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Cytochrome oxidase I sequences reveal possible cryptic diversity in the cosmopolitan symbiotic copepod *Nesippus orientalis* Heller, 1868 (Pandaridae: Siphonostomatoida) on elasmobranch hosts from the KwaZulu-Natal coast of South Africa

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ABSTRACT

Over the past decade, numerous molecular phylogenetic studies uncovered cryptic diversity within the Copepoda, yet very few investigations focused on symbiotic copepods. Here we report mitochondrial DNA cytochrome oxidase I diversity in the cosmopolitan elasmobranch symbiont *Nesippus orientalis* off the KwaZulu-Natal coast of South Africa. Analysis of partial COI sequences of copepods sampled from a diversity of shark hosts, revealed the presence of two divergent clades. Diversity within the clades does not appear to be structured based on host species, host individual, geographic locality or time of sampling. However, divergence between the two clades seems to be related to host species. Phylogenetic analyses of representatives from the two clades, along with *Nesippus* spp., *Caligus* spp. and *Lepeophtheirus* spp. outgroups, further supports the distinction between the two clades. Future molecular phylogenetic investigations of widespread copepod symbionts most likely will reveal far greater levels of biodiversity than currently recognized.

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1. Introduction

The genus *Nesippus* Heller, 1868 includes five nominal species, i.e. *N. orientalis* Heller, 1868; *N. crypturus* Heller, 1868; *N. vespa* Kirtesinghe, 1964; *N. tigris* Cressey, 1967 and *N. nana* Cressey, 1970 (Boxshall and Halsey, 2004; Cressey, 1967, 1970). All species are cosmopolitan in their distribution and occur in the mouths, on the gill arches and in the nasal passages of a variety of elasmobranch hosts (Cressey, 1967, 1970; Dippenaar and Jordaan, 2006).

Nesippus orientalis can be easily distinguished from the other species by the presence of dorsal plates on the fourth thoracic segment (Cressey, 1967). They are commonly found on a number of shark species (Cressey, 1967, 1970; Dippenaar, 2004; Dippenaar

and Jordaan, 2006), often in clusters on the gill arches and in the mouth (Dippenaar and Jordaan, 2006).

The life cycle of *N. orientalis* is unknown, however, Wilson (1907) already stated that the life cycles of representatives of Pandaridae are similar to those of the Caligidae and this suggestion is strengthened by the monophyletic grouping of Pandaridae and Caligidae (Huys et al., 2007; Dippenaar, 2009). The general life cycle of caligids consists of two free-living nauplius stages, followed by one infective copepodid stage and four parasitic chalimus stages, two parasitic preadult stages and the parasitic adults (Kabata, 1981). Even though small deviations from the general life cycle are found (see Lin and Ho, 1993), information about their life cycles (see Lin and Ho, 1993; Schram, 1993; Todd, 2007) can be used to deduce that the life cycle of *N. orientalis* individuals likely comprise a short free-living phase, while all other stages will be attached to a host. Information about the biology of members of the Caligidae (see Boxshall, 1974; Anstensrud, 1990; Todd, 2007) can be used to speculate that males of *N. orientalis* can probably

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copulate with several females, while the females may be polyan-drous. Individuals of *N. orientalis* attach to the gill arches and in the mouth of their hosts and the females in particular do not seem to move vast distances due to their orderly way of attachment (Dippenaar and Jordaan, 2006). Based on these observations and the occurrence of young among adults (Dippenaar and Jordaan, 2006), it thus appears unlikely that they would move from one host to another, even when hosts come into physical contact with one another.

Since it is impractical to track the planktonic stages of marine invertebrate larvae in the water column (Todd, 1998), especially of those species that as adults live symbiotically on elasmobranch hosts, analyses of the degree and scale of genetic differentiation among populations offer an indirect method of accessing the scale of larval dispersal (Todd, 1998). Knowlton (1993, 2000) also recognized the potential of molecular genetics to identify sibling or cryptic species (i.e. species difficult to distinguish using traditional techniques), which often exist among members of the Crustacea and Copepoda (see e.g. Burton and Lee, 1994; Bucklin et al., 1999; Rocha-Olivares et al., 2001). In this respect, mitochondrial

genomes contain markers suitable for studies of population genetics and evolution (Shao and Barker, 2007), with the cytochrome c oxidase subunit I (COI) gene reported to be successfully employed for species recognition (Hebert et al., 2003; Waugh, 2007). Even though COI markers get saturated quickly around 0.3 substitutions per site, determined with uncorrected pairwise distances, a fairly good approximation can still be made to delimit species using this method (Lefebvre et al., 2006). Under the phylogenetic species definition, the COI gene has a proposed species delimitation threshold of 0.16 substitutions per site in crustaceans (Lefebvre et al., 2006), and has proven to be specifically efficient in copepods (Bucklin et al., 1999; Hill et al., 2001; Øines and Heuch, 2005).

Few studies have been conducted to assess the genetic variation within and between populations of symbiotic siphonostomatoids. A study on the genetic heterogeneity within populations of *Lepeophtheirus europaeensis* Zeddam, Berrebi, Renaud, Raibaut and Gaborion, 1988 parasitic on two host species, reported incipient sympatric speciation (De Meeüs et al., 1992). More recent studies on *Caligus elongatus* Nordmann, 1832 revealed the presence of two distinct genotypes possibly distinguishing two sibling species

Table 1
A list of the 43 COI haplotypes (see Fig. 2), obtained from *Nesippus orientalis* specimens, collected from 19 different host individuals (host nr), host species, the localities (see Fig. 1) where the hosts were caught, the dates when they were caught and their GenBank accession Nos.

Haplotype	Host nr	Shark species	Locality	Coordinates	Date caught	GenBank Accession Nos.
<i>Clade I</i>						
GHDN01	1	<i>Sphyrna mokarran</i>	Durban	29.51S 31.00E	11/2000	GQ369463
GHDN02	1	<i>Sphyrna mokarran</i>	Durban	29.51S 31.00E	11/2000	GQ369464
GHDN03	1	<i>Sphyrna mokarran</i>	Durban	29.51S 31.00E	11/2000	GQ369465
BTRB01	2	<i>Carcharhinus limbatus</i>	Richards Bay	28.48S 32.06E	10/2002	GQ369466
BTRB02	2	<i>Carcharhinus limbatus</i>	Richards Bay	28.48S 32.06E	10/2002	GQ369467
BTRB03	2	<i>Carcharhinus limbatus</i>	Richards Bay	28.48S 32.06E	10/2002	GQ369468
ZMRB01	3	<i>Carcharhinus leucas</i>	Richards Bay	28.48S 32.06E	10/2002	GQ369469
ZMRB02	3	<i>Carcharhinus leucas</i>	Richards Bay	28.48S 32.06E	10/2002	GQ369470
<i>Clade II</i>						
GWRB01	4	<i>Carcharodon carcharias</i>	Richards Bay	28.48S 32.06E	10/2002	GQ369471
RTRB03	5	<i>Carcharias taurus</i>	Richards Bay	28.48S 32.06E	11/1999	GQ369480
CHPE01	6	<i>Sphyrna lewini</i>	Port Edward	31.03S 30.14E	06/1998	GQ369502
CSMG02	7	<i>Carcharhinus brachyurus</i>	Margate	30.52S 30.21E	11/2004	GQ369505
GWLB01	8	<i>Carcharodon carcharias</i>	Leisure Bay	31.01S 30.14E	03/1999	GQ369474
RTRB06	10	<i>Carcharias taurus</i>	Richards Bay	28.48S 32.06E	11/1997	GQ369483
RTRB02	5	<i>Carcharias taurus</i>	Richards Bay	28.48S 32.06E	11/1999	GQ369479
RTRB04	5	<i>Carcharias taurus</i>	Richards Bay	28.48S 32.06E	11/1999	GQ369481
RTSB03	11	<i>Carcharias taurus</i>	Scottburgh	30.17S 30.45E	11/2002	GQ369492
GWRB03	4	<i>Carcharodon carcharias</i>	Richards Bay	28.48S 32.06E	10/2002	GQ369473
GWLB03	8	<i>Carcharodon carcharias</i>	Leisure Bay	31.01S 30.14E	03/1999	GQ369476
RTRB09	12	<i>Carcharias taurus</i>	Richards Bay	28.48S 32.06E	11/1999	GQ369486
RTRB01	5	<i>Carcharias taurus</i>	Richards Bay	28.48S 32.06E	11/1999	GQ369478
RTZN01	13	<i>Carcharias taurus</i>	Zinkwazi	29.12S 31.27E	10/1998	GQ369493
SHDN01	14	<i>Sphyrna zygaena</i>	Durban	29.51S 31.00E	10/2002	GQ369500
GWLB02	8	<i>Carcharodon carcharias</i>	Leisure Bay	31.01S 30.14E	03/1999	GQ369475
SFSR02	9	<i>Isurus oxyrinchus</i>	Salt Rock	29.30S 31.15E	11/2002	GQ369495
RTSB02	11	<i>Carcharias taurus</i>	Scottburgh	30.17S 30.45E	11/2002	GQ369491
RTRB10	12	<i>Carcharias taurus</i>	Richards Bay	28.48S 32.06E	11/1999	GQ369487
SHDN02	14	<i>Sphyrna zygaena</i>	Durban	29.51S 31.00E	10/2002	GQ369501
RTRB07	10	<i>Carcharias taurus</i>	Richards Bay	28.48S 32.06E	11/1997	GQ369484
RTRB11	12	<i>Carcharias taurus</i>	Richards Bay	28.48S 32.06E	11/1999	GQ369488
SHAB01	15	<i>Sphyrna zygaena</i>	Anstey's Beach	29.55S 31.01E	11/2002	GQ369497
SHMZ01	16	<i>Sphyrna zygaena</i>	Mzamba	31.05S 30.10E	08/1995	GQ369499
RTRB05	5	<i>Carcharias taurus</i>	Richards Bay	28.48S 32.06E	11/1999	GQ369482
SHAB02	15	<i>Sphyrna zygaena</i>	Anstey's Beach	29.55S 31.01E	11/2002	GQ369498
CHAM01	17	<i>Sphyrna lewini</i>	Amanzimtoti	30.03S 30.53E	07/1998	GQ369503
RTSB01	11	<i>Carcharias taurus</i>	Scottburgh	30.17S 30.45E	11/2002	GQ369490
SFSR01	9	<i>Isurus oxyrinchus</i>	Salt Rock	29.30S 31.15E	11/2002	GQ369494
RTRB08	10	<i>Carcharias taurus</i>	Richards Bay	28.48S 32.06E	11/1997	GQ369485
GWUM02	18	<i>Carcharodon carcharias</i>	Umlhanga Rocks	29.43S 31.05E	11/2000	GQ369477
CSMG01	7	<i>Carcharhinus brachyurus</i>	Margate	30.52S 30.21E	11/2004	GQ369504
SFSR03	9	<i>Isurus oxyrinchus</i>	Salt Rock	29.30S 31.15E	11/2002	GQ369496
RTRB12	19	<i>Carcharias taurus</i>	Richards Bay	28.48S 32.06E	11/1999	GQ369489
GWRB02	4	<i>Carcharodon carcharias</i>	Richards Bay	28.48S 32.06E	10/2002	GQ369472

Hosts: GH = Great Hammerhead, BT = Blacktip, ZM = Zambezi, GW = Great White, RT = Raggedtooth, CH = Scalloped Hammerhead, SH = Smooth Hammerhead, SF = Shorfin Mako, CS = Copper Shark; Localities: RB = Richards Bay, ZN = Zinkwazi, SR = Salt Rock, UM = Umlhanga Rocks, DN = Durban, AB = Anstey's Beach, AM = Amanzimtoti, SB = Scottburgh, MG = Margate, LB = Leisure Bay, PE = Port Edward, MZ = Mzamba.

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occurring sympatrically, both on individual hosts and from the same geographical area (Øines and Heuch, 2005; Øines and Schram, 2008).

This is the first report of an intraspecific phylogenetic study of the cosmopolitan *N. orientalis* based on COI DNA sequences. We sampled the copepods from a range of elasmobranch hosts off a 324 km section of the east coast of South Africa (Dudley and Cliff, 1993). Multiple copepods were analysed from the same host individuals; from multiple individuals of the same host species caught at the same locality but at different times; from the same host species caught at different localities along the coast; and from a range of shark species. Our aim was to determine intraspecific differentiation and whether geographic distance and/or host species affected the extent

of differentiation. Given the cosmopolitan distribution of the species and the range of highly mobile shark hosts, from which the species has been recorded, one might hypothesize that passive dispersal by free-living life stages, in addition to shark host movements, may lead to genetic homogeneity among the symbionts.

2. Materials and methods

2.1. Selection of material

Nesippus orientalis individuals were collected from various sharks caught in the nets of the Natal Sharks Board along the KwaZulu-Natal coast of South Africa over a period of 10 years

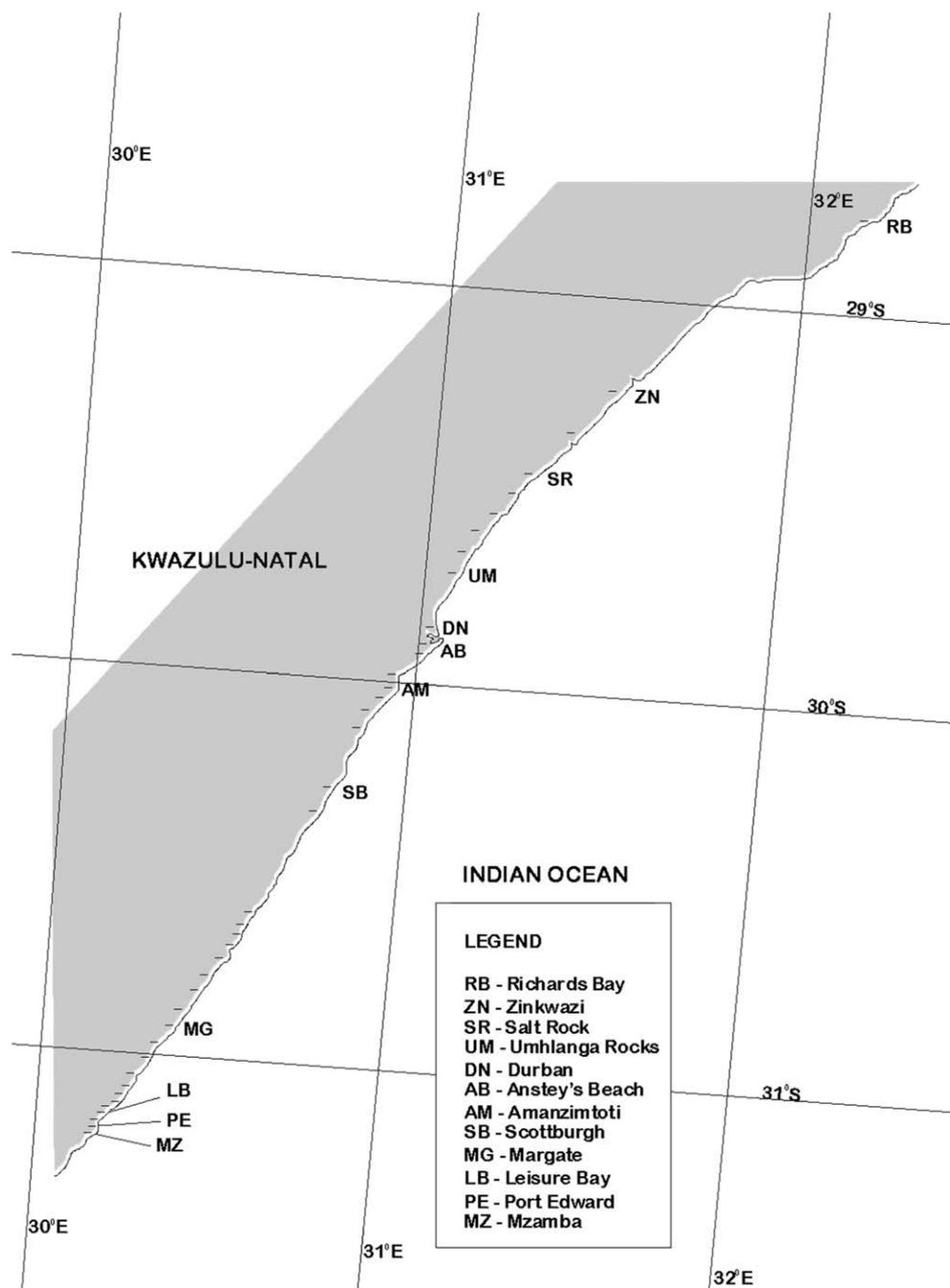


Fig. 1. A map of the east coast of South Africa, indicating the beaches where hosts of *Nesippus orientalis* specimens were caught in the Natal Sharks Board's nets. (Abbreviations used in Table 1, Figs. 2 and 3: RB = Richards Bay, ZN = Zinkwazi, SR = Salt Rock, UM = Umhlanga Rocks, DN = Durban, AB = Anstey's Beach, AM = Amanzimtoti, SB = Scottburgh, MG = Margate, LB = Leisure Bay, PE = Port Edward, MZ = Mzamba.)

(Table 1, Fig. 1). Collected specimens were preserved in 70% EtOH. Female specimens of *N. orientalis* were selected from each of nine different host species (i.e. *Carcharodon carcharias* Linnaeus, 1758; *Isurus oxyrinchus* Rafinesque, 1810; *Carcharias taurus* Rafinesque, 1810; *Carcharhinus brachyurus* Günther, 1870; *C. limbatus* Valenciennes, in Müller and Henle, 1839; *C. leucas* Valenciennes, 1839; *Sphyrna lewini* Griffith and Smith, in Cuvier, Griffith and Smith, 1834; *S. mokarran* Rüppell, 1837 and *S. zygaena* Linnaeus, 1758), all members of the Galeomorphii, caught at various localities along the KwaZulu-Natal coast (Fig. 1, Table 1). Additionally, female individuals of *N. vespa* Kirtisinghe, 1964 collected from *Rhina ancylostoma* Bloch and Schneider, 1801 were selected as outgroup. Host nomenclature and systematics conform to Compagno (1999). A stereomicroscope was used to verify the species identification of the selected individuals according to the presence of dorsal plates on the fourth thoracic segment (Cressey, 1967).

2.2. DNA extraction

DNA was extracted using the DNeasy kit for animal tissues according to the manufacturer's instructions (Qiagen) and with final elution using ddH₂O.

2.3. Polymerase chain reaction

The partial mitochondrial *COI* gene was amplified via the polymerase chain reaction (PCR) (Saiki et al., 1988), using the primers LCO1490 and HCO2198 (Folmer et al., 1994). Two microlitres of the diluted DNA solution was added to a 25 μ l PCR reaction containing: 1X *Taq* buffer, 2.5–4 mM MgCl₂, 2 mM dNTPs (Promega), 12.5 pmol of each primer and 0.75–1 units of Supertherm *Taq* polymerase (Southern Cross Biotechnology) and ddH₂O to make up the total reaction volume. Cycling parameters in a Geneamp[®] PCR System 9700 (Applied Biosystems) included the following: an initial denaturation at 95 °C (3 min), followed by 35–40 cycles of 95 °C (30 s) denaturation, 45–55 °C (30 s) annealing and 72 °C (45 s) extension. A final extension at 72 °C (5 min) completed the amplification. Amplified samples were refrigerated.

Five microlitres of PCR products were electrophoresed through a 1.5% agarose gel and visualized with ethidium bromide under ultraviolet light. PCR products were purified using ethanol/ammonium acetate precipitation or the High Pure[™] PCR Product Purification Kit (Boehringer Mannheim). Purified products were eluted in ddH₂O.

2.4. Sequencing

Cycle sequencing was performed in 10 μ l volumes, containing approximately 100 ng of purified DNA as template, 1.6–3.2 pmol primer (either LCO1490 or HCO2198) and 2 μ l of ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Cycle sequencing was performed in a Geneamp[®] PCR System 9700 and nucleotide sequences were determined through an ABI 3100 automated sequencer (Applied Biosystems). Sequences were generated once from both strands of DNA and the consensus of each individual was obtained through alignment and inspection in Sequence Navigator 1.01 (Applied Biosystems). No ambiguous bases were identified. Consensus sequences were aligned, under default settings, using Clustal X (Thompson et al., 1997). Nucleotide sequences were unambiguously translated to amino acid sequences using MacClade 4.0 (Maddison and Maddison, 2001) and thus confirmed as of mitochondrial origin. Longer sequences were trimmed so that all sequences used in the analyses were of equal length. Sequences were deposited in GenBank under accession numbers GQ369463–GQ369507.

2.5. Analyses

Unique mtDNA haplotypes were identified by constructing a haplotype network following a statistical parsimony approach as implemented in TCS version 1.21 (Clement et al., 2000), based on 95% confidence of connections among haplotypes (Templeton et al., 1992). The analysis inferred an ancestral allele according to coalescence theory (Crandall and Templeton, 1993). Tajima's test for neutrality (Tajima, 1989) and indices of DNA polymorphism, i.e. haplotype (Nei, 1987) and nucleotide diversity (Tajima, 1983), were estimated in DnaSP version 4 (Rozas et al., 2003).

Phylogenetic analyses included representatives of the divergent lineages identified within *N. orientalis* (two haplotypes from clade I and four haplotypes of clade II), as well as sequences generated for *N. vespa* (GQ369506 and GQ369507) in the present study. The following outgroup sequences from GenBank were also included for comparative purposes: *Nesippus crypturus* (FJ447379) and representatives of the sister group Caligidae (Huys et al., 2007; Dippenaar, in press), i.e. *Caligus diaphanus* (EF065616), *C. clemensi* (AM235887), *C. elongatus* (AY386273), *Lepeophtheirus salmonis* (EU288263), *L. pectoralis* (AY861364) and *L. hippoglossi* (AY861362). Uncorrected pairwise genetic distances among the taxa were estimated in PAUP version 4* (Swofford, 2002). Model parameters, such as base frequencies, Ti:Tv ratio, proportion of invariable sites (I) and the α value of the gamma distribution (rate variation among sites) were estimated as part of a maximum likelihood (Felsenstein, 1981) search in PAUP. The estimated parameters were used in a neighbour-joining (Saitou and Nei, 1987) and maximum likelihood estimation of phylogenetic relationships using PAUP*. Bootstrap (Felsenstein, 1985) support for nodes was based on 1000 replicates.

Bayesian inference was conducted using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). We used random starting trees and flat priors. Two independent MCMC runs of 1 million generations were done and split frequencies recorded every 1000th generation. This stabilized at 0.003 indicating convergence. Tree sampling was done every 100th generation and a burn-in of 1000 sampled trees ensured sampling from the region of stationarity.

3. Results

Not all DNA extractions or PCR amplifications were successful. Since some copepods were collected from hosts that were caught, frozen and defrosted again, it may have resulted in the degeneration of the copepod DNA thus resulting in unsuccessful DNA extraction. The final data set comprised 43 ingroup sequences of 525 nucleotides, with 114 variable sites defining 35 unique haplotypes (Fig. 2). Transitions accounted for about 68% of the variable sites, with three sites having both a transition and transversion. Substitution ratios at the different codon positions were 10.5% (1st), 0.9% (2nd) and 88.6% (3rd), respectively. Most of the substitutions occurring among the ingroup were synonymous with only two non-synonymous substitutions. A first codon position transition occurred in SFSR03 from GGG to AGG at position 61 resulting in a change in the coded amino acid from Glycine to Serine. RTRB05 had a second codon position transversion from GTG to GGG in position 74, changing the coded amino acid from Valine to Glycine.

Tajima's test was non-significant ($P > 0.1$). Overall, haplotype ($h = 0.988$, $SD = 0.009$) and nucleotide diversity ($\pi = 6.47\%$, $SD = 1.2\%$) estimates were high, indicating considerable diversity within this wide-ranging species over the range sampled off the South African coast. The TCS analysis could join haplotypes separated by a maximum of nine steps based on the 95% confidence criterion. The most striking observation from the haplotype network

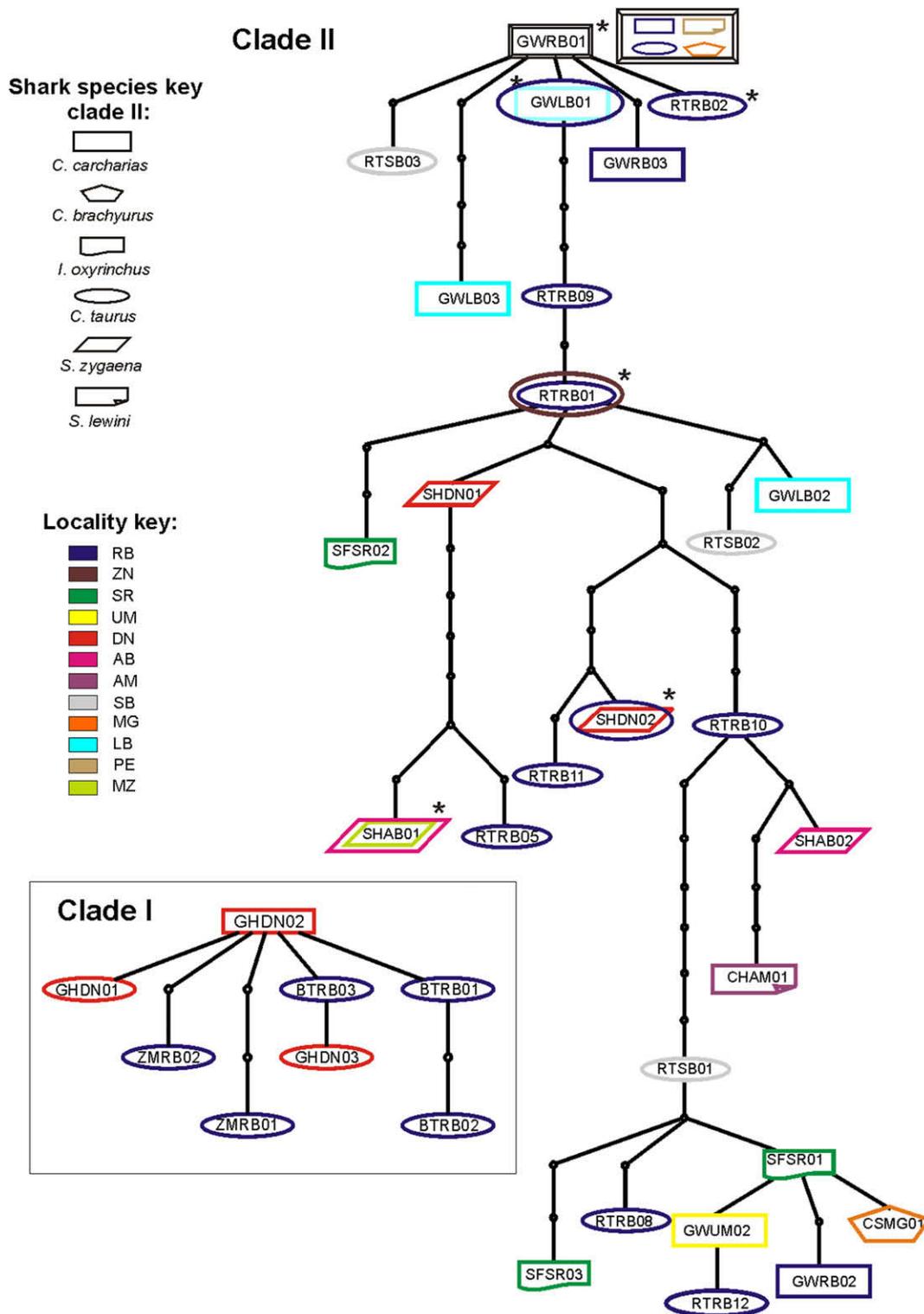


Fig. 2. TCS network depicting the relationships among the 35 unique *Nesippus orientalis* COI haplotypes separated into two distinct clades: clade I consists of eight haplotypes from eight individuals and three different hosts (*S. mokarran*, *C. limbatus*, *C. leucas*); clade II consists of 27 haplotypes from 35 individuals collected from six different shark hosts (*S. lewini*, *S. zygaena*, *C. brachyurus*, *I. oxyrinchus*, *C. taurus* and *C. carcharias*). Each connecting line represents a single mutational change and small circles represent unsampled or extinct haplotypes. Sampling localities (see Fig. 1) are colour coded for both clades. For clade II, the different shark species are distinguished by different shapes for each detected *Nesippus* haplotype. Shared haplotypes are indicated by asterisks.

was the presence of two divergent clades that could not be joined with confidence by the TCS analysis: clade I comprised eight haplotypes, represented by copepods ($n = 8$) collected from Great hammerhead (*Sphyrna mokarran*), Blacktip (*Carcharhinus limbatus*) and Zambezi (*C. leucas*) shark hosts from Durban and Richards Bay

(central and northern KwaZulu-Natal coast); clade II comprised 27 haplotypes, including individuals ($n = 35$) collected from throughout the sampled region from six different shark hosts (Scalloped hammerhead (*Sphyrna lewini*), Smooth hammerhead (*S. zygaena*), Copper shark (*Carcharhinus brachyurus*), Shortfin mako

(*Isurus oxyrinchus*), Raggedtooth (*Carcharias taurus*) and Great white (*Carcharodon carcharias*). Diversity within the two clades was high in terms of haplotype diversity (clade I $h = 1$; clade II $h = 0.982$) indicating few shared alleles, while relatively low levels of nucleotide diversity (clade I $\pi = 0.59\%$; clade II $\pi = 1.47\%$) were estimated.

All individuals within clade I had unique haplotypes. Within clade II there were only a few instances of haplotype sharing, i.e. the inferred ancestral haplotype GWRB01 ($n = 4$) was shared by individuals from a Great white and Raggedtooth from Richards Bay, a Scalloped hammerhead from Port Edward and a Copper shark from Margate (north and south coasts); haplotype GWLB01 ($n = 2$) was recorded from a Great white caught off Leisure Bay and a Raggedtooth from Richards Bay (north and south coasts); RTRB02 ($n = 2$) from the same Raggedtooth host individual from Richards Bay; RTRB01 ($n = 2$) was represented by copepods from Raggedtooth sharks from Richards Bay and Zinkwazi (north coast); SHDN02 ($n = 2$) was recorded from a Smooth hammerhead from Durban and a Raggedtooth from Richards Bay and SHAB01 ($n = 2$) from Smooth hammerheads from Anstey's Beach and Mzamba. Neither the pattern of haplotype sharing, nor the relationships among haplotypes within clade II, appeared to be associated with the geographic location of the shark specimen, with particular host species, the particular sampled host individual, or with the sampling date (Fig. 2, Table 1).

In the final data set there were 12 instances, where 2 or 3 individuals from the same host individual were analysed (Table 1). Some of these copepods were genetically quite similar, especially in clade I (average number of mutations among copepods from the same host individual = 2.7 steps). In clade II there were, however, several instances, where genetically distinctive haplotypes were sampled from the same host individual (10–28 mutational steps, see Fig. 2).

The uncorrected sequence divergence estimates among the five selected haplotypes (representing the two clades) and the outgroup taxa are summarized in Table 2. In contrast to the estimates within the two clades (clade I, 0.8%; clade II 0.8–2.5%), the divergence between the two *N. orientalis* clades (average divergence 17.44%) was within the same range as among the three *Nesippus* species (~16.5–22.3%), as well as among the three species of both *Caligus* and *Lepeophtheirus* (~17–19.3%).

Model parameters were as follows: base frequencies ($A = 0.26$, $C = 0.13$, $G = 0.18$, $T = 0.43$); Ti/Tv ratio 6.69; proportion of invariable sites 0.49 and $\alpha = 0.51$, indicating some rate heterogeneity among sites. The phylogenetic analyses yielded congruent topologies in most respects. However, the relationships among the *Nesippus* species, including the two clades within *N. orientalis*, were

unresolved in the ML topology while both ML and NJ clustered *C. diaphanus* and *C. elongatus* as sister taxa. Only the Bayesian phylogram is presented in Fig. 3, but the NJ and ML support values are also indicated. From the sequence divergence estimates and their reciprocal monophyly in the phylogeny (78% NJ bootstrap support, 1.0 posterior probability), clades I and II within *N. orientalis* appeared to represent two distinct sister lineages.

4. Discussion

The overall diversity of *N. orientalis* individuals, along the sampled area of the east coast of KwaZulu-Natal, is high as it is evident from the relatively high estimates of the haplotype ($h = 0.988$) and nucleotide ($\pi = 6.47\%$) diversities. This may be indicative of a stable population with a large long-term effective population size or an admixed sample of individuals from historically separated populations (Avice, 2000), subjected to drift and limited gene flow.

However, according to the haplotype network (Fig. 2) and the phylogeny (Fig. 3) the partial COI sequences examined among *N. orientalis* specimens clearly represent two highly supported and distinct lineages. The divergence between the two clades (17.44%) is clearly at a level expected for interspecific rather than intraspecific relationships in crustaceans. This value is comparable to the more than 17% and 18% sequence divergence between conspecific populations of the harpacticoid copepods, *Tigriopus californicus* (Burton, 1998; Burton and Lee, 1994, respectively), is lower than that obtained for conspecific populations of *Cletocamptus deitersi* (25%) (Rocha-Olivares et al., 2001), but higher than the comparable K2P distance for conspecific populations of the siphonostomatoid *Caligus elongatus* (12%) (Øines and Schram, 2008). Sequence divergence of 16–22% exists between previously defined *Nesippus* species (Table 2) which agrees with previous findings of 13–22% sequence divergence in congeneric species of copepods (Bucklin et al., 1999). Therefore, clades I and II probably represent separate species under the phylogenetic species concept. Even with the more than 17% divergence between the two clades of *N. orientalis* individuals, no amino acid substitutions occurred between the two clades with two amino acid substitutions occurring within clade II (0.8–2.5% divergence). Isofemale lines of *T. californicus* showed 15.4% divergence which all constituted synonymous substitutions (Burton and Lee, 1994), while Burton (1998) found only two amino acid substitutions between populations with a 22% divergence. From this it is clear that the sequence divergence and number of amino acid substitutions are probably not related and does not agree with Lefebvre et al. (2006) that there is mostly no amino acid divergence within species. These

Table 2
Uncorrected pairwise percentage sequence divergence among *Nesippus orientalis* haplotypes and relative to outgroups used in the present study, based on partial sequences of the mitochondrial DNA cytochrome oxidase subunit 1 gene. Intra-clade divergences for *Nesippus orientalis* clades I and II are in bold. Genbank accession numbers: *Caligus diaphanus* (EF065616), *C. clemensi* (AM235887), *C. elongatus* (AY386273), *Lepeophtheirus salmons* (EU288263), *L. pectoralis* (AY861364), *L. hippoglossi* (AY861362), *Nesippus crypturus* (FJ447379), *N. vespa* (GQ369507), GHDN02 (GQ369463), ZMRB01 (GQ369469), RTZN01 (GQ369493), GWRB01 (GQ369471), SHAB01 (GQ369497), RTSB01 (GQ369490).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	<i>L. salmonis</i>	–												
2	<i>L. pectoralis</i>	18.18	–											
3	<i>L. hippoglossi</i>	19.45	17.18	–										
4	<i>C. diaphanus</i>	21.92	20.52	19.56	–									
5	<i>C. clemensi</i>	19.77	20.85	16.82	17.00	–								
6	<i>C. elongatus</i>	22.49	23.40	22.36	19.29	18.75	–							
7	<i>N. crypturus</i>	21.45	22.74	18.65	19.96	16.82	22.54	–						
8	<i>N. vespa</i>	20.83	20.56	22.72	21.12	20.02	22.43	18.24	–					
9	GHDN02	21.97	22.52	18.44	21.89	20.29	21.94	16.45	17.71	–				
10	ZMRB01	22.40	22.73	19.03	22.09	20.68	22.14	16.47	17.93	0.81	–			
11	RTZN01	24.61	24.08	23.45	21.66	21.87	24.55	18.79	22.14	17.51	17.77	–		
12	GWRB01	24.43	23.81	23.05	21.19	22.18	24.65	18.40	21.17	17.09	17.33	0.80	–	
13	SHAB01	25.31	23.27	23.09	21.01	23.31	25.28	18.60	22.28	17.41	17.64	1.93	2.21	–
14	RTSB01	24.07	24.20	22.85	21.68	22.67	24.80	18.23	22.28	17.25	17.48	1.13	1.26	2.53

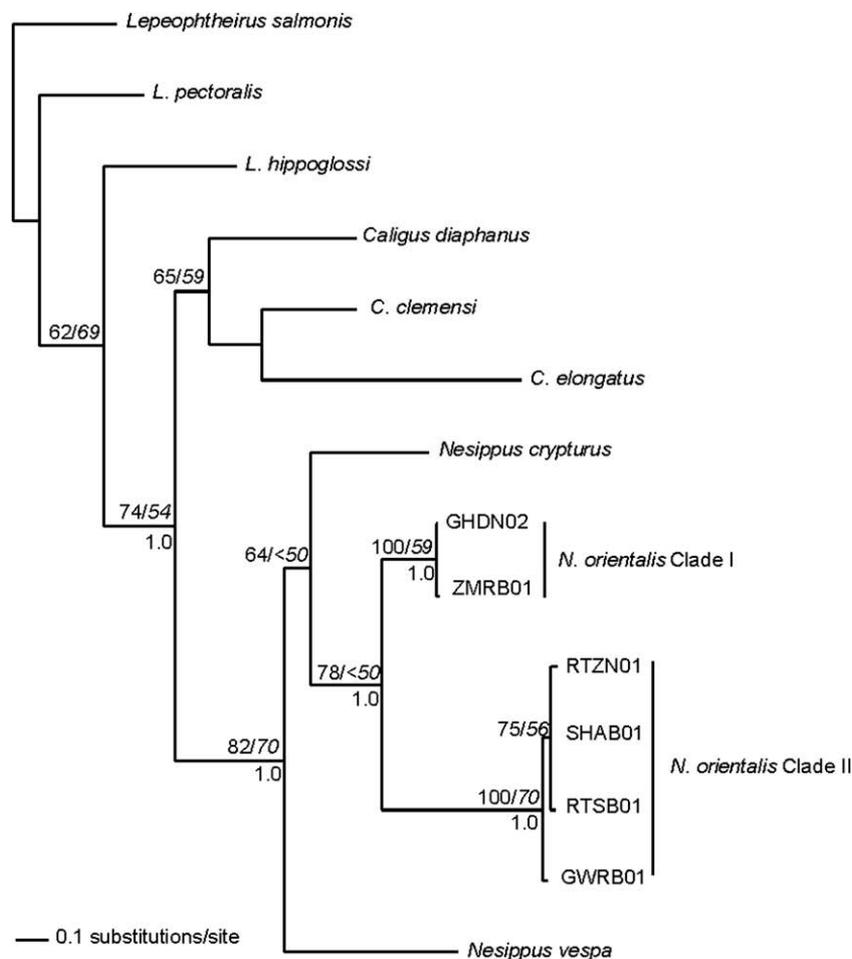


Fig. 3. Phylogenetic relationships among *Nesippus* species sampled from the South African coast based on Bayesian analysis of the partial mtDNA COI gene. *Caligus* and *Lepeophtheirus* species were specified as outgroups. Bootstrap support values (NJ/ML) and Bayesian posterior probability values (≥ 0.95) are indicated at nodes. Genbank accession numbers: *Caligus diaphanus* (EF065616), *C. clemensi* (AM235887), *C. elongatus* (AY386273), *Lepeophtheirus salmonis* (EU288263), *L. pectoralis* (AY861364), *L. hippoglossi* (AY861362), *Nesippus crypturus* (FJ447379), *N. vespa* (GQ369507), GHDN02 (GQ369463), ZMRB01 (GQ369469), RTZN01 (GQ369493), GWRB01 (GQ369471), SHAB01 (GQ369497), RTSB01 (GQ369490).

findings, together with the knowledge that most genetic analyses of species boundaries in Crustacea confirm or reveal the existence of cryptic species (Knowlton, 2000), necessitate a thorough morphological re-examination of *N. orientalis* specimens collected from *S. mokarran*, *C. limbatus* and *C. leucas*. Morphological information obtained from such a study will establish whether individuals in clade II indeed represent a cryptic/sibling species or rather a pseudo-sibling species (i.e. due to inadequate study of morphological features (Knowlton, 1993)) to those in clade I. If indeed these are cryptic species, one is likely not to observe any morphological differences.

The distinction between the two lineages was not related to locality or date of capture. Specimens of clade I were collected from hosts belonging to two different families, i.e. Carcharhinidae (*C. limbatus* and *C. leucas* caught during 2002 at Richards Bay) and Sphyrniidae (*S. mokarran* caught during 2000 at Durban) (Fig. 2, Table 1). Specimens of clade II were collected from hosts representing four different families, i.e. Odontaspidae (*C. taurus* caught during 1997 and 1999 at Richards Bay, at Zinkwazi during 1998 and at Scottburgh during 2002); Lamnidae (*I. oxyrinchus* caught during 2002 at Salt Rock and *C. carcharias* caught during 1999 at Richards Bay, at Umhlanga Rocks during 2000 and at Leisure Bay during 2002); Carcharhinidae (*C. brachyurus* caught at Margate during 2004), and Sphyrniidae (*S. lewini* caught during 1998 at Amanzimtoti and Port Edward, and *S. zygaena* caught during 1995 at Mzamba and at Durban and Anstey's Beach during 2002). Although there

was overlap between the host ranges of the copepods with respect to the shark families that the two clades were recorded from, there appears to be host selection differences between the copepods from the two lineages. Future research should focus on providing a more detailed assessment of the distribution and host range of the two lineages and if host behavioural differences may impact host selection. This may be possible since it is presumed that the currents caused by the movements or respiration of hosts are among the factors directing a copepod to them, while chemoreception may be involved on the part of the copepod (Kabata, 1981). Even though factors that limit species distributions might also be expected to demarcate genetically distinct lineages within species (Burton, 1998), no such factors are currently known for *N. orientalis* individuals.

Fairly high diversity levels were found within each clade (clade I $h = 1$; clade II $h = 0.982$) with no shared haplotypes in clade I and only a few shared haplotypes in clade II and relatively low levels of nucleotide diversity (clade I $\pi = 0.59\%$; clade II $\pi = 1.47\%$). This may be indicative of rapid population growth from an ancestral population with a small effective population size which had sufficient time for the recovery of the haplotype variation through mutation (Avice, 2000). Haplotype relationships within the two clades did not appear to follow an easily identifiable pattern. For example, four specimens collected from four different hosts, from three different localities on the north and south coast during four different years, shared the same haplotype (the inferred ancestral

haplotype of clade II, see Fig. 2, Table 1). The sharing of the ancestral haplotype (GWRB01) specifically and haplotypes GWLB01 and RTRB01 could reflect the retention of ancestral polymorphisms since they were more central haplotypes in the network, while the sharing of haplotypes RTRB02, SHDN02 and SHAB01 could indicate recent dispersal due to the short free-living stage and/or host movements and interactions. It is, however, also possible that more extensive sampling, especially the analyses of more individuals from the same host individuals, may reveal more instances of shared haplotypes and haplotype frequency differences as observed in other siphonostomatoid parasites (see for example Øines and Heuch, 2007).

The diversity within the two clades (average divergence = ~1.6%) was an order of magnitude less than that estimated between them, but the level of differentiation within the clades compares well with previous studies. It is higher than that found among isofemale lines of *T. californicus* from six populations (<1%) (Burton and Lee, 1994) and among geographically separated populations of *Calanus helgolandicus* (0.5–0.8%) (Hill et al., 2001), similar to that found within each type of *Cletocamptus deitersi* (0.2–1.7%) (Rocha-Olivares et al., 2001) and less than that among conspecific *Pseudocalanus* individuals ($\pm 2\%$) and between *Calanus pacificus* subspecies (3%) (Hill et al., 2001). These values confirm previous conclusions by Avise (2000) and Waugh (2007) that the general intraspecific divergence in mitochondrial genes (specifically COI) is usually less than 1% and rarely more than 2%, while intraspecific variation is generally less than 10% of that observed between species.

Understanding of processes underlying phylogeographic patterns requires extensive temporal and spatial sampling. However, in populations of *T. californicus* phylogeographic patterns were more adequately described using few sequences from many populations than it would have been using many sequences from few populations (Burton, 1998). The current sampling was sufficient to indicate high diversity and the possible existence of cryptic species in *N. orientalis* individuals. More extensive sampling may be needed to attempt to understand the processes underlying this diversity. However, increased sampling of *N. orientalis* specimens from more hosts, will be exceptionally daunting, given the life history of *N. orientalis* and the need for destructive sampling of hosts. Furthermore, doing laboratory crossings between individuals from the two lineages to determine whether they are reproductively isolated, will be impossible due to their hosts and infection site on the hosts. Therefore, determining the taxonomic position of the two lineages will have to rely on morphology and sequence divergence. The partial COI gene sequenced in the current study proved to be efficient in distinguishing diversity on the intraspecific and interspecific levels. However, even though species delimitation based on COI alone is done as part of DNA barcoding (Hebert et al., 2003; Waugh, 2007) it is imperfect and should be combined with at least one nuclear gene to be more robust (Lefebure et al., 2006).

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