESS = 2,460), and the mode was zero (ESM, Fig. S2). These results indicate that the data fit the assumptions of a strict molecular clock, and therefore the results of the strict molecular clock analysis were used for species delimitation. For the strict molecular clock, a maximum clade credibility (MCC) tree was constructed from 18,000 sampled trees (after removal of the first 10% as burnin) using TreeAnnotator v1.6.1. This tree was analysed using the general mixed Yule-coalescent (GMYC) model with both single (Pons et al., 2006) and multiple (Monaghan et al., 2009) thresholds, as implemented in the R package 'splits'.

Table 1 COI and ITS clans and macro-morphology associated with collected specimens

Specimen (code)	Collection date	COI clan	ITS clan	Number of tentacles	Length of pneumatophore
New Brighton 2 (NB2)	13-Feb-05	1	I	Multiple	Small
New Brighton 3 (NB3)	13-Dec-05	1	I	Multiple	Small
New Brighton 4 (NB4)	13-Dec-05	1	I	Multiple	Small
New Brighton 5 (NB5)	13-Dec-05	1	I	Multiple	Small
New Brighton 7 (NB7)	13-Dec-05	1	I	Multiple	Small
New Brighton 8 (NB8)	13-Dec-05	1	I	Multiple	Small
New Brighton 9 (NB9)	13-Dec-05	1	I	Multiple	Small
New Brighton 10 (NB10)	13-Dec-05	1	I	Multiple	Small
New Brighton 22 (NB22)	06-Jan-08	1	I	Multiple	Small
New Brighton 32 (NB32)	06-Jan-08	1	I	Multiple	Small
New Brighton 103 (NB103)	16-Jan-06	2	–	Multiple	Small
New Brighton 109 (NB109)	16-Jan-06	2	I	Multiple	Small
Foxton 1 (F1)	19-Dec-06	1	I	Multiple	Small
Foxton 2 (F2)	19-Dec-06	1	I	Multiple	Small
Foxton 3 (F3)	19-Dec-06	1	I	Multiple	Small
Foxton 5 (F5)	19-Dec-06	1	I	Multiple	Small
Himatangi Beach 1 (HB1)	10-Dec-06	1	I	Multiple	Small
Himatangi Beach 2 (HB2)	10-Dec-06	1	I	Multiple	Small
Himatangi Beach 3 (HB3)	10-Dec-06	1	I	Multiple	Small
Himatangi Beach 5 (HB5)	10-Dec-06	1	I	Multiple	Small
Taranaki 1 (T1)	02-Feb-06	–	I	Multiple	Small
Taranaki 2 (T2)	02-Feb-06	1	I	Multiple	Small
Taranaki 3 (T3)	02-Feb-06	1	I	Multiple	Small
Taranaki 4 (T4)	02-Feb-06	1	I	Multiple	Small
Riversdale 1 (R1)	19-Feb-06	3	III	Single	Small
Riversdale 2 (R2)	19-Feb-06	1	I	Multiple	Small
Riversdale 3 (R3)	19-Feb-06	1	I	Multiple	Small
Riversdale 4 (R4)	19-Feb-06	3	III	Multiple	Large
Riversdale 13 (R13)	29-Nov-06	3	III	Multiple	Small
Riversdale 18 (R18)	29-Nov-06	3	III	Single	Small
Riversdale 20 (R20)	29-Nov-06	1	I	Multiple	Small
Hokitika 1 (H1)	26-Nov-05	1	I	Single	Small
Hokitika 2 (H2)	26-Nov-05	1	I	Multiple	Small
Hokitika 3 (H3)	26-Nov-05	1	I	Single	Small
Hokitika 4 (H4)	26-Nov-05	1	I	?	Small
Hokitika 53 (H53)	03-Mar-06	1	I	Single	Small
Mt Maunganui 1 (MM1)	20-Mar-06	3	III	Multiple	Large
Mt Maunganui 2 (MM2)	20-Mar-06	3	III	Multiple	Large
Mt Maunganui 3 (MM3)	20-Mar-06	3	III	Multiple	Large
Mt Maunganui 4 (MM4)	20-Mar-06	3	III	Multiple	Large
Western Australia 1 (WA1)	16-Feb-07	2	II	Single	Small
Western Australia 2 (WA2)	16-Feb-07	1	I	Single	Small
Western Australia 3 (WA3)	16-Feb-07	1	I	Single	Small
Western Australia 6 (WA6)	16-Feb-07	1	I	Single	Small

Table 1 continued

Specimen (code)	Collection date	COI clan	ITS clan	Number of tentacles	Length of pneumatophore
Brisbane 7 (B7)	24-Nov-07	2	II	Single	Small
Brisbane 9 (B9)	24-Nov-07	2	II	Single	Small
Brisbane 10 (B10)	24-Nov-07	2	II	Single	Small
Nelson 1 (N1)	18-Dec-07	2	I	Multiple	Small
Nelson 2 (N2)	18-Dec-07	2	I	Multiple	Small
Nelson 3 (N3)	18-Dec-07	2	–	Multiple	Small
Abel Tasman 2 (AT2)	07-Mar-08	1	I	Single	Small
East Auckland 10 (AE10)	07-Dec-07	1	I	Multiple	Small
East Auckland 14 (AE14)	05-Feb-08	1	–	?	Small
East Auckland 15 (AE15)	09-Mar-08	1	I	Multiple	Small
West Auckland 2 (AW2)	15-Nov-07	1	I	Multiple	Small

For number of tentacles, "?" indicates that the original tentacle number is unknown. For length of pneumatophore, "small" = <5 cm and "large" = >5 cm

Results

The macro-morphological features of each specimen are displayed in Table 1. The best-fit models of nucleotide sequence substitution estimated by MODELTEST are, for COI, the K81uf+I+Γ model (nucleotide frequencies = A: 0.3839, C: 0.1416, G: 0.1683, T: 0.3062, proportion of invariant sites = 0.2100, gamma shape parameter = 0.7202, rA-C = 1.000, rA-G = 9.8019, rA-T = 3.7476, rC-G = 3.7476, rC-T = 9.8019, rG-T = 1.000), and for ITS1, the TVM+Γ model (nucleotide frequencies = A: 0.2699, C: 0.2089, G: 0.2385, T: 0.2828, proportion of invariant sites = 0, gamma shape parameter = 0.3396, rA-C = 1.0423, rA-G = 0.7742, rA-T = 1.3478, rC-G = 0.2027, rC-T = 0.7742, rG-T = 1.000).

Cytochrome *c* oxidase I

Three clans are identified from the COI sequences (Fig. 2) with a minimum bootstrap support of 82%. Clan 1 contains specimens from all Australasian locations sampled, except Mount Maunganui. Clan 2 contains specimens from the entire geographic range sampled, including Kauai and Midway. Specimens from Mt Maunganui, and some of those from Riversdale, form a distinct clan (clan 3) that appears to have substantial internal structure (large within-clan pairwise genetic distances) when compared to the other clans, although the relationships between groups within this clan have only moderate bootstrap support (66–78%). This clan also has the smallest geographic range of all the clans and is only found in the northeastern and eastern areas of the North Island of New Zealand. The Genbank sequence labelled '*P. physalis* (Atlantic Ocean)' does not group with any of these clans and forms an isolated branch of its own.

Pairwise genetic distances between clans vary between 7.3 and 12.6% (Table 2). Pairwise genetic distances within clans are all <1.5% except for clan 3, which has a mean pairwise distance of 6.1%, despite having the smallest geographical range. There is evidence of a barcoding gap (i.e. a distinct gap between the distributions of within-clan and between-clan distances (Meyer & Paulay, 2005)) for clans 1 and 2, but not clan 3 (Fig. 3).

Whilst this article was in review three additional COI sequences were published for *Physalia* spp. (Ortman et al., 2010). We reanalysed the data including these sequences and all three of them fell into clans we found in our original analysis, therefore they do not appear to represent additional unsampled genetic diversity. Two sequences labelled as *Physalia physalis* (GQ120032 for isolate Phph01 and GQ120033 for isolate Phph02, both from the Northern Gulf of Mexico) grouped very closely with the other Genbank sequence from the Atlantic labelled *Physalia physalis* (AY937374, which is identical to GQ120032). A sequence labelled as *Physalia* sp. BO-2009 (GQ120034 for isolate Phph03, from the Sargasso Sea) grouped within clan 2, close to specimens

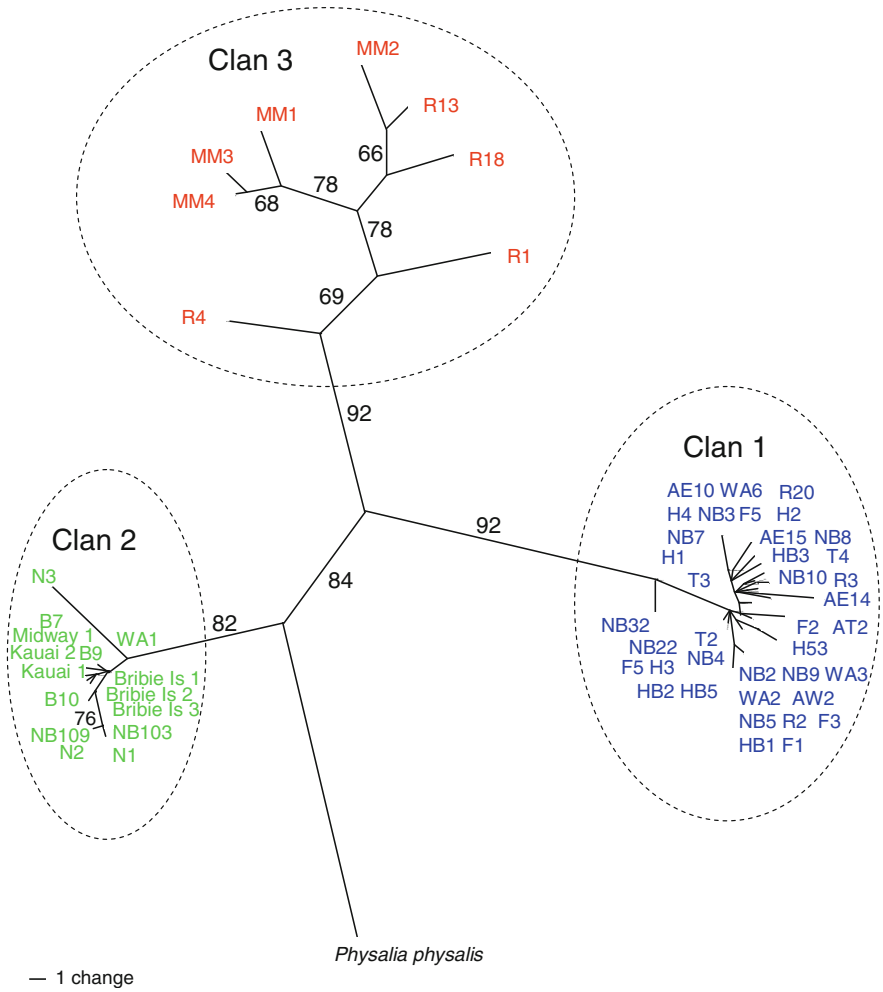

Fig. 2 Unrooted ML tree for sequences of 566 bp of the mitochondrial COI gene from 61 specimens of *Physalia* constructed using a heuristic search with starting trees obtained by stepwise addition and branch swapping by TBR as implemented in PAUP*4 (Swofford, 2003). Parameters for the ML model were determined by MODELTEST using the AIC (Posada & Crandall, 1998). The model with the highest Akaike weight was K81uf+I+Γ (see main text for parameter values). *Numbers on branches* indicate bootstrap support (1,000 replicates). Clans are coloured to differentiate them more readily. The tip labelled '*Physalia physalis*' represents the GenBank sequence AY937374 from an Atlantic Ocean specimen. The tips labelled 'Bribie Is' (Brisbane), 'Midway' and 'Kauai' represent sequences provided by Brenden Holland. Other tips are labelled with specimen codes listed in Table 1. The *scale bar* represents one nucleotide substitution (equivalent to an uncorrected *P*-distance of ∼0.18%)

from Hawaii. This extends the geographical range of this clan into the Atlantic Ocean. All locality information for these sequences was kindly provided by Brian Ortman (pers. comm.). A phylogenetic tree depicting the results of these analyses is available as ESM (Fig. S4).

The single threshold GMYC analysis (ESM, Fig. S4) identified clan 1 as a single species, distinct from clans 2 and 3 and from the Genbank sequence labelled '*P. physalis* (Atlantic Ocean)', and split clans 2 and 3 into two and six distinct species, respectively. The multiple threshold GMYC analysis (not shown) split clans 1, 2 and 3 into two, three and five distinct species, respectively. Due to the localised nature of our geographical sampling, and controversy over the effects of incomplete sampling on the results of GMYC analyses (Lohse, 2009; Papadopoulou et al., 2009), these results should be treated with extreme caution. Nevertheless, they do suggest that the genetic

Table 2 Mean pairwise genetic distances (uncorrected *P*-distances) within and between clans of *Physalia*

COI		ITS	
Clan	Sequence divergence (%)	Clan	Sequence divergence (%)
Clan 1	1.1	Clan I	0.8
Clan 2	1.3	Clan II	1.2
Clan 3	6.1	Clan III	0.9
Clan 1 and 2	9.4	Clan I and II	2.0
Clan 1 and 3	11.6	Clan I and III	1.8
Clan 2 and 3	11.6	Clan II and III	2.2
Clan 1 and *P. physalis*	11.6		
Clan 2 and *P. physalis*	7.3		
Clan 3 and *P. physalis*	12.6		

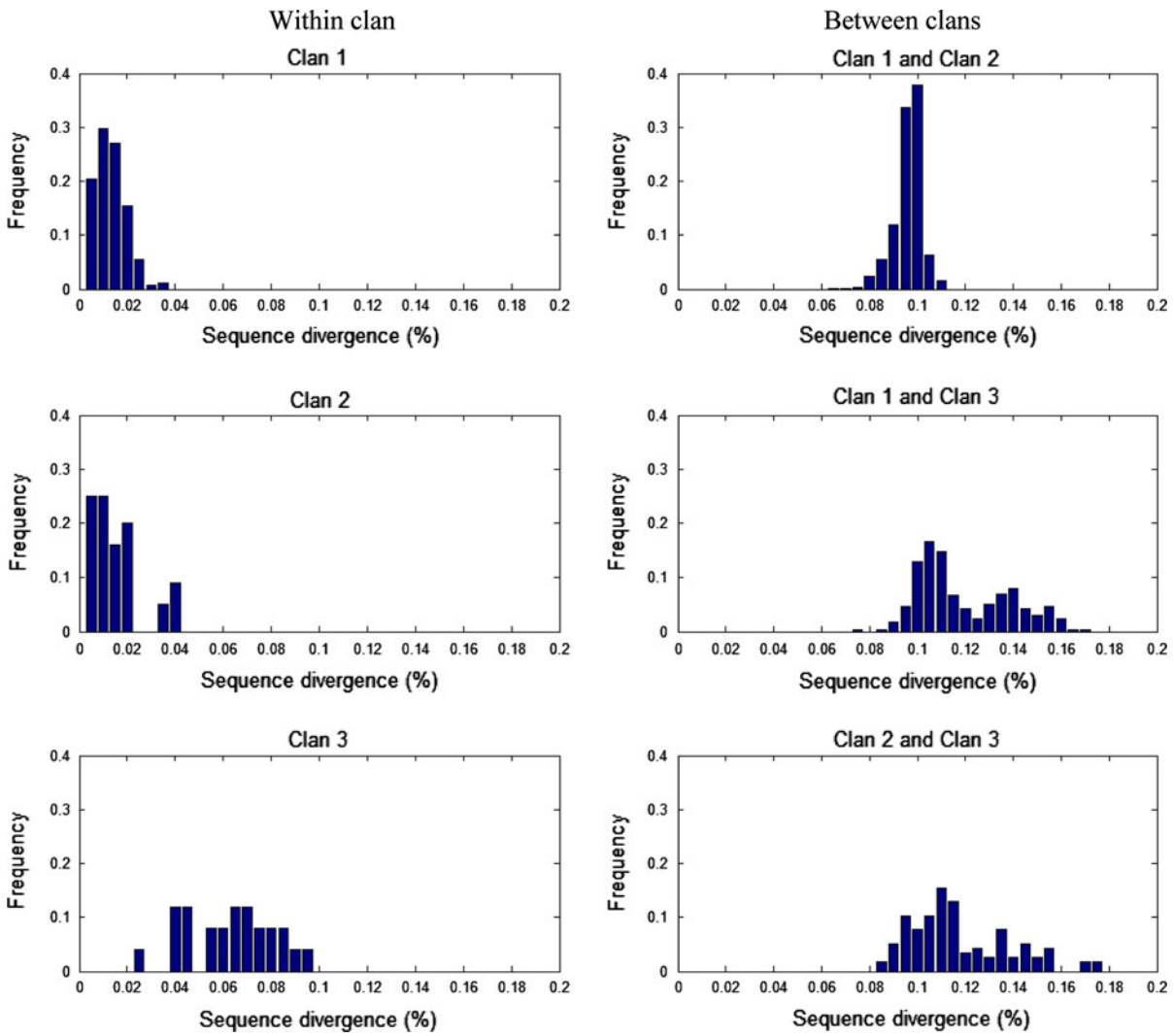

Fig. 3 Frequency distributions of within-clan (*left*) and between-clan (*right*) pairwise genetic distances (uncorrected *P*-distances) for sequences of 566 bp of the mitochondrial COI gene from 61 specimens of *Physalia*

diversity amongst *Physalia* in New Zealand coastal waters is partitioned into discrete clusters that could represent distinct species. A definitive answer will have to await more complete geographical sampling and a fuller understanding of the relationship between sampling effort and species delimitation.

Internal transcribed spacer

The ITS1 tree (Fig. 4) is similar to the COI tree but there are important differences. Three distinct groups are identified, however it was not possible to obtain voucher ITS1 sequences for *P. physalis* or *P. utriculus*, so this information is missing. One of these groups, corresponding to specimens from Western Australia and Brisbane, is paraphyletic, but this appears to be due to conflict over the position of the Western Australian specimen (WA1) (which we suspect may be of hybrid origin; see below). If this specimen is removed from the analysis then three distinct convex (non-polyphyletic) groups are identified (see below). For this reason we refer to these three groups in the ITS

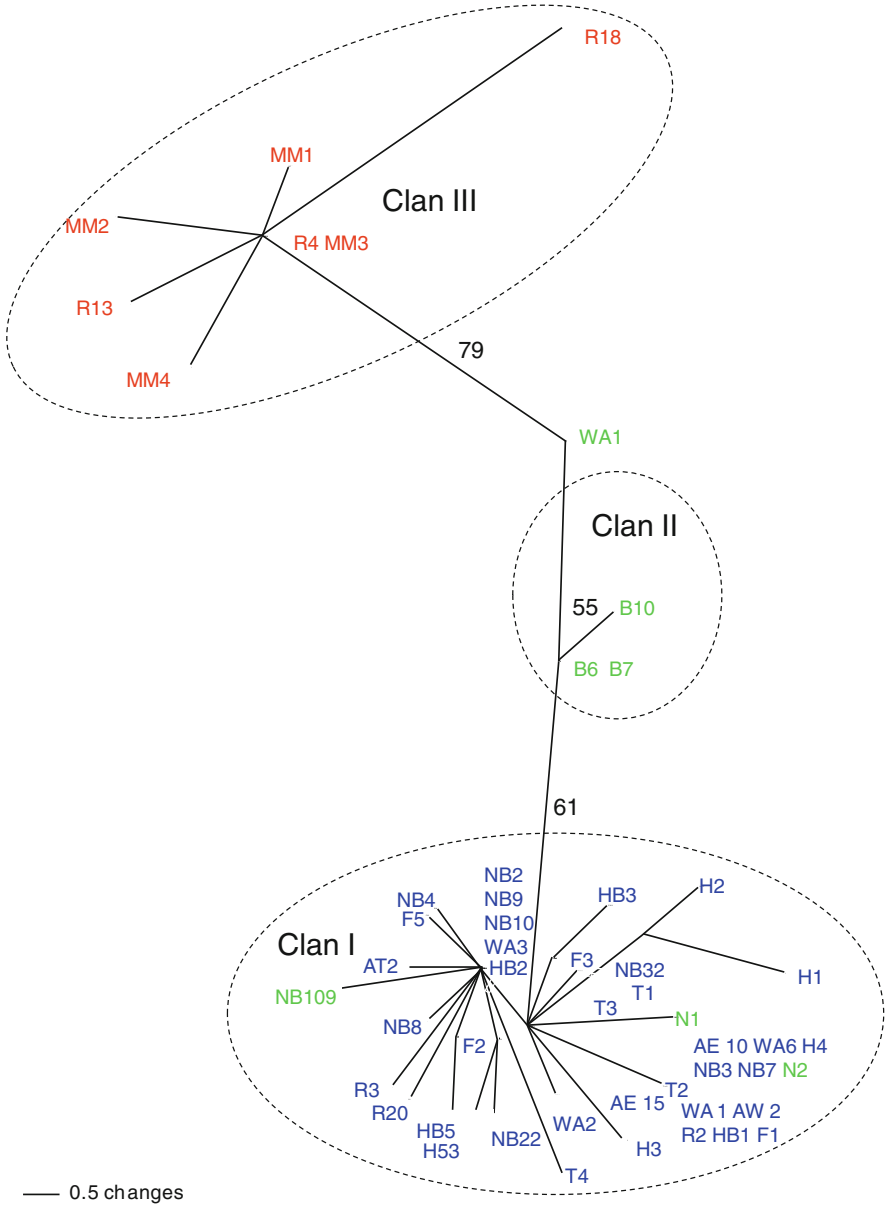

Fig. 4 Unrooted ML tree for sequences of 456 bp of the ITS1 of the nuclear ribosomal cistron from 52 specimens of *Physalia* aligned using PRANK (Loytynoja & Goldman, 2005) with default parameters and constructed using a heuristic search with starting trees obtained by stepwise addition and branch swapping by TBR as implemented in PAUP*4 (Swofford, 2003). Parameters for the ML model were determined by MODELTEST using the AIC (Posada & Crandall, 1998). The model with the highest Akaike weight was TVM+Γ (see main text for parameter values). *Numbers on branches* indicate bootstrap support (1,000 replicates). *Colours* indicate COI clans (Fig. 2). Tips are labelled with specimen codes listed in Table 1. The *scale bar* represents 0.5 nucleotide substitutions (equivalent to an uncorrected *P*-distance of ~0.11%). (Color figure online)

tree as clans, despite the fact that inclusion of WA1 makes one of these groups paraphyletic. This allows us to examine the correspondence between COI and ITS clans. To avoid confusion between COI and ITS clans, ITS clans are referred to by Roman numerals. The only ITS clan that is identical to a COI clan and has moderate bootstrap support (79%) is clan 3/III. ITS clan I contains specimens from both COI clans 1 and 2. The specimens from COI clan 2 found in ITS clan I were collected from Nelson and New Brighton and are the only specimens from COI clan 2 found in New Zealand. The remainder of COI clan 2, with individuals from Western Australia and Brisbane, form ITS clan II (although this clan is only convex if WA1 is removed from the analysis; see above). The pairwise genetic distances between and within ITS clans are similar, with mean pairwise distances within clans ranging from 0.8 to 1.2%, and mean pairwise distances between clans ranging from 1.8 to 2.2% (Table 2) and significant overlap between the distributions (i.e. no barcoding gap) (Fig. 5).

Split decomposition neighbour networks (Figs. 6, 7) support the ML findings and highlight the clan structure found in the ML analysis. Moreover, the networks show that there is some conflict between ITS clans II and III, particularly regarding the placing of

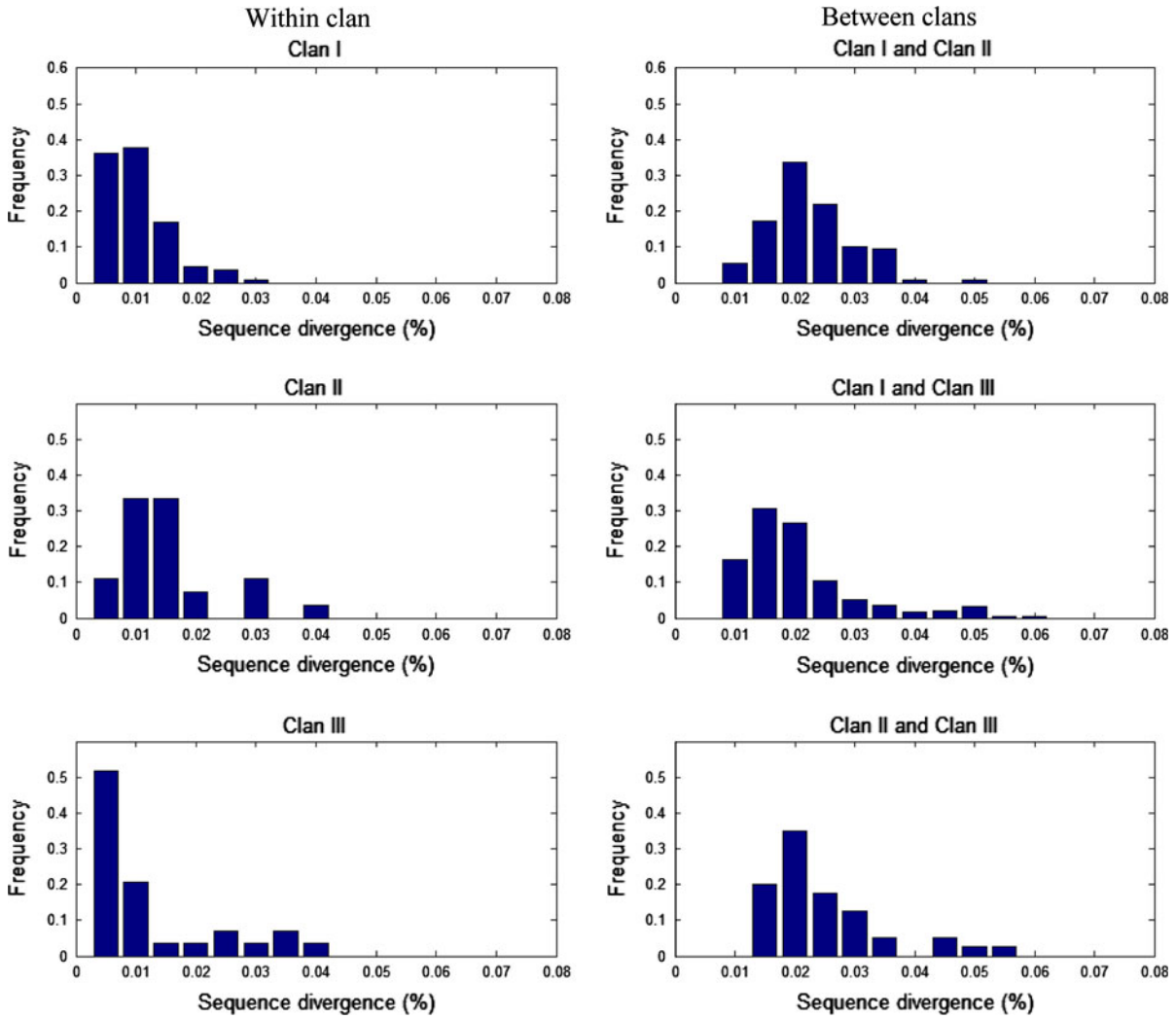

Fig. 5 Frequency distributions of within-clan (*left*) and between-clan (*right*) pairwise genetic distances (uncorrected *P*-distances) for sequences of 456 bp of the ITS1 of the nuclear ribosomal cistron from 52 specimens of *Physalia* aligned using PRANK (Loytynoja & Goldman, 2005) with default parameters

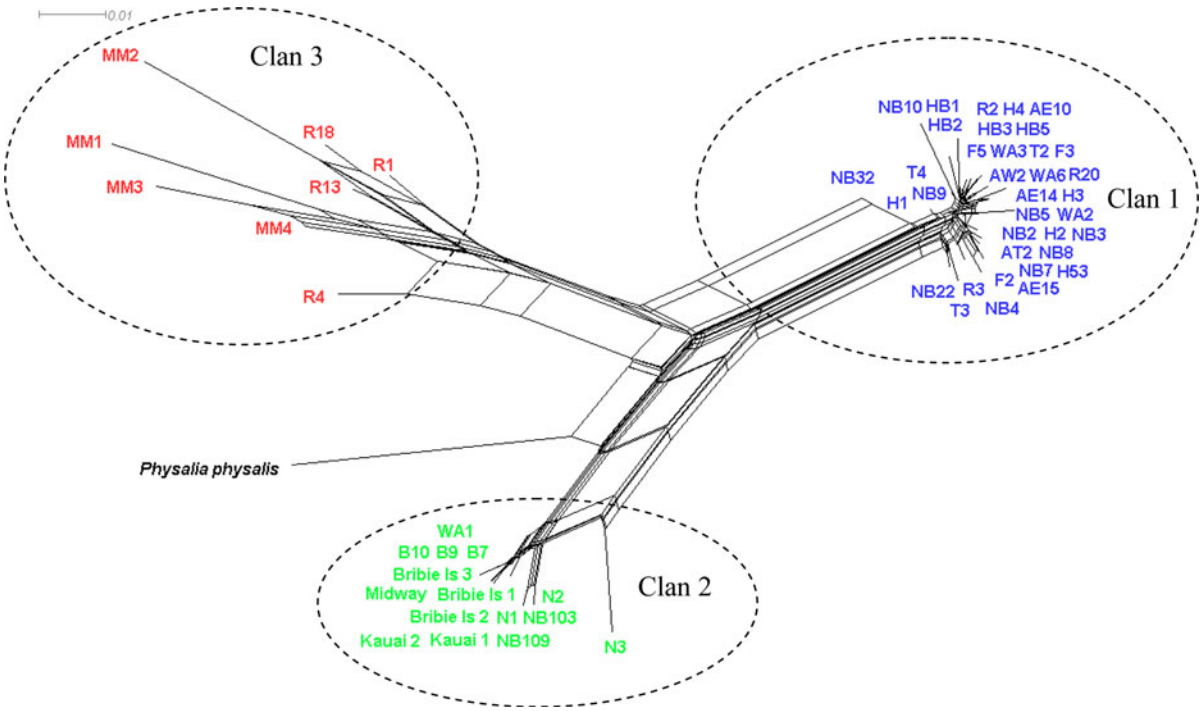

Fig. 6 Split decomposition neighbour network constructed using SplitsTree4 (Hudson & Bryant, 2006) for sequences of 566 bp of the mitochondrial COI gene from 61 specimens of *Physalia*. The tip labelled '*Physalia physalis*' represents the GenBank sequence AY937374 from the Atlantic Ocean. The tips labelled 'Bribie Is' (Brisbane), 'Midway' and 'Kauai' represent sequences provided by Brenden Holland. Other tips are labelled with specimen codes listed in Table 1

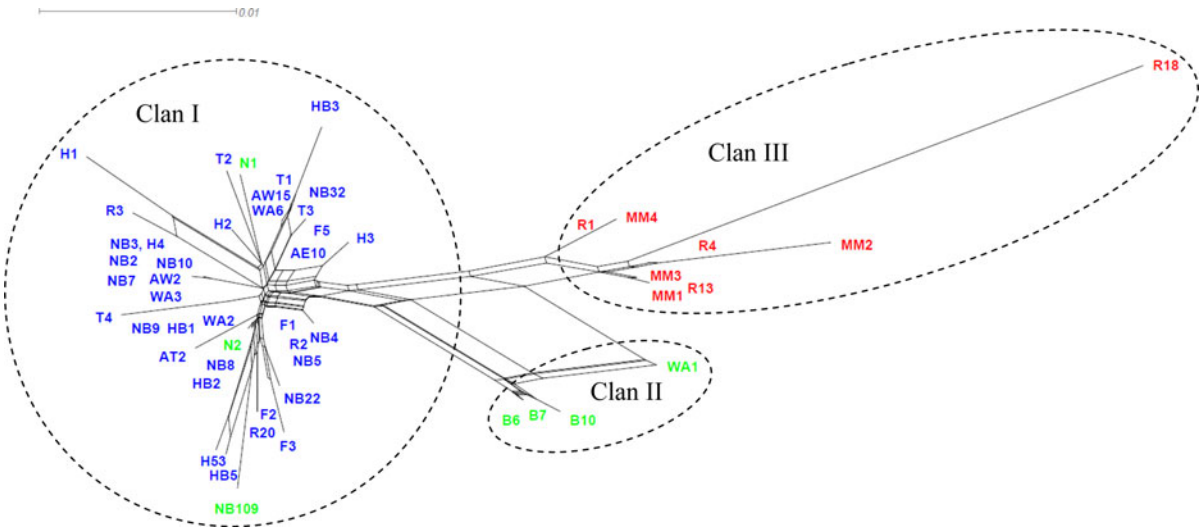

Fig. 7 Split decomposition neighbour network constructed using SplitsTree4 (Hudson & Bryant, 2006) for sequences of 456 bp of the ITS1 of the nuclear ribosomal cistron from 52 specimens of *Physalia* aligned using PRANK (Loytynoja & Goldman, 2005) with default parameters. Tips are labelled with specimen codes listed in Table 1

WA1. Results of the sliding window analysis indicate that a window length of 200 bp is optimal to assess potential conflict within the ITS sequences. Percentage bootstrap support for the inclusion of WA1 in ITS clan II or ITS clan III is shown in Fig. 8. This analysis shows clear conflict for the inclusion of WA1 in either ITS clan II or ITS clan III. Bootstrap support for the inclusion of WA1 in ITS clan II decreases below 50% for windows starting at positions 72–132, with a corresponding rise above 50% in support for the inclusion of WA1 in ITS clan III, i.e. the middle third of this sequence supports the placement of WA1 in ITS clan II, whilst both ends support the placement of WA1 in ITS clan III. This conflict appears to be the cause of the paraphyly of ITS clan II (Fig. 4). When WA1 is removed from the analysis, the remainder of ITS clan II becomes convex with a bootstrap support of 91% (not shown).

Discussion

As for many species with ambiguous taxonomy, molecular techniques have proved suitable for investigating the phylogenetic structure of *Physalia* in New Zealand coastal waters and have generated a surprisingly complex taxonomic picture. Nevertheless, the results provide a solid base for further molecular and morphological investigation of this genus at both local and global scales. This is necessary to resolve the taxonomic structure of the genus.

New Zealand species

It appears that *Physalia* that inhabit New Zealand coastal waters may represent a species complex, for which it is not currently possible to determine the exact number of species. Specimens with similar macro-morphological features group together in clans 1/I and 2/II and therefore these features cannot be used to diagnose these clans as species. All clan 3/III specimens have a single tentacle and small pneumatophore (Table 2), but this may be because of the small number of individuals examined. The lack of specimens from clans 1/I and 2/II conforming to a distinct morphology highlights the ambiguity of the characteristics that have been used to identify species in the past, however, a more in-depth analysis is beyond the skill of these authors and the scope of this study.

An integrated taxonomy approach requires the use of multiple taxon identification tools to differentiate species. From the molecular results obtained here it is

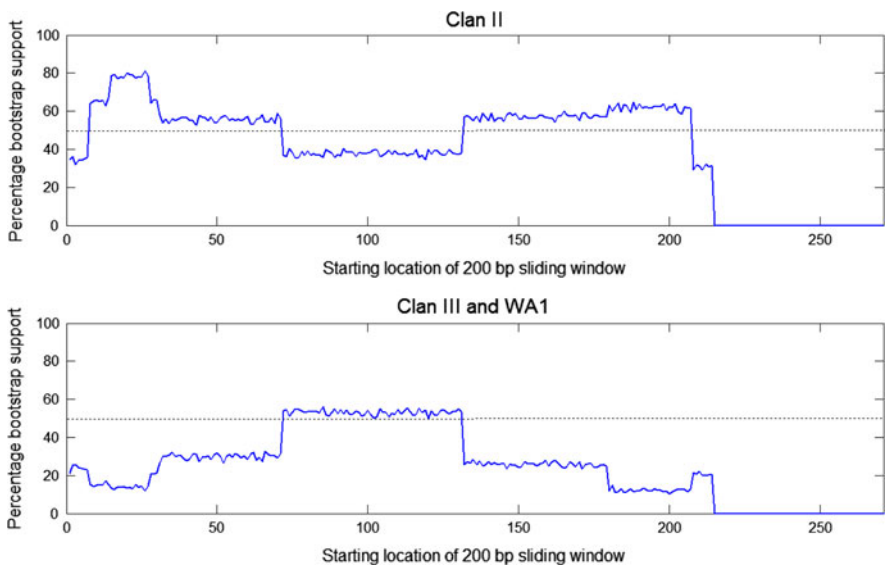

Fig. 8 Percentage parsimony bootstrap support across *Physalia* ITS sequences for conflicting nodes representing alternative placing of WA1. *Top* clan II (B6–7, B10 and WA1), *bottom* clan III and WA1 (MM1–4, R1, R4, R13, R18 + WA1) using a 200 bp sliding window (1,000 replicates). For each window, 1,000 parsimony bootstrap replicates were performed using the fast stepwise addition option in PAUP*4. Bootstrap support for each node was extracted using a MATLAB 7.6.0 script

hypothesised that a *Physalia* complex inhabits New Zealand coastal waters that could consist of multiple species. Clan 3/III is well supported for both genes and unlike other clans shows substantial internal structure, and the concordance between COI and ITS indicates that it has not hybridised with other clans. These are all indications that this clan could represent a distinct species, at least according to the genotypic cluster species definition (Mallet, 1995), however, an investigation of the genetic diversity of the genus at a global scale is necessary to confirm this as it is possible that individuals with intermediate genotypes live outside the geographical range sampled. Pairwise genetic distances between the clans all considerably exceed most threshold cut-off values commonly used to flag potential new species in the DNA barcoding literature (usually between 2 and 5%; see, for example, Hebert et al. (2010)). Pairwise genetic distances within COI clan 3 are also large, suggesting that there may even be multiple species within this clan, although a higher rate of molecular evolution is also a possibility. However, cytonuclear discordance between clans 1/I and 2/II creates uncertainty for these clans as separate species.

Clan 3/III was the only one found in the northeast of the North Island of New Zealand, a locality that raises the possibility of a separate source area. Brodie (1960) released over 10,000 float cards to assess the surface ocean currents around New Zealand and it is reasonable to assume that *Physalia* would display similar movement patterns to these cards as they are likely to be influenced by wind and currents in a similar way. Cards released from the North Cape drifted down the east coast of the North Island and via the East Auckland Current to the East Cape. Past the East Cape the East Current continues south until it meets the Canterbury Current in the south of the North Island (Gardner, 1961). This pattern of currents may explain why clan 3/III was found in East Auckland, and why Riversdale had both clans 1/I and 3/III present.

Cytonuclear discordance

The current global initiative to use COI as a standalone species discriminator has been predominately successful across most taxa attempted (Ward et al., 2005) but it is recognised by the DNA barcoding community that there will be exceptions. In particular, groups that exhibit hybridisation, ancestral polymorphism and pseudogenes pose potential problems for barcoding (Bensasson et al., 2001). The cytonuclear discordance between COI and ITS1 found in this study could be the result of (1) incomplete lineage sorting and paralogy of ITS1 due to the 4-fold greater coalescence time for this nuclear marker, meaning that insufficient time has elapsed since speciation for reciprocal monophyly of ITS1 to have been established (Rich et al., 1997), although no incidences of multiple ITS1 sequences were found in any of the specimens we sequenced (all PCR products produced a single band on agarose gels, and no multiple peaks were apparent in any of the sequence chromatograms) and ITS1 was chosen specifically for its mechanisms of concerted evolution, which tend to keep all copies of the gene within an individual genome homogenous; (2) hybridisation between clans 1/I and 2/II; or (3) ancestral mitochondrial polymorphism. Hybridisation is common in cnidarians (van Oppen et al., 2000) and therefore this possibility is worthy of further investigation, particularly in view of the possible hybrid origin of the ITS sequence of the Western Australian specimen (see above). McFadden (1999) and McFadden & Hutchinson (2004) suggested that Octocorallia possess many traits that predispose them to hybridisation including closely related, morphologically similar species with overlapping ranges and reproductive periods. It appears that *Physalia* share the majority of these traits. Clans have significant geographical overlap and Totton (1960) concluded that although there was morphological variation, it was not enough in his opinion to indicate multiple species within the genus. There also appears the possibility of reproductive overlap as gonodendra (reproductive structures) are produced continuously on new individuals (Totton, 1960) and therefore it is assumed that there is a steady supply of gametes. Moreover, individual specimens were collected from all clans throughout the sampling period indicating that gametes from different clans could be present at the same time. An alternative explanation for the cytonuclear discordance is that ancestral polymorphism has been maintained in some lineages within the genus. For this to have occurred, the ancestral population from which COI clans 1 and 2 are descended would need to have had two mitochondrial alleles, one of which became fixed in clan 2 (or the other is not represented in our sample), whilst clan 1 retained both. Although this is a plausible scenario and cannot be discounted, we consider it less likely than the other

two hypotheses. These results highlight the need to include multiple genes, particularly from the nuclear genome, along with COI to gain an overall view of the phylogeny of the group before ascertaining whether COI can be used as a species discriminator.

Conclusions

This study attempts to test the null hypothesis that only a single species of *Physalia* occurs in New Zealand coastal waters, using molecular techniques. Although our haplotype sampling is limited, the results indicate that there is substantial cryptic diversity. Whether this cryptic diversity reflects deep evolutionary history within a morphologically conservative taxon, or a more recent origin of genetic diversity in a taxon that has not shown an equal degree of morphological change, cannot be answered by our data, and this question should be a focus of future research. These alternatives imply very different rates of molecular evolution. Higher-level phylogenies (e.g. Dunn et al., 2005) show much variability in branch-lengths between siphonophore taxa, suggesting that it is at least possible that rates in *Physalia* could be high enough to generate the degree of genetic diversity seen here within a single species. However, the amount of genetic diversity contained within the small geographic region we have sampled suggests that, on a global scale, there may be a great deal more diversity within this genus than has previously been recognised, and we therefore recommend similar studies in other parts of the world where *Physalia* is found. Despite the local scale of our geographical sampling, these results suggest that a taxonomic revision may be necessary, as the current taxonomy does not appear to accommodate our findings unless the single species currently recognised, *Physalia physalis*, contains an extraordinary level of intra-specific diversity.

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