UNIVERSITY OF CALIFORNIA, SAN DIEGO

Speciation in the Open Ocean: the Phylogeography of the Oceanic Copepod Family Eucalanidae

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Oceanography

by

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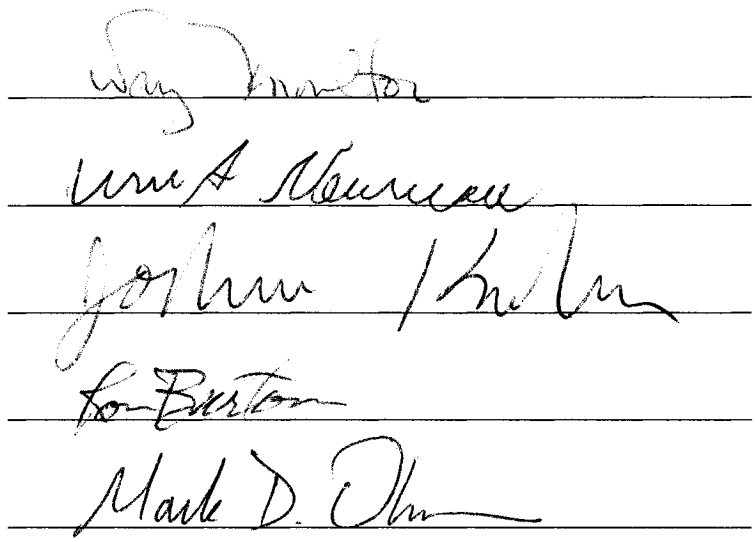

Chair

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To

Grzegorz M. Dziadurski

For all his support

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ABSTRACT OF THE DISSERTATION

Speciation in the Open Ocean: the Phylogeography of the Oceanic Copepod Family Eucalanidae

by

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Doctor of Philosophy in Oceanography
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In this dissertation I address how open ocean plankton populations can diverge genetically, and ultimately form new species. Research focuses on the oceanic calanoid copepod family Eucalanidae. A global phylogenetic study of the Eucalanidae revealed substantial cryptic diversity at the species level. Genetic data from both mitochondrial and nuclear gene loci support 13 new genetic lineages within the Eucalanidae. These new lineages range from 1.6% to 23.2% divergent from their closest relatives (16S rRNA, p-distances) suggesting that although some diverged relatively recently, other represent quite ancient speciation events. *Rhincalanus nasutus* was found to be a cryptic species complex, with at least 7 genetically distinct, predominantly allopatric populations worldwide. A molecular phylogeny for the family supports monophyly of the Eucalanidae, all four eucalanid genera, and the 'pileatus' and 'subtenuis' species groups.

A distinct genetic form of *Eucalanus hyalinus* s. l. was identified in subtropical waters worldwide. The species name *Eucalanus spinifer* T. Scott was removed from synonymy with *E. hyalinus* and applied to the smaller form, elevating the number of species in *Eucalanus* to a total of six. Adult females of the sister species can be distinguished morphologically by morphometric, shape, and size characters. Results suggest that species originally identified through molecular markers will also be distinguishable by morphological characters.

A global population genetic study of the sympatric sister species *Eucalanus hyalinus* and *E. spinifer* demonstrated that oceanic zooplankton species can be highly genetically structured on macrogeographic spatial scales, despite experiencing extensive gene flow within features of the large-scale ocean circulation. Habitat discontinuities at the boundaries of subtropical gyres, and continental landmasses, acted as effective barriers to gene flow for both species. The sister species differed in their global population genetic structures as well as in their oceanographic distributions. Species-specific differences in habitat were an important factor determining patterns of dispersal between populations of each species worldwide.

Two unique spliceosomal introns were discovered in the nuclear gene elongation factor 1-α in *Rhincalanus* and *Eucalanus*, and the locus was found to have limited phylogenetic utility due to difficulty in identifying orthologous, functional gene copies.

Chapter I

Introduction to the Dissertation

Speciation has been a topic of central interest to biologists since Darwin published his *The Origin of Species* in 1859. Myriad models of the speciation process have been proposed (Bush 1994; Coyne and Orr 1997; Doebeli and Dieckmann 2000; Rice and Hostert 1993), and they can be broadly classified into three main geographic types: the allopatric, parapatric, and sympatric models. Speciation under allopatry is envisioned to occur when populations become geographically isolated, undergo a process of differentiation, and then do not introgress upon secondary contact. This model, with E. Mayr (1942, 1959b) as its strongest proponent, was long thought to be the most important, if not the only, way by which new species could arise in nature. One variant of this model is the peripheral isolates model, termed peripatry, in which one of the isolated populations is envisioned to be a small population. The parapatric model (Endler 1977; Felsenstein 1981) highlights the importance of a gene flow – selection balance, and makes the point that speciation can occur without strict geographic isolation, if the magnitude and geographic distance of gene flow are sufficiently small to be overcome by the force of selection. The third model, speciation in sympatry, envisions populations that diverge while maintaining broad regions of biogeographic range overlap. Ecological specialization and/or divergence in reproductive characters are the key features that allow differentiation to proceed (Diehl and Bush 1989; Doebeli and Dieckmann 2000, 2003). The importance of these different modes of speciation continues to be debated, but authors working mainly

with terrestrial organisms consider the allopatric family of models as most likely to contribute the bulk of new species arising in nature (Bush 1975; Chesser and Zink 1994; Lynch 1989).

The process of speciation is poorly understood in the marine pelagic environment. In marine systems, the geographic isolation necessary in the initial step of population differentiation for allopatric or peripatric speciation may rarely occur. This is particularly true for high-dispersal taxa that are transported long distances in ocean currents, either as adults or during a long planktonic larval stage. Such high-dispersal taxa are also typically characterized by large population sizes and extensive biogeographic ranges, in addition to high gene flow between populations. Marine holozooplankton populations represent an extreme case of such taxa, and yet we know that speciation must have occurred in the open ocean, due to the existence of many clades that are entirely oceanic in distribution. How, then, do such populations speciate? Have we underestimated the potential for geographic isolating barriers in the marine pelagos? Or does speciation in the open ocean more often occur under parapatric or sympatric models?

These questions are not new, and zooplankton taxonomists and biogeographers have long been seeking clues that would answer this paradox. Monographic works on several zooplanktonic taxa (Berner 1957; McGowan 1960; Brinton 1962; Brodsky 1965; Lang 1965; Frost and Fleminger 1968; Frost 1969; Fleminger 1973, 1975; Fleminger and Hulsemann 1974; Mullin and Evans 1976; Nishida, 1985; Markhaseva,

1996) have elucidated patterns in biogeographic distributions, and in some cases morphological divergence, which shed light on this problem.

Although much of the plankton research of the 1960s and 70s focused on describing the biogeographic distributions of planktonic species and in elucidating how their distributions may be linked to oceanographic water masses, a number of authors speculated on possible speciation modes and isolating mechanisms. A few major conclusions emerged from this work. These include: (1) the speculation that speciation by allopatric geographic isolation is the dominant mode of speciation in planktonic organisms (McGowan 1971; Fleminger 1975; Fleminger 1986), (2) the hypothesis that glacial-interglacial cycles of the Pleistocene have been an important environmental factor controlling genetic connectivity, and therefore speciation, between planktonic populations (which implies that extant species are relatively young; Brinton 1957, 1962; Fleminger 1986), (3) the proposition that strong upwelling regions (Fleminger 1986), oligotrophic waters (Park 1994), coastal geographic features (e.g., Gulf of California; Fleminger 1975; Fleminger et al. 1982), and strong environmental gradients (Fleminger and Hulseman 1987) serve as physical isolating barriers that cause speciation, and (4) the observation that reinforcement may play an important role in the development of morphological pre-zygotic isolating mechanisms and the maintenance of species boundaries for sympatric, congeneric, oceanic species (Frost and Fleminger 1968; Frost 1969). In addition, work by Brinton (1957, 1962), Lang (1965), and Frost (1969), among others, demonstrated that planktonic populations in separate hemispheres are not connected via submergence of animals

into deep water in tropical latitudes. These observations presented a new conundrum to resolve: whether or not, and to what extent, disjunct populations in subtropical or temperate latitudes are genetically connected. Many of the hypotheses, speculations, and paradoxes presented by these monographic biogeographic plankton studies can be addressed with genetic data, and research reported in this dissertation bears directly on the validity of some of these concepts (e.g., extant species' ages appear to substantially predate Pleistocene events).

Another line of investigation that provides insight into population divergence and speciation in the open ocean is the study of population genetic structure of zooplanktonic taxa. Studies of mtDNA haplotype distributions in oceanic marine zooplankton populations have found high levels of gene flow over broad spatial scales. Oceanic copepods, such as *Nannocalanus minor* (Bucklin et. al., 1996), *Calanus finmarchicus* (Kann and Wishner 1996; Bucklin et al. 2000; Bucklin et al. 1996; Bucklin and Kocher 1996), and *Undinula darwini* (Afanas'yev 1989) as well as the oceanic euphausiid *Meganyctiphanes norvegica* (Bucklin et al. 1997), exhibit little or no genetic structure across mesoscale oceanographic features, and only mild structure across ocean basins or current systems. Deeply divergent intraspecific phylogroups do not appear to be a common feature of these populations (but see Peijnenburg et. al. 2004), and observations of intraspecific levels of variation across ocean basins of 0.5-1% at the mitochondrial cytochrome oxidase I (CO1) gene locus (Bucklin and Lajeunesse 1994; Bucklin et. al. 2003) appear to be the norm. Within planktonic foraminifera, Darling et. al (2000) and Norris and de Vargas (2000) identified multiple

cryptic species and found identical SSU rRNA haplotypes distributed on opposite sides of the globe in three species (but see Darling et. al. 2004). These results suggest either ongoing or relatively recent gene flow between geographically very distant populations. Results from intertidal or estuarine copepods are, however, rather different. Studies by Lee (2000) and Burton (1998) on populations or nascent species of neritic *Eurytemora affinis* and the splash-pool copepod *Tigriopus californicus* find levels of genetic divergence of 18-19% at COI, demonstrating that deep divergences can and do develop given sufficiently low levels of genetic exchange between populations.

These studies suggest that oceanic zooplankton populations are typically characterized by high levels of gene flow among conspecific populations on meso- to ocean basin spatial scales. Given this pattern, how do populations of oceanic species initially become divergent?

The long-term objective of this research was to understand the process of speciation in open ocean planktonic organisms, and to elucidate the biogeographic, oceanographic, and morphological factors that may facilitate population divergence in oceanic populations. The research focused on one globally distributed, ecologically important family of calanoid copepods, the Eucalanidae. Specific objectives were 1) to develop a model system in which all cryptic species had been identified, 2) to infer a well-resolved species-level phylogeny for the family including all extant ESUs (evolutionary significant units), 3) to test two central hypotheses regarding the degree of biogeographic range overlap and morphological divergence between sister species

pairs, 4) to determine whether new cryptic species, initially identified by genetic data, would be differentiable by conventional morphological, taxonomic characters, and 5) to identify the oceanographic and geological features that act as important barriers to gene flow between conspecific populations of oceanic species. In order to address these research objectives, I conducted a global study of the biogeography, phylogenetics, population genetics, and morphology of species in the family Eucalanidae (Fig. 1, Appendix A).

Development of a model system in which speciation events can be confidently identified is an essential first step in being able to test evolutionary questions about the speciation process. Research presented in **Chapter II** laid these foundations for the larger objectives of the research. The family Eucalanidae comprised 23 described species in four circumglobal genera prior to my dissertation (Fleminger, 1973; Geletin 1976; Bjornberg, 1986a, 1986b; Bradford-Grieve 1994; Prusova et. al. 2001). Of these 23 previously described species, 20 were included in the phylogenetic analysis of the family in **Chapter II**. An additional 12 novel genetic lineages were identified, including representatives from all four eucalanid genera and all three major ocean basins worldwide. Of these new lineages, four are considered cryptic species, and the remaining eight may also deserve specific status following consideration of ecological and morphological characters. New genetic lineages were in some cases highly divergent from their closest relatives (e.g., *Pareucalanus* sp. and *P. sewelli*), and were found to occur in allopatry, parapatry, and sympatric relative to their closest congeners.

In **Chapter III**, I carefully examine and formally describe one of these new cryptic species. The name *Eucalanus spinifer* T. Scott 1894 is removed from synonymy with *E. hyalinus* (Claus, 1866) and is applied to the smaller of the two genetic forms, increasing the number of species in the genus *Eucalanus* to a total of six. Descriptions of both adult females and males are included, and characters are presented by which to distinguish sister species *E. spinifer* and *E. hyalinus*. This research provides some of the first evidence that species initially identified by genetic data will, at least in some cases, also be identifiable by morphological criteria. Research on this sister species pair is continued in **Chapter IV**.

In **Chapter IV**, I examine the global population genetic structure of a pair of circumglobal, oceanic species in order to identify the oceanographic or geological features that act as effective barriers to gene flow for open ocean plankton species. The specific objectives of this chapter are 1) to determine the spatial scale over which oceanic plankton species achieve panmixia on evolutionary timescales, 2) to identify the oceanographic features that act as barriers to gene flow, and 3) to examine whether the presence and efficacy of barriers to gene flow are congruent across a sympatric, circumglobal, sister species pair. The sister species were found to exhibit a common pattern of substantial genetic structure on large, macrogeographic spatial scales (between ocean basins and hemispheres), in combination with relative genetic homogeneity within subtropical gyre systems. Water mass boundaries at the edge of subtropical central waters and continental land masses were observed to act as effective barriers to gene flow for both species, although the impact of specific

barriers on the population genetic structure of each species varied across the species pair. The sister species were also observed to differ in their oceanographic distributions, despite broad sympatry over large portions of their biogeographic ranges. I suggest that these species-specific differences in distribution are a primary factor determining global population genetic structure.

In **Chapter V** I examine the molecular evolution of the nuclear gene elongation factor 1-α in the Eucalanidae, and discuss its possible utility for phylogenetic inference in studies of copepods. The nuclear gene was found to occur in multiple functional copies in the Eucalanidae, in addition to a number of apparently non-functional pseudogene copies. One observed intron was shared with other arthropod taxa (Brady and Danforth, 2004), and two new introns were observed which appear likely to have arisen recently, and independently, within the family Eucalanidae. The gene contained sufficient variation to be useful as a phylogenetic marker, but close genetic similarity between functional and non-functional copies made it difficult to identify orthologous gene copies with confidence, limiting the gene's potential use in phylogenetic research.

The final chapter of the dissertation, **Chapter VI**, summarizes the findings of the dissertation, discusses implications of the results, and outlines future research directions. In particular, I outline future efforts to test biogeographic and morphological hypotheses in relation to the eucalanid molecular phylogeny.

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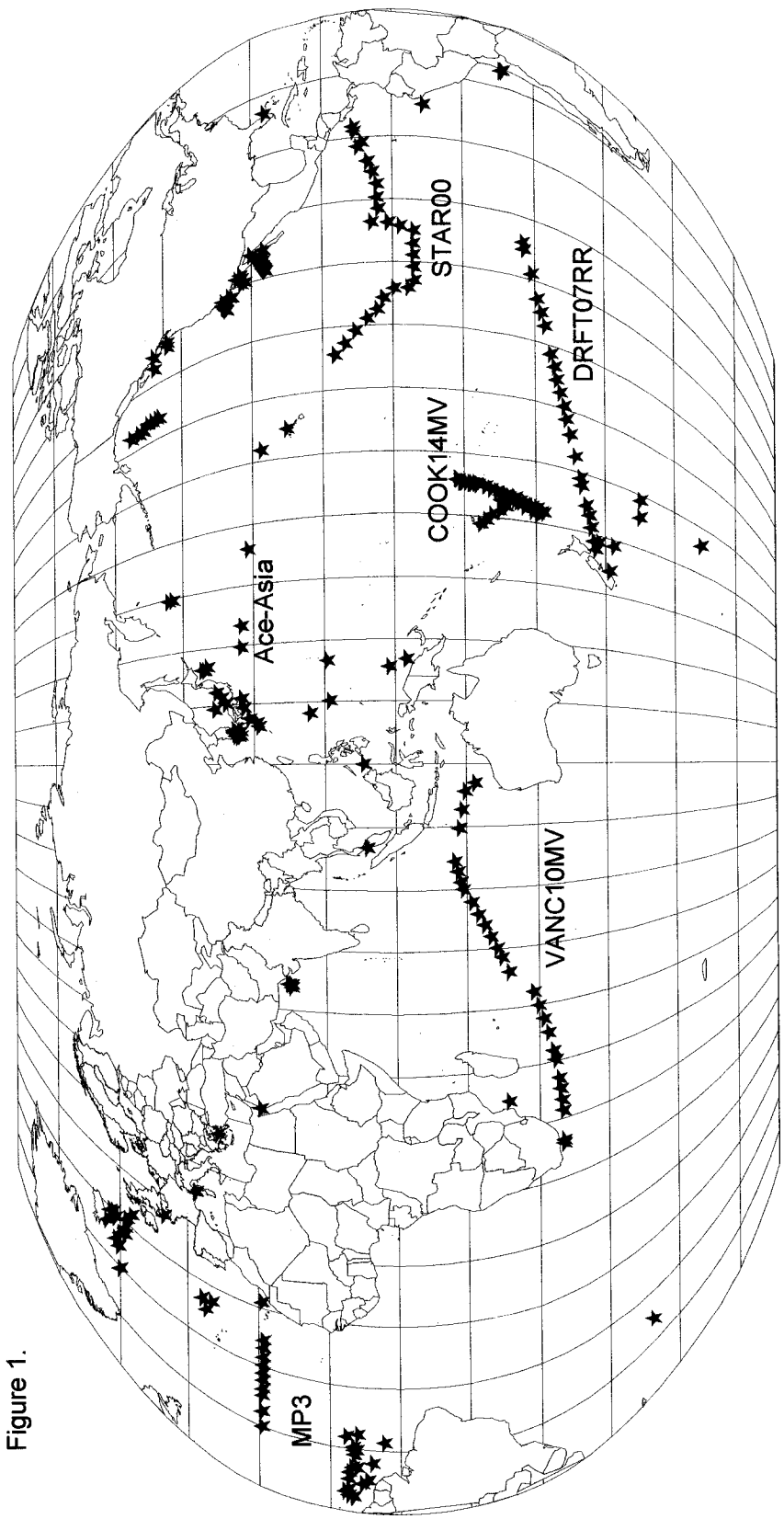

Figure 1. Geographic distribution of ethanol-preserved and frozen plankton samples acquired and used in the dissertation. Locations listed in Appendix A.

Cryptic speciation on the high seas; global phylogenetics of the copepod family Eucalanidae

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Few genetic data are currently available to assess patterns of population differentiation and speciation in planktonic taxa that inhabit the open ocean. A phylogenetic study of the oceanic copepod family Eucalanidae was undertaken to develop a model zooplankton taxon in which speciation events can be confidently identified. A global survey of 20 described species (526 individuals) sampled from 88 locations worldwide found high levels of cryptic diversity at the species level. Mitochondrial (16S rRNA, CO1) and nuclear (ITS2) DNA sequence data support 12 new genetic lineages as highly distinct from other populations with which they are currently considered conspecific. Out of these 12, at least four are new species. The circumglobal, boundary current species *Rhincalanus nasutus* was found to be a cryptic species complex, with genetic divergence between populations unrelated to geographic distance. 'Conspecific' populations of seven species exhibited varying levels of genetic differentiation between Atlantic and Pacific basins, suggesting that continental landmasses form barriers to dispersal for a subset of circumglobal species. A molecular phylogeny of the family based on both mitochondrial (16S rRNA) and nuclear (ITS2, 18S rRNA) gene loci supports monophyly of the family Eucalanidae, all four eucalanid genera and the 'pileatus' and 'subtenuis' species groups.

Keywords: copepods; open ocean; speciation; cryptic species; Eucalanidae; *Rhincalanus nasutus*

1. INTRODUCTION

Marine invertebrate species often exhibit high levels of gene flow between populations owing to effective transport of planktonic larvae on ocean currents (e.g. Palumbi 1992; Lessios *et al.* 1997; Bierne *et al.* 2003*a*,*b*). This high gene flow would be expected to oppose the processes of population differentiation and speciation. Large-scale studies to examine marine speciation in near-shore invertebrates include research on sea urchins (Palumbi *et al.* 1997; Lessios *et al.* 2001), gastropods (Reid *et al.* 1996), mussels (Ladoukakis *et al.* 2002; Bierne *et al.* 2003*a*), giant clams (Benzie & Williams 1997) and starfish (Williams 1997; Williams & Benzie 1998) among others. However, little attention has been focused on marine species that inhabit the vast expanse of the open ocean. How does speciation take place in the open ocean, where barriers to dispersal are particularly difficult to discern, and where species are planktonic throughout their entire life cycle? Few data are currently available to answer this question, despite its importance to understanding the universality of evolutionary processes in high gene flow systems. Recent work on oceanic Foraminifera (de Vargas *et al.* 1999, 2001, 2002; Stewart *et al.* 2001), coccolithophores (Sáez *et al.* 2003) and deep sea fishes (Miya & Nishida 1997) suggests that greater specificity in ecological and oceanographic habitat preferences than previously supposed may be an important component of differentiation in the open ocean.

Pelagic copepods are remarkably diverse despite many biological characteristics that should inhibit the speciation process. Oceanic copepod species typically have large population sizes, which extend over vast geographic ranges, and are planktonic throughout their entire life cycle. Many have the ability to tolerate large vertical gradients in environmental properties, which may predispose them to being able to survive in a variety of oceanographic environments. Despite these characteristics they are by far the most diverse taxon of the marine zooplankton, with approximately 1800 marine calanoid species reported worldwide (Mauchline 1998). However, sibling species are common in marine taxa (Knowlton 1993, 2000; de Vargas *et al.* 2003), and recent observations of copepod 'populations' exhibiting high levels of genetic divergence despite morphological conservatism suggests that for this group reproductive isolation may be uncoupled from morphological divergence (Bucklin *et al.* 1996, 1998; Rocha-Olivares *et al.* 2001; Lee & Frost 2002). This raises the possibility that copepod species, particularly those with circumglobal biogeographic distributions, may include multiple lineages that are evolutionarily distinct.

The calanoid copepod family Eucalanidae is an oceanic taxon whose member species are likely to have arisen in the open sea. Member species are ecologically prominent in subtropical, tropical, equatorial and temperate–boreal waters of the world ocean. Eucalanids can be extremely abundant, particularly in low-oxygen regions, and sometimes constitute almost 100% of the calanoid zooplankton fauna (Muniza & Kazmi 1995). The family comprises 23 described species in four circumglobal genera (*Eucalanus*, *Rhincalanus*, *Pareucalanus*, *Subeucalanus*). Early work by Lang (1965) and Fleminger (1973) established global descriptions for biogeographic distributions of species within the family and recognized four species groups among the 17 species within the genus *Eucalanus s. l.* These included the 'subtenuis', 'pileatus', 'elongatus' and 'attenuatus' species groups, all distinguished by characteristic features of the distribution of integumental pores on the exoskeleton as well as by the shape and position of seminal receptacles. Geletin (1976) elevated two of these

Table 1. Eucalanid taxa considered in this analysis, with their approximate biogeographic distributions (modified from Lang (1965) and Fleminger (1973)) and number of individuals analysed for DNA sequences of 16S rRNA, ITS2,18S rRNA and CO1. (For species in which multiple genetic lineages were identified, the original species descriptor and date are included with the first reference to the described species in the table. Type specimens were not examined and it is not currently known which of the genetic lineages corresponds to the original species description. Abbreviations for locations: C., central; CA, California Current; NA, North Atlantic; SWP, southwest Pacific; Sulu, Sulu Sea; K/PH, Kuroshio Current and Philippine Sea; PAC, Pacific; EP, eastern Pacific; WP, western Pacific.)

			no. of individuals sequenced			
genus	species (clade)	biogeographic distributions	16S	ITS2	18S	CO1
Eucalanus	*bungii* Giesbrecht, 1892	boreal, sub-polar, N. Pacific	18	4		10
	californicus Johnson, 1938	transition zone, N. Pacific	34	4		11
	elongatus (Dana, 1849)	tropical, Pacific and Indian	4	3		
	hyalinus (1) (Claus, 1866)	tropical–subtropical, circumglobal	45	3	2	> 250
	hyalinus (2)	tropical–subtropical, circumglobal	26	3		> 250
	inermis Giesbrecht, 1892	eastern tropical Pacific	20	4	2	9
Rhincalanus	*cornutus* Dana, 1849	tropical–subtropical, Atlantic	17	4		
	rostrifrons (EP) Dana, 1852	tropical–subtropical, E. + C. Pacific	18	4	2	20
	rostrifrons (WP)	tropical–subtropical, W. Pacific	19	5		
	gigas Brady, 1883	southern ocean, circumpolar	19	3	2	
	nasutus (CA) Giesbrecht, 1888	California Current	12	4		31
	nasutus (Peru)	Humboldt Current	10	4		6
	nasutus (Sulu)	Indo-west Pacific	8	4		5
	nasutus (K/PH)	Kuroshio Current, Philippine Sea	18	3		10
	nasutus (SWP)	southwest Pacific, subtropical	35	3		8
	nasutus (NA)	northern N. Atlantic	12	3	2	3
Pareucalanus	sp.	tropical–subtropical, circumglobal	22	6		
	sewelli (NA) (Fleminger, 1973)	tropical–subtropical, Atlantic	20	4		
	sewelli (PAC)	tropical–subtropical, Pacific	15	4		
	attenuatus (Dana, 1849)	equatorial, Pacific and Indian	23	7	2	
	parki (Fleminger, 1973)	temperate, N. Pacific	12	3		
	langae (Fleminger, 1973)	temperate, southern ocean	10	3	2	
Subeucalanus	*crassus* (NA) (Giesbrecht, 1888)	tropical–subtropical, Atlantic	8	3		
	crassus (SWP)	tropical–subtropical, Pacific	3	—	1	
	sp.	Korean Strait, East China Sea	8	4		
	longiceps (Matthews, 1925)	boreal–temperate, southern ocean	10	—	2	
	monachus (Giesbrecht, 1888)	tropical–subtropical, Atlantic	15	2		
	subtenuis (Giesbrecht, 1888)	tropical, circumglobal	35	2		
	mucronatus (Giesbrecht, 1888)	tropical, Indian and W. Pacific	5	2		
	pileatus (NA) (Giesbrecht, 1888)	tropical–subtropical, Atlantic	4	4		
	pileatus (PAC)	tropical–subtropical, Pacific	5	4		
	subcrassus (Giesbrecht, 1888)	tropical, Indo-Pacific	21	4		

species groups to generic status: the genera *Subeucalanus* (comprising 'subtenuis' and 'pileatus' species groups) and *Pareucalanus*. Bradford-Grieve (1994) subsequently included *Pareucalanus peruanus* (Volkov 1971), and Prusova *et al.* (2001) described a new species, *Subeucalanus flemingeri*, from the Persian Gulf. Including the four species in *Rhincalanus*, this brings the current total to 23 described species in the family (table 1). Bjornberg (1972, 1986) also proposed that the family Eucalanidae may be polyphyletic, based on observations of naupliar swimming behaviour and general morphology. She concluded that there were two distinct lineages in the family, and proposed that *Pareucalanus* and *Subeucalanus* be placed in their own family, the Subeucalanidae, in the superfamily Centropagoidea. Such a placement could imply colonization of oceanic waters by an ancestrally coastal/neritic group rather than *in situ* diversification in the open ocean, and is therefore an important hypothesis to test in the present study.

One eucalanid species of particular interest is the cosmopolitan *R. nasutus*. *Rhincalanus nasutus* is often very abundant in the surface waters of boundary currents and upwelling zones in all three major ocean basins, and is largely absent from central oligotrophic waters (Schmaus & Lehnhofer 1927; Lang 1965; Castro *et al.* 1993). The species is eurybathic (0–4800 m), and has centres of abundance at lower epipelagic and upper mesopelagic depths (Grice & Hulsemann 1965; Lang 1965; Roe 1972; Ohman *et al.* 1998). The species has never been examined on a global spatial scale.

The present study seeks to develop an oceanic zooplankton taxon for which the phylogeny is well resolved and cryptic species have been identified. This is a necessary first step to examining speciation in the open sea, and this study will serve as the foundation for ongoing work on evolution and speciation within the Eucalanidae. I sequenced mitochondrial and nuclear DNA from 20 species of eucalanid copepods from around the world to

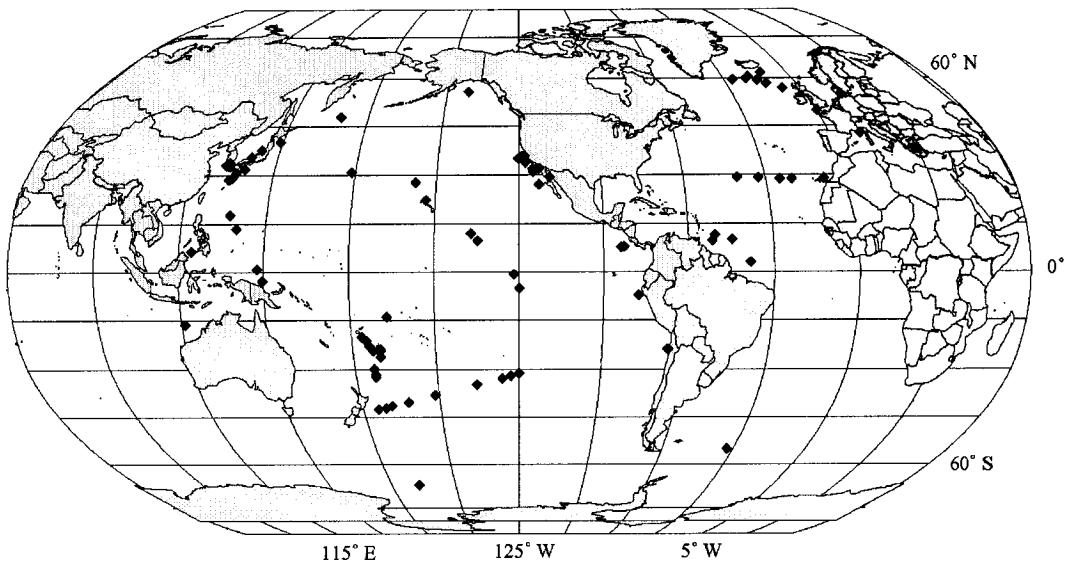

Figure 1. Collection sites for eucalanid specimens sequenced in this study.

address the following questions. (i) Do morphological species correspond to genetic clades? And are there genetic subdivisions within species? (ii) Do continents function as barriers to gene flow in circumglobal species? (iii) Do evolutionary relationships among eucalanid species match expectations based on morphological similarity? (iv) Are the Eucalanidae monophyletic?

2. MATERIAL AND METHODS

(a) *Sample collection and specimen identification*

Eucalanids were collected from a total of 88 locations worldwide (figure 1). Table 1 lists the species analysed, their approximate biogeographic distributions, and sample sizes for mitochondrial 16S ribosomal RNA (16S rRNA), nuclear internal transcribed spacer 2 (ITS2), nuclear 18S ribosomal RNA (18S rRNA) and mitochondrial cytochrome oxidase 1 (CO1) sequences. Specific sampling localities can be found in electronic Appendix A (available on The Royal Society's Publications Web site). The number of collecting localities for each species varied according to its biogeographic distribution, ranging to a maximum of 23 sampling locations for the circumglobal species *R. nasutus*. A total of 526 individuals were sequenced (16S rRNA) from 20 out of the 23 described species in the family. Samples on four major cruises were collected with 333 μm mesh plankton nets towed obliquely to either 200 m or 800–1000 m depth. The remaining samples were collected with a variety of sampling gear. Samples were either preserved in 95% ethyl alcohol, or frozen immediately in liquid nitrogen or a −80 °C freezer.

Specimens were individually sorted and identified to species following Fleminger (1973), Bradford-Grieve (1994) and Lang (1965). Species requiring integumental pore analysis for accurate identification, including *Pareucalanus attenuatus*, *P. sewelli*, *Subeucalanus subtenuis*, *S. mucronatus*, *S. crassus*, *S. pileatus* and *S. subcrassus*, were identified by removing the anterior portion of the prosome for DNA sequencing, and subjecting the posterior prosome and urosome to integumental pore analysis (as described in Fleminger 1973). In a few cases, digestion of the whole specimen of *S. pileatus* and *subcrassus* was necessary to obtain sufficient DNA for amplification. In these cases, voucher specimens of these species from the same samples were identified by integumental pore analysis.

(b) *DNA extraction, PCR and sequencing*

The 16S rRNA locus was used to screen individuals within a species for the presence of cryptic genetic lineages. Any lineages identified were then included in the ITS2 dataset by sequencing 2–7 individuals from the new lineage. Limited data for CO1 are presented here, primarily to enable comparison with other copepod groups. A pilot study demonstrated limited usefulness of CO1 as a phylogenetic marker owing to high levels of saturation.

DNA was extracted from individual copepods using either a lysis buffer protocol (Lee & Frost 2002) or the QIAGEN DNeasy tissue kit. Primers and conditions used in PCR amplification reactions can be found in electronic Appendix B. DNA sequencing was carried out on either an ABI 373 or MegaBACE 500 automated DNA sequencer.

Additional RNA/cDNA experiments were undertaken for CO1 with *E. hyalinus* 2 ($n = 2$) and *R. nasutus* (CA, $n = 3$, SWP, $n = 4$) to ensure that sequences obtained from genomic DNA based PCR reactions were expressed gene products. Synthesized cDNA was PCR amplified, cloned, sequenced and compared with sequences obtained from genomic DNA based PCR amplifications. ITS2 cloning experiments were also undertaken with two eucalanid species (*S. subtenuis* and *S. mucronatus*), in which three clones containing post-PCR ITS2 inserts were sequenced for each of two individuals. New sequence data obtained in this study can be found under GenBank accession numbers AY335822–AY335899 and AY371083–AY371094.

(c) *Sequence alignment and phylogenetic reconstruction*

Multiple sequence alignments were performed using CLUSTALW (Thompson *et al.* 1994), followed by manual editing as necessary in MACCLADE (Maddison & Maddison 2000). A series

of five alignments of the 16S rRNA data were generated using a variety of gap opening and extension costs. Alignments were then compared with published secondary structures for arthropod mitochondrial 16S rRNA genes, including *Eurytemora affinis* (Lee 1997), *Artemia salina*, *Drosophila melanogaster* (Cannone *et al.* 2002), as well as insect structures published by Buckley *et al.* (2000). Indels present within the eucalanid sequences were largely confined to helices 61, 75 and 84 in Domains IV and V (notation as in Buckley *et al.* 2000). A total of 81 sites that could not be confidently aligned were deleted, resulting in a 245 bp final alignment. The final 16S rRNA dataset contained 131 variable sites, 125 of which were parsimony informative. A genus-level 16S rRNA alignment was also generated for *Rhincalanus* sequences, from which no characters were deleted. The final ITS2 alignment (511 bp in length) contained 16 indels, 13 of which were a single nucleotide in length. The 18S rRNA sequences were highly conserved, and the final alignment was unambiguous.

Phylogenetic analyses for all datasets were conducted with maximum-parsimony (MP), maximum-likelihood (ML) and Bayesian methods. MP and ML analyses were performed with PAUP* 4.10b (Swofford 2002). MP analysis for each dataset was repeated 1000 times with random sequence addition, to explore tree space for multiple optima. For ML and Bayesian analyses, the appropriate model of molecular evolution was selected by the Akaike Information Criterion as implemented in MODELTEST (Posada & Crandall 1998). Parameter values from MODELTEST were used as a starting point, with subsequent refinement through an iterative process including: (i) heuristic searching of tree space; and (ii) re-estimation of parameter values based on the new tree topology. This process was repeated until tree topologies were stable. Final models and parameters can be found in table 3 in electronic Appendix B. Node stability was estimated in MP and ML analyses by performing 1000 replicates of the non-parametric bootstrap with 10 or 100 random sequence additions per replicate.

Bayesian analyses were conducted with MRBAYES (Huelsenbeck & Ronquist 2001). All analyses were performed with uninformative priors. Four chains were used per run (three heated and one cold), and each analysis was repeated three times, twice for two million generations, with the final analysis running for 10 million generations. All three analyses of the same dataset produced identical tree topologies.

Phylogenetic analyses were conducted on each of the datasets individually. The 16S rRNA and ITS2 data were then combined, tested for incongruence by the incongruence length difference (ILD) test, and analysed again as a combined dataset. The combined dataset consisted of 756 aligned nucleotide positions, 282 of which were parsimony informative. The ILD test was conducted with 1000 test replicates, with 100 random sequence additions per replicate. The ILD test was only marginally non-significant ($p = 0.062$). Combined results are presented in figure 2*a*, and results for separate analyses can be found in electronic Appendix D.

Several calanoid species were included here as outgroup taxa. New sequence data for *Labidocera trispinosa*, *Labidocera jollae*, *Centropages bradyi*, *Candacia bipinnata* and *Candacia* sp. were included as representative taxa of the Centropagoidea. Gene sequences of *Calanus pacificus* (AF295333), *Calanus hyperboreus* (AF227971), *Calanus finmarchicus* (AF367719), *Calanus propinquus* (AY118066), *Calanoides acutus* (AY118071), *Metridia lucens* (AF293440), *Haloptilus ocellatus* (AY118069) and *Ctenocalanus citer* (AY118078) were retrieved from GenBank to serve as outgroups in the Megcalanoidea, Arietelloidea and Clausocalanoidea (Bucklin *et al.* 1995, 2003; S. Grabbert, A. C. Bucklin, S. B. Smolenack and H. U. Dahms, unpublished data).

3. RESULTS

(a) *Molecular phylogeny of the Eucalanidae*

The three gene loci (16S rRNA, ITS2, 18S rRNA) varied considerably in their rate of molecular evolution, and each proved useful for resolving different nodes within the family phylogeny. The 16S rRNA locus exhibited the highest levels of divergence, ranging between 1.6% and 42% (uncorrected p-distances; see electronic Appendix C), and was best able to differentiate species and subspecies lineages. All previously described species exhibited fixed DNA substitutions at 16S rRNA, differentiating them from all other species. Intraspecific variation at 16S rRNA was observed, with haplotypes ranging from 0.1% to 1.7% divergent. Values close to this maximal value of 1.7% were only observed in circumglobal species *Eucalanus hyalinus* 1, *Pareucalanus* sp. and *Subeucalanus subtenuis*. Intraspecific variation within a region was typically observed to be between 0.1% and 1.0%. The youngest pair of previously described sister species, *E. californicus* and *E. bungii*, exhibited a 3% difference at 16S rRNA (table 2*d*). No variation was observed at ITS2 within individuals or within species, and the locus was most useful for resolving intermediate–deep nodes within the family.

Molecular evolutionary relationships among eucalanid species were consistent across the three gene loci and three phylogenetic reconstruction methods employed. The four genera currently included in the family Eucalanidae, *Eucalanus*, *Pareucalanus*, *Rhincalanus* and *Subeucalanus*, were all recovered as monophyletic groups in both mitochondrial and nuclear analyses. The highest levels of bootstrap support for monophyly at the genus level can be observed in the combined dataset (figure 2*a*), with all bootstrap and posterior probability support values at 100%. The 'pileatus' and 'subtenuis' species groups originally designated by Fleminger (1973) within the *Subeucalanus* genus were also consistently recovered as distinct groups across loci and methods employed. A high level of confidence for this result can be observed in all datasets, with bootstrap values ranging between 95% and 100% for these groupings across all phylogenetic methods.

Relationships among species within genera were also largely consistent with expectations based on morphological similarity. High levels of statistical support were found for sister species relationships of *E. californicus* and *bungii* (100%), *P. langae* and *parki* (98%), *S. subtenuis* and *mucronatus* (92%), *P. sewelli* and *attenuatus* (70%), and *R. rostrifrons* and *cornutus* (98%, all values for ML, combined data), as expected given greater morphological similarity between these species pairs. The ITS2 and 16S rRNA results differ only in their resolution of these sister species nodes. However, the relationships between the latter two species pairs are complicated by the discovery of two new genetic lineages, which may reflect more recent speciation events. To the best of the author's knowledge, there were no previous morphological hypotheses for relationships at deeper nodes within species groups, so the results in figure 2 represent the first assessment of patterns of divergence for these species.

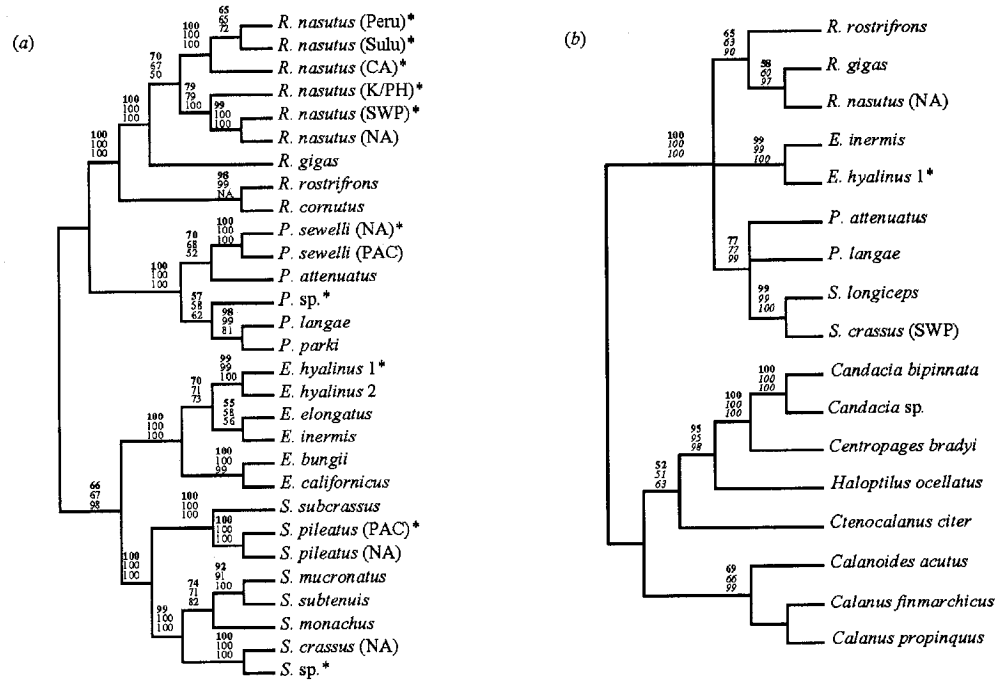

Figure 2. Results for phylogenetic analysis of (*a*) combined data including 16S rRNA + ITS2, and (*b*) 18S rRNA. Values above each node correspond, from top to bottom, to bootstrap support from ML and MP analyses, with the final values corresponding to posterior probability support from the Bayesian analysis. Values in bold are ML bootstrap values. Asterisks denote new lineages. The position of two additional new lineages, *S. crassus* (SWP) and *R. rostrifrons* (WP), can be found in the 16S rRNA analysis in figure 4*a*, electronic Appendix D.

(b) *Testing for monophyly of the Eucalanidae*

Results from outgroup analysis with all three gene loci were consistent with monophyly of the Eucalanidae. In the 18S rRNA analysis (figure 2*b*), all eucalanid and centropagoid species (*Candacia bradyi*, *Candacia bipinnata* and *Centropages* sp.) were included as ingroup taxa, and species from calanoid superfamilies Arietelloidea, Megacalanoidea and Clausocalanoidea were designated as outgroup taxa. One hundred per cent support was observed for monophyly of the Eucalanidae across all three analytical methods. Centropagoid species *C. bipinnata*, *C.* sp. and *C. bradyi* were found to group together in a monophyletic clade with other calanoid superfamily representatives, rejecting the hypothesis of Bjornberg (1972, 1986) of close relationships among Centropagoid, *Pareucalanus* and *Subeucalanus* species. Outgroup analysis results for 16S rRNA and ITS2 datasets were also found to be consistent with monophyly of the Eucalanidae.

(c) *Cryptic taxa*

Twelve new genetic lineages were discovered within the family Eucalanidae. The term 'lineage' is applied here to any population characterized by unexpectedly high levels of genetic divergence from other conspecifics. All such lineages exhibit fixed differences at mitochondrial loci from other conspecific populations, and all but one also display differences at nuclear ITS2 that appear to be fixed. The observed genetic distances between these new lineages and their closest relatives ranged between 1.6% and 23.2% (uncorrected p-distance) at 16S rRNA (table 2). These distances range from slightly less divergent than the youngest sister species pair known to be reproductively isolated, *E. californicus* and *E. bungii* (3%; table 2*d*), to divergences comparable to the deepest intrageneric nodes within the family (e.g. *E. inermis* and *E. californicus*, 19.7%; electronic Appendix C). Genetic distances were also determined at the CO1 locus, but only for species within *Eucalanus* and *Rhincalanus*. New lineages ranged from 5.1% to 24.3% divergent at CO1 from other conspecific populations (table 2). Genetically distinct lineages were found in all four eucalanid genera, and in both Atlantic and Pacific ocean basins.

Out of the 12 new genetic lineages identified here, at least four are cryptic species. Owing to the current absence of supporting ecological and morphological data, the criteria used here to designate new lineages as cryptic species are very conservative. All four cryptic species: (i) show fixed differences at both mitochondrial and nuclear loci; (ii) occur in close geographic proximity to their closest congeners; and (iii) exhibit genetic divergences greater than 3% at 16S rRNA from all other conspecific populations. Criterion (iii) was chosen to reflect the observed genetic divergence between *E. californicus* and *E. bungii*, the least genetically divergent pair of previously described sister

Table 2. Genetic differentiation of new lineages in the Eucalanidae, based on 326 bp of 16S rRNA, 511 bp of ITS2 and 518 bp of CO1.
((*a*) Divergence of cryptic species from their closest congener, (*b*) divergence of lineages with moderate to high fixed differences, (*c*) genetic differentiation between conspecific 'populations' in Atlantic and Pacific ocean basins, and (*d*) genetic differentiation of *Eucalanus californicus* and *Eucalanus bungii*, the least genetically divergent pair of previously described sister species in the family. Location abbreviations as in table 1. A dash represents no data.)

	uncorrected p-distance estimates		
genus species	16S (%)	ITS2 (%)	CO1 (%)
(*a*)			
Pareucalanus sp.–*P. sewelli* (NA)	18.1–23.2	1.8	—
Subeucalanus sp.–*S. crassus*	18.6–18.9	3.4	—
Eucalanus hyalinus 1–*E. hyalinus* 2	7.9–8.7	0.2	13.5–16.6
Rhincalanus nasutus clade 1–clade 2	15.4–18.2	3.2	20.0–24.3
(*b*)			
R. rostrifrons (WP)–*rostrifrons* (EP)	6.7–7.6	0.0	—
Rhincalanus nasutus (CA)–*nasutus* (Peru)	1.6–2.7	0.2	5.1–6.6
R. nasutus (CA/Peru)–*nasutus* (Sulu)	6.2–6.4	0.4	10.1–14.7
R. nasutus (SWP)–*nasutus* (NA)	3.6–4.0	0.2	8.8–10.1
R. nasutus (SWP/NA)–*nasutus* (K/PH)	8.7–10.3	0.8	18.3–19.7
(*c*)			
Pareucalanus sewelli	2.4	0.6	—
Rhincalanus nasutus (NA–SWP)	3.6–4.0	0.2	8.8–10.1
Subeucalanus pileatus	9.6–10.3	0.4	—
Subeucalanus crassus (NA–SWP)	3.1–4.0	—	—
Eucalanus hyalinus 1	0.5–1.7[a]	—	0.0–2.5[a]
Eucalanus hyalinus 2	0.3–0.6[a]	—	0.5–4.2[a]
Pareucalanus sp.	0.0–1.7[a]	—	—
(*d*)			
E. californicus–bungii	3	0.8	13.5

[a] Not fixed differences between populations.

species in the Eucalanidae (table 2*d*). This value is used as a general guideline for the level of genetic differentiation that can accompany speciation within this family. Criterion (ii) is included as a guideline for populations likely to have come into secondary contact, and which therefore do not fall into the ambiguous category of allopatrically distributed populations that may or may not be 'potentially interbreeding' populations *sensu* Mayr (1963*a*,*b*).

The four species identified here as cryptic species are highly divergent from their closest relatives at both 16S rRNA and CO1 (table 2*a*). The new species *Pareucalanus* sp. is inferred to have diverged before the *P. sewelli–P. attenuatus* split (figure 2; electronic Appendix D), and in the 16S rRNA analysis is found with moderate bootstrap support to be a sister species to the clade containing the three lineages, *P. sewelli* (NA), *P. sewelli* (PAC) and *P. attenuatus*. The position of *Pareucalanus* sp. is poorly resolved in the combined dataset (figure 2*a*). It was found to co-occur with *P. sewelli* in the tropical Atlantic, and it co-occurs with both *sewelli* and *attenuatus* in the western tropical Pacific (electronic Appendix A). *Eucalanus hyalinus* 2, the sister species to *hyalinus* 1 (99% support, ML), was found to co-occur with *hyalinus* 1 in subtropical and temperate waters of both Atlantic and Pacific basins (electronic Appendix A; E. Goetze, unpublished data).

A new genetic clade was discovered in the *Subeucalanus* 'subtenuis' species group which includes three genetic lineages within the nominal species *S. crassus*. One 'crassus' lineage, *Subeucalanus* sp., is considered a cryptic species, and is genetically very divergent from the remaining 'crassus' lineages (18.6%, 16S rRNA). This species is identified on the basis of specimens collected only in the Korean Strait region, and little is currently known about its biogeographic distribution. It is, however, clear that both *S. crassus* (PAC) and *Subeucalanus* sp. occur in the western Pacific (electronic Appendix A). The two most divergent clades within the *R. nasutus* species complex (see below) are also included in table 2*a*, as it is certain that there are a minimum of two species within the complex. Both major clades are present in both Atlantic and Pacific ocean basins (figure 3*a*,*c*), and the observed genetic differentiation between the two clades is high (15.4–18.2% at 16S rRNA, 20–24.3% at CO1).

Out of the remaining eight genetically distinct lineages, five exhibit moderate to high levels of fixed differences from their closest relatives, but, in the absence of morphological and ecological data, are not classified as cryptic species here (table 2*b*). In the first case, *Rhincalanus rostrifrons*, two divergent lineages are identified at 16S rRNA (6.7–7.6%), but no differences between the lineages are observed at ITS2. The two lineages occur in close proximity in the western tropical Pacific (electronic Appendix A). The four remaining lineages are members of the *R. nasutus* species complex described below.

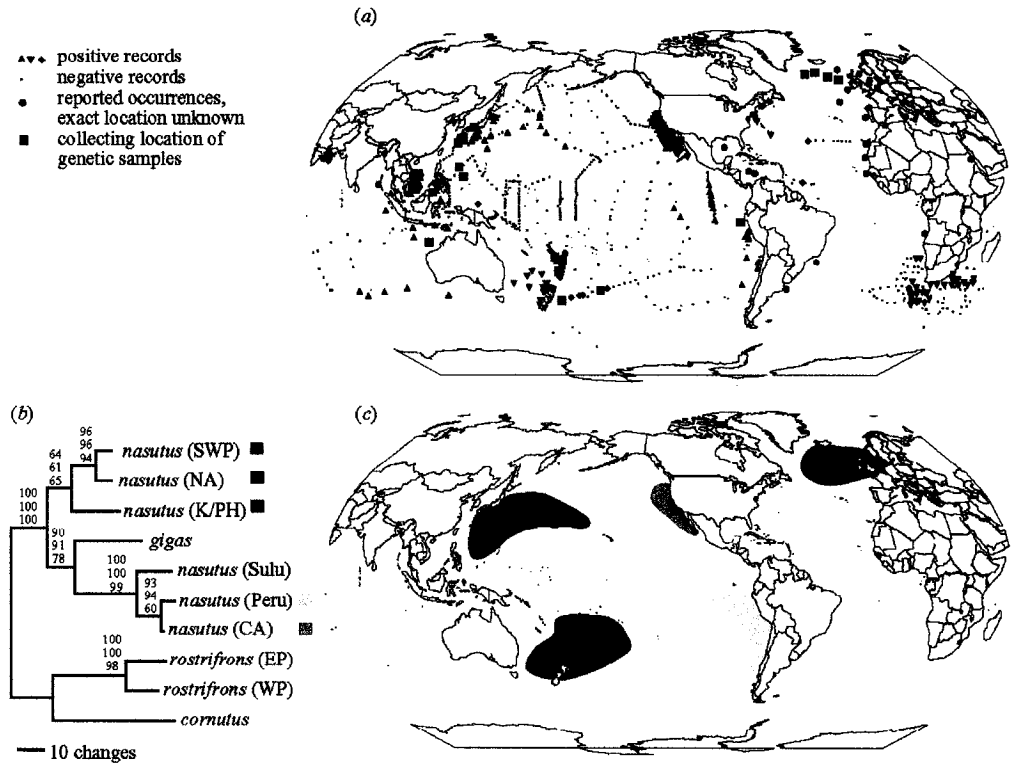

Figure 3. Geographic distribution of genetic lineages within the *Rhincalanus nasutus* species complex. (*a*) Geographic distribution of recorded locations for *R. nasutus*, and collection locations of specimens sequenced in this study. Blue squares represent genetic sample locations, red triangles and diamonds represent positive records for which exact locations are known, green circles represent positive records where *R. nasutus* has been reported to occur, but for which no exact positions are known, and black dots are negative records for *R. nasutus*. Literature sources for recorded locations include: Lang, (1965, red triangles); Bradford-Grieve (1994), CalCOFI atlases (Bowman & Johnson 1973; Fleminger 1964), Grice & Hart (1962), De Decker (1984), Deevey & Brooks (1977), Muniza & Kazmi (1995) all of which are marked by red inverted triangles. Green circles mark records from Madhupratap *et al.* (1981), Campos Hernandez (1980), Razouls & de Bovée (1999), Scotto di Carlo *et al.* (1984), Weikert & Koppelmann (1993), Poulet *et al.* (1996), and red diamonds mark new distributional data from this study. (*b*) Phylogram from ML analysis of the genus-level *Rhincalanus* sequence alignment (16S rRNA). Bootstrap values above each node listed as in figure 2. Location abbreviations as in table 1. (*c*) A preliminary interpretation of the geographic distribution of genetic lineages, based on the locations of known population centres and evolutionary relationships among sequenced specimens. Colour coding for lineages included in (*b*).

(d) *The* Rhincalanus nasutus *species complex*

Six genetically distinct lineages were identified within circumglobal *R. nasutus*, corresponding to populations in the California Current, the Humboldt Current, the Sulu Sea, the southwestern subtropical Pacific, the Kuroshio Current and Philippine Sea and the northern North Atlantic (figure 3*b*,*c*). All lineages show unique fixed differences at both mitochondrial and nuclear loci, with genetic distances between lineages ranging from 1.6% to 18.2% at 16S rRNA (table 2*a*,*b*). Levels of genetic divergence between *nasutus* lineages in all cases but one are as great or greater than that observed between *E. californicus* and *E. bungii*.

The *R. nasutus* species complex contains two main clades (figure 3*b*). One clade consists of the California, Humboldt and Sulu Sea lineages, and the second clade includes lineages from the Kuroshio Current/Philippine Sea, southwestern subtropical Pacific and the northern North Atlantic. Levels of genetic divergence between the two clades are high (15–18% 16S rRNA, 20–24% CO1), and are roughly equivalent to that between *Rhincalanus gigas* and either clade (electronic Appendix C). In the genus-level phylogenetic analysis (figure 3*b*), high bootstrap support (90–100%) was found for the placement of the Antarctic species *R. gigas* within the *nasutus* species complex, as a member of the California–Peru–Sulu lineage. In the family-level analyses (figure 2) the placement of *R. gigas* was ambiguous, with weak bootstrap support for *gigas* outside the *nasutus* complex in the ITS2 results.

(e) *Gene flow between ocean basins*

The presence of gene flow between 'populations' currently considered conspecific in Atlantic and Pacific basins appears to differ between eucalanid species. Eight species in the Eucalanidae have circumglobal distributions, of which seven were sampled in both Atlantic and Pacific

Oceans. Four of these species, *P. sewelli*, *S. crassus*, *S. pileatus* and *R. nasutus*, all show fixed differences at 16S rRNA between ocean basins (table 2*c*), with divergences ranging between 2.4% and 10.3%. Fixed DNA substitutions were also observed in the nuclear genomes of *S. pileatus*, *P. sewelli* and *R. nasutus*, the three species for which ITS2 data are also available. These divergences signify a total absence of gene flow between lineages in Atlantic and Pacific basins for these four species.

The three remaining circumglobal species, *E. hyalinus* 1, *E. hyalinus* 2 and *Pareucalanus* sp., all appear to have either exchanged genes in the recent past, or to experience ongoing gene flow between ocean basins. These three species exhibit levels of genetic differentiation between ocean basins (0.0–1.7%, 16S rRNA) that are typical of intraspecific variation.

4. DISCUSSION

(a) *Morphological species versus genetic clades*

This is one of the first phylogenetic studies to screen systematically and globally for the presence of cryptic species within an oceanic zooplankton taxon. Our results demonstrate that indeed, sibling species are common in the sea (Knowlton 1993). Validating species boundaries with molecular markers will be an essential first step to any study of a morphologically conservative marine group in which a taxonomically complete phylogeny is required. The high levels of inter-population genetic divergence and intra-population genetic cohesiveness of the 12 new genetic lineages identified here suggest that these 'populations' are distinct on both ecological and evolutionary time-scales. Results demonstrate a total absence of gene flow between these 12 lineages and other populations with which they are currently considered conspecific.

While it is not a trivial matter to determine which of these lineages should be considered new species based on genetic data alone, several species concepts can provide guidance as to what the requirements should be for a population to be recognized as a valid species. Under the Phylogenetic Species Concept (Cracraft 1989), all 12 lineages identified here would be considered new species. Designation of new species under the Biological Species Concept (Mayr 1942, 1963*a*) is more ambiguous given the absence of data on reproductive compatibility. However, at least five of these lineages occur in close enough geographic proximity to allow interbreeding. The fixed genetic differences observed in mitochondrial and nuclear genomes of four lineages demonstrate that they have not done so. In addition to these four cryptic species, eight new lineages are also likely to deserve species-level recognition following further consideration of differentiation in morphological or ecological characters. In the meantime, these lineages should be recognized as demographically and evolutionarily distinct from other conspecific populations, regardless of their current taxonomic status. Future work on the Eucalanidae will include examination of all genetically distinct lineages for differentiation in morphological characters.

The biogeographic distributions of these new lineages are incompletely known, given the limitations of sampling in the current dataset. However, it appears that examples can be found in which they occur in sympatry (*E. hyalinus* 1 and 2, *Pareucalanus* sp. and *sewelli*/*attenuatus*), parapatry (*R. rostrifrons* (EP and WP)) and allopatry (*R. nasutus*, *S. pileatus* (NA–PAC), *P. sewelli* (NA–PAC), *S. crassus* (NA–SWP)) relative to their closest congeners. Preliminary results suggest that the 10 lineages that appear in allo- and parapatry fractionate what was originally thought to be a circumglobal or large-scale biogeographic range. For example, new lineages discovered in *P. sewelli*, *S. pileatus* and *R. rostrifrons* may have distributions restricted to one ocean basin, with a sister lineage found in a second ocean basin. The two cryptic species that co-occur in sympatry with their closest congeners may also partition oceanographic habitat by depth or water mass preferences. For example, the presence of a sibling species previously cryptic within *P. sewelli* may help to explain curious observations of bimodal vertical distributions of populations in the eastern North Atlantic (Roe 1972, fig. 1, under species name *attenuatus*), as well as the presence of two distinct size groups within adult females (Fleminger 1973, fig. 16) and especially adult males of *P. sewelli* (Roe 1972, fig. 4).

Six of the genetically distinct lineages mentioned above were identified within *R. nasutus*, which has previously been considered a single circumglobal species (Schmaus & Lehnhofer 1927; Lang 1965; Castro *et al.* 1993; Bradford-Grieve 1994). The high levels of interpopulation genetic divergence observed in this taxon suggests that it is very probably a species complex. Although putative cosmopolitan marine species are increasingly being identified as cryptic species complexes (Scholin *et al.* 1995; Klatau *et al.* 1999; de Vargas *et al.* 1999, 2001, 2002; Lee 2000; Lazoski *et al.* 2001), there have been few examples for species that extend well offshore of the coastal zone. A literature compilation of recorded locations for *R. nasutus* (figure 3*a*) demonstrates that the species can and does occur in oceanic regions as far as 4000 km offshore. Given such an oceanographically broad distribution, it would be reasonable to expect high levels of gene flow at ocean basin scales. The genetic data, however, strongly support the conclusion that lineages centred in different coastal boundary currents do not exchange genes.

Furthermore, genetic relationships among *nasutus* lineages do not follow expectations based on surface ocean circulation. Dominant gyre flow fields in subtropical waters of each hemisphere might be expected to induce a more recent shared evolutionary history for lineages in eastern and western boundary currents of each hemisphere than with lineages in other parts of the globe. However, observed sister lineage pairs include the northern North Atlantic and southwestern subtropical Pacific, and the California and Peru populations (figure 3*b*,*c*). The close California–Peru relationship demonstrates more recent connectivity between these eastern boundary current lineages in the Pacific than between east–west boundary currents within each hemisphere. The sister lineage relationship between the northern North Atlantic and southwestern subtropical Pacific supports a more recent shared evolutionary history across both hemispheres and ocean basins than between clade 2 lineages in the Pacific (Kuroshio/Philippine Sea and southwest subtropical Pacific). Results from the *R. nasutus* species complex emphasize that marine species need not be neritic or coastal in distribution for allopatric populations to diverge genetically on large spatial scales, and secondly, that

evolutionary relationships between lineages may not reflect present-day surface ocean currents.

(b) *Phylogeny of the Eucalanidae, and congruence with morphological taxonomy*

Relationships among previously described eucalanid species, as inferred from both mitochondrial and nuclear DNA data, were highly congruent with results from previous morphological studies of the family. Analysis of DNA sequence data from three gene loci consistently recovered strong support for four monophyletic genera in the family Eucalanidae, with member species following designations by Fleminger (1973), Geletin (1976) and Bradford-Grieve (1994). The subgeneric clades of the 'pileatus' and subtenuis' species groups (Fleminger 1973) within *Subeucalanus* were also very well supported by the current genetic dataset. Mitochondrial and nuclear DNA data support specific-level differentiation for all 20 previously described species in the family that were sampled here.

The family Eucalanidae was also found to be monophyletic, with no support for the hypothesis of Bjornberg (1972, 1986) of two major lineages within the family. Results for all three gene loci suggest that the genera *Subeucalanus* and *Pareucalanus* are more closely related to the genera *Rhincalanus* and *Eucalanus* than to members of the calanoid superfamily Centropagoidea. Implications from this result are twofold; first, that diversification of the *Pareucalanus* and *Subeucalanus* genera was not associated with expansion of a predominantly coastal group, the Centropagoidea, into oceanic waters. Second, that naupliar morphological and behavioural evolution may be more plastic on evolutionary time-scales than previously thought (Fryer 1984; Dahms 2000). Results presented here suggest that phylogenetic relationships inferred from adult characters more closely approximate the evolutionary history of this family.

(c) *Barriers in the plankton: formidable or permeable?*

Oceanographic barriers to dispersal have long been thought to play an important role in the process of speciation in oceanic zooplankton, despite the fact that it is often remarked that it is difficult to envision how this occurs in the open ocean (McGowan 1971; Pierrot-Bults & van der Spoel 1979). Recent genetic data from planktonic Foraminifera have called this view into question, with observations of SSU or ITS haplotypes shared between ocean basins in *Orbulina universa*, *Globigerinella siphonifera* II and *Globorotalia truncatulinoides* (de Vargas *et al.* 1999, 2002, 2003), and between poles in *Globigerina bulloides*, *Turborotalia quinqueloba* and *Neogloboquadrina pachyderma* (Darling *et al.* 2000). Such observations have suggested that barriers to gene flow, such as subtropical fronts, tropical waters (for cold water species) or continental landmasses, may be highly permeable or non-existent for planktonic organisms, with the ability to establish successful populations outside the distributional range as the only key factor controlling expansion of a biogeographic range (Norris 2000; Norris & de Vargas 2000).

Results presented here suggest a qualification of this view, whereby barriers to dispersal are permeable for some species and formidable to others. Levels of genetic differentiation between Atlantic and Pacific 'populations' of seven circumglobal eucalanid species vary from 10.3% fixed sequence divergence (*S. pileatus* NA, PAC) to intraspecific haplotype sharing of 16S rRNA or CO1 haplotypes between ocean basins (*E. hyalinus* 1, *Pareucalanus* sp.; table 2*c*). Four out of seven species exhibit a total absence of gene flow between 'populations' in the Atlantic and Pacific, while the remaining three demonstrate relatively recent or ongoing genetic exchange between basins. Given that all seven species have successful populations established in both ocean basins, it appears unlikely that individuals effectively disperse, but fail to recruit in the local population. Rather, continental landmasses do appear to act as a barrier to dispersal in some, but not all species. Similar results are observed for circumglobal *Clausocalanus jobei* and *C. pergens* (Bucklin *et al.* 2003), suggesting that species specificity in patterns of gene flow on circumglobal scales may be a general phenomenon in zooplanktonic species. Additionally, the four species that exhibit an absence of gene flow between ocean basins all have distributions that extend to 40° S latitude, suggesting that the extent of the southern edge of the distributional range does not appear to be the key factor determining success in dispersal around Cape Horn (cf. Fleminger & Hulsemann 1973). It is currently unclear what characteristic of the life history, ecological specificity, evolutionary history and/or biogeography of a species determines its ability to disperse across such semi-permeable barriers as the continental landmasses of the Americas.

This paper is dedicated to Abraham Fleminger, whose life's work on the evolution of calanoid copepods continues to be an inspiration to the author. His careful morphological analysis of the Eucalanidae serves as a foundation for the work presented here. The following people very kindly provided specimens from disparate regions of the global ocean: G. Mitchell and S. Storms, P. Fiedler and V. Philbrick, M. Galbraith, B. Frost, S. Ohtsuka, S. Nishida and R. Machida, Y.-S. Kang, M. Landry and C. Allen, J. Grieve, V. Andersen, S. Gasparini, X. Irigoien, E. Carpenter and A. Subramaniam, R. Harris and T. Smith, R. Escribano and P. Hidalgo Diaz, P. Ayon, and R. Wilson and T. Baiz. A. Yayanos and D. Capone welcomed E.G. aboard their cruises during 2001. Grace Wyngaard and Sarah Gerken kindly provided PCR primers for ITS2. M. D. Ohman, J. M. Flowers, C. S. Willet, R. Burton, N. Knowlton, C. de Vargas, W. Newman and J. Kohn provided discussion, encouragement, technical advice and comments on the manuscript. The work was supported by NSF grants OCE02-21063 and OCE01-10300, a UC ship funds grant and an EPA STAR graduate fellowship.

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As this paper exceeds the maximum length normally permitted, the author has agreed to contribute to production costs.

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These are electronic appendices to the paper by Goetze 2003 Cryptic speciation on the high seas; global phylogenetics of the copepod family Eucalanidae. *Proc. R. Soc. Lond.* B **270**, 2321–2331. (DOI 10.1098/rspb.2003.2505.)

Electronic appendices are refereed with the text. However, no attempt is made to impose a uniform editorial style on the electronic appendices.

Electronic Appendix A.

Sampling locations of specimens sequenced for each species, with corresponding date and number of specimens sequenced (16s rRNA). Conventions for signs in lat/long are: latitude, + is North, - is South, longitude + is East, - is West. Locations are as follows: CA = California, PE=Peru, SU= Sulu Sea, K/PH= Kuroshio Current and Philippine Sea, SWP = southwest Pacific, NA = North Atlantic, PAC= Pacific, EP= Eastern Pacific, WP= Western Pacific. Date conventions are Month/Day/Year.

Genus species (clade)	Station	Latitude	Longitude	Date	No. specimens
Eucalanus bungii	D9-0	48.34	164.43		8
	ST. 29	40.59	144.42	9/1/01	1
	OSO1053	56.42	-145.85	6/29/01	6
	IOS2026	51.19	-127.15	8/23/00	3
E. californicus	67.85	35.62	-124.54	8/8/01	10
	67.60	36.27	-122.47	8/7/01	5
	117.65	27.62	-117.21	10/14/0	15
	ST. 6	29.52	130.47	5/26/01	1
	LJC	32.51	-117.16	6/20/00	3
E. elongatus	ST. 26	7.00	120.00		4
E. hyalinus(1)	AA-2	28.21	-162.14	3/16/01	8
	ST.3	31.16	131.52	5/24/01	3
	MP3-18	10.57	-49.39	7/10/01	6
	DRFT07-08	-34.02	-140.03	12/23/0	16
	DRFT07-06	-32.03	-130.60	12/21/0	12
E. hyalinus(2)	67.70	36.12	-123.48	1/8/01	1
	67.80	35.78	-124.19	1/8/01	1
	67.90	35.45	-124.90	1/8/01	2
	93.60	31.51	-119.34	3/29/02	4
	MP3-12	29.57	-45.026	7/2/01	5
	Dyfamed	43.25	7.52	6/29/01	3
	FL2	62.47	-15.95	5/15/01	1
	C	60.08	-19.84	5/13/01	1
	EC3	61.19	-22.95	5/12/01	1
	DRFT07-04	-30.40	-124.47	12/19/01	1
	DRFT07-08	-34.02	-140.03	12/23/01	2
	DRFT07-18	-40.53	-173.00	12/28/01	4
E. inermis	Mejillones	-23.00	-71.00		3
	Antofagasta	-23.30	-71.0		8
	M00-49	12.49	-141.59	9/4/00	6
	GC98 11/1 Est:14	Gulf of California		12/1/98	3
Rhincalanus cornutus	MP3-34	3.59	-43.07	8/2/01	10
	MP3-12	29.57	-45.03	7/2/01	4
	MP3-14	12.04	-55.26	7/8/01	3
R. rostrifrons(EP)	M00-94	8.22	-88.47	9/27/00	5
	M00-61	-0.1	-126.32	9/10/00	4
	M00-49	12.49	-141.59	9/4/00	4
	COOK14-41	-13.17	-171.43	10/27/01	5
R. rostrifrons(WP)	AA-19	32.26	134.23	4/3/01	5
	COOK11-4	1.425	143.43	8/15/01	11

Genus species (clade)	Station	Latitude	Longitude	Date	No. specimens
	314/01	33.0	128.4	8/1/01	3
R. gigas	SOFEX	-66.24	-171.0	2/13/02	12
	FROST-1	-54.00	-38.00		7
R. nasutus(CA)	93.30	32.51	-117.32	3/28/02	8
	GC98 11/1 Est:14	Gulf of California		12/1/98	2
	LJC	32.51	-117.16	6/20/00	2
R. nasutus(PE)	PAYON-1	-6.47	-82.33	8/31/01	10
R. nasutus(SU)	ST. 26	7.00	120.00		8
R. nasutus(K/PH)	PM1	36.10	135.51	5/22/01	3
	ST-3	31.16	131.52	5/22/01	7
	ST-11	29.10	129.48	5/28/01	1
	COOK11-2	18.20	132.44	8/11/01	6
	COOK11-3	14.03	135.28	8/12/01	1
R. nasutus(SWP)	COOK14-04	-22.14	-178.26	10/7/01	3
	COOK14-05-2	-23.52	-177.05	10/7/01(2)	1
	COOK14-06	-23.31	-175.13	10/8/01	4
	COOK14-07	-23.13	-174.44	10/9/01	4
	COOK14-09	-23.50	-174.26	10/10/01	1
	COOK14-25	-29.14	-177.36	10/19/01	10
	COOK14-23	-31.60	-177.21	10/17/01	2
	DRFT07-13	-37.27	-156.01	12/26/01	4
	DRFT07-20	-41.58	-178.34	12/31/01	8
R. nasutus(NA)	BC7	58.96	-16.98	5/8/01	2
	EC3	61.19	-22.95	5/12/01	2
	ADH	57.49	-11.53	5/21/01	6
	I3	60.53	-24.35	5/22/02	2
Pareucalanus sp.	AA-8	31.24	173.92	3/22/01	3
	AA-19	32.26	134.23	4/3/01	3
	COOK11-05	-2.24	145.22	8/16/01	1
	COOK14-03	-20.52	-179.29	10/7/01	2
	COOK14-05	-23.52	-177.05	10/7/01	3
	COOK14-06	-23.31	-175.42	10/8/01	3
	ST. 26	7.00	120.00		1
	MP3-14	12.038	-55.26	8/2/01	1
	MP3-18	10.57	-49.39	7/10/01	2
	MP3-34	3.59	-43.07	8/2/01	1
	CAN1	29.37	-13.48	6/22/01	2
P. sewelli(NA)	AS1	38.47	25.049	6/14/01	8
	CAN1	29.37	-13.48	6/22/01	3
	MP3-12	29.57	-45.03	7/2/01	2
	MP3-14	12.04	-55.26	7/8/01	5
	MP3-18	10.57	-49.39	7/10/01	2
P. sewelli(PAC)	GC98 11/1 Est:14	Gulf of California		12/1/98	3
	M00-94	8.22	-88.47	9/27/00	3
	DRFT07-11	-36.05	-149.29	12/24/01	3
	DRFT07-15	-39.37	-166.42	12/28/01	6
P. attenuatus	AA-8	31.24	173.92	3/22/01	2
	AA-19	32.26	134.23	4/3/01	5
	M00-65	-4.44	-124.33	9/12/00	8
	M00-94	8.22	-88.47	9/27/00	1
	ZP480/483	22.75	-158.0	7/24/00	2
	COOK11-05	-2.237	145.22	8/16/01	2
	COOK14-03	-20.52	-179.23	10/7/01	1
	COOK14-05	-23.52	-177.05	10/7/01	2

Genus species (clade)	Station	Latitude	Longitude	Date	No. specimens
P. parki	ST E2	38.10	138.41	5/27/01	3
	PM1	36.10	135.51	5/22/01	1
	90.60	32.25	-119.58	4/2/02	3
	93.3 40.0	32.31	-118.13	3/29/02	1
	93.3 60.0	31.51	-119.34	3/29/02	1
	77.70	34.23	-122.15	6/28/02	2
	Fm9	43.13	-125.10	4/6/02	1
P. langae	COOK14-03	-20.52	-179.29	10/7/01	1
	COOK14-06	-23.31	-175.42	10/8/01	2
	DRFT07-04	-30.40	-124.46	12/19/01	1
	DRFT07-05	-31.21	-127.50	12/20/01	1
	DRFT07-15	-39.37	-166.42	12/28/01	3
	DRFT07-19	-41.24	-175.40	12/30/01	2
Subeucalanus crassus(NA)	PLYM-1	50.25	-4.05	11/27/01	8
S. crassus(SWP)	COOK14-21	-31.05	-177.05	10/16/01	3
S. sp.	205/03	34.25	127.9	10/13/01	8
S. longiceps	DRFT07-18	-40.53	-173.02	12/28/01	6
	DRFT07-19	-41.24	-175.40	12/30/01	4
S. monachus	CAN1	29.37	-13.48	6/22/01	7
	MP3-03	29.15	-25.28	6/27/01	8
S. mucronatus	ST. 26	7.00	120.00		5
S. subtenuis	M00-51	10.30	-139.11	9/5/00	8
	M00-96	8.45	-87.38	9/28/00	8
	ST-3	31.16	131.52	5/24/01	4
	314/01	33.0	128.4	6/1/01	5
	314/04	33.5	127.0	8/1/01	6
	COOK11-02	18.195	18.20	8/11/01	4
S. pilaetus(NA)	MP3-23	10.32	-56.32	7/19/01	4
S. pilaetus(PAC)	M00-94	8.22	-88.46	9/27/00	5
	205/03	34.25	127.9	10/13/01	5
S. subcrassus	COOK14-02	-19.32	179.27	10/7/01	5
	COOK14-03	-20.53	-179.29	10/7/01	5
	M00-94	8.22	-88.46	9/27/00	5
	205/03	34.25	127.9	10/13/01	6
Outgroup Taxa:					
Candacia bipinnata	86.7 60	32.99	-120.35	4/4/02	(18S only)
Candacia sp.	COOK14-38	-15.25	-172.14	10/27/01	(18S only)
Centropages bradyi	93.3 28	32.91	-117.40	3/28/02	4
Labidocera jollae	93.3 28	32.91	-117.40	3/28/02	4
L. trispinosa	LJC	32.51	-117.16	3/24/01	7

Electronic Appendix B. Supplemental Methods.

PCR primers and amplification conditions:

for 16S rRNA (*ca.* 380 bp fragment, 3' half) 16SAR-L, 16SCB (Braga *et al.* 1999), 16S_PAR1 [5'- GCTAAGGTAGCATAATAATTAGTT –3'] (genus *Pareucalanus*), or 16S_SUB2 [5' –AAGTGCTAAGGTAGCATAAT –3'] (genus *Subeucalanus*)

for COI (~585 bp fragment) COI_VH , COI_VL (Folmer *et al.* 1994) as well as eucalanid – specific COI_RN1 [5'-GTAGT(AGCT)GTAAC(AT)GCTCATGC-3']

for nuclear ITS2 (~520 bp length) ITS3F [5'- GCATCGATGAAGAACGCAGC –3'] (White *et al.* 1990) and ITS10R [5'- TACGGGCCTATCACCCTCTACG-3'] (Gerken and Wyngaard, pers. comm.)

for nuclear 18S rRNA, those listed in Spears *et al.* (1992), as well as eucalanid-specific 18S_F691 [5'-GGTTTCCCGGAAGCTTCCTGCC-3'] and 18S_INT3R [5'-GCCTCACTAAGGTGAAACCGCG-3'].

Amplification conditions for all loci consisted of: 30 secs at 95°C, 30 secs at 50-55°C, 1 min at 72°C for 35-40 cycles, and an extension step for 5 min at 72°C. Amplification products were cleaned using a QIAGEN PCR product purification protocol, and 1-5 μl of PCR product was used as template DNA in a cycle sequencing reaction.

Primer References:

(1) Braga, E., R. Zardoya, *et al.* (1999). "Mitochondrial and nuclear rRNA based copepod phylogeny with emphasis on the Euchaetidae (Calanoida)." *Mar. biol* 133(1): 79-90.

(2) Folmer, O., M. Black, *et al.* (1994). "DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates." *Mol. mar. biol. biotechnolog* 3(5): 294-299.

(3) White, T. J., T. Bruns, *et al.* (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols*. M. A. Innis, D. H. Gelfand and J. J. Sninsky. New York, Academic Press.

(4) Spears, T., L. G. Abele, *et al.* (1992). "The monophyly of Brachyuran crabs: A phylogenetic study based on 18s rRNA." *Syst. Biol.* 41(4): 446-461.

Table 3. Final models and parameter values used in maximum likelihood and Bayesian analyses. "Combined" data includes 16S and ITS2 loci, and "Rhincal_16S" denotes the genus-level analysis of the *Rhincalanus* 16S data with no deleted nucleotide positions. Models are TrN (Tamura Nei '93), SYM (symmetrical), and TVM (transversion), where additions of I and G reflect inclusion of a proportion of invariable sites (I, pinv) and gamma distributed rate heterogeneity across nucleotide sites (G, shape parameter = alpha).

Locus	model	Substitution matrix	alpha	pinv.
16S	TrN+I+G	[1.0, 6.1610, 1.0, 1.0, 9.5745]	0.9468	0.4104
ITS2	SYM+G	[1.456, 5.453, 2.105, 0.844, 2.537]	0.2262	0
18S	SYM+I+G	[0.704, 3.859, 1.643, 0.121, 2.152]	0.5458	0.4444
Combined	TVM+I+G	[1.174, 5.994, 1.992, 1.028, 5.994]	0.7844	0.4851
Rhincal_16S	TVM+G	[0.702, 16.818, 3.635, 2.237*e-7, 16.818]	0.2194	0

Electronic Appendix C.

Uncorrected p-distance estimates between all included eucalanid taxa for 16S rRNA and ITS2 datasets. Values above the diagonal represent divergence at ITS2, values below the diagonal at 16S rRNA. Values in **bold** are distance estimates between new genetic lineages and their closest congeners. Data for *R. rostrifrons* (SEP) at ITS2 is listed as NA, due to identical sequence at this locus for the two *rostrifrons* lineages.

	crassus NA	*crassus* SWP	*S. sp.*	*subcra.*	*pileat.* NA	*pileat.* PAC	*mucron*	*subten.*	*longic.*	*monac*	*langae*	*parki*	*sp.*	*sewelli* (NA)	*sewelli* (PAC)	*attenu.*	*nasut.* (CA)	*nasut.* (Peru)	*nasut.* (Sulu)	*nasut.* (K/PH)	*nasut.* (SWP)	*nasut.* (NA)	*gigas*	*rostrif.* (NWP)	*rostrif.* (SEP)	*cornu.*	*hyal. 1*	*hyal. 2*	*bungii*	*californ*	*elongat*	*inermis*
crassus (NA)	-		**3.4**	14.4	14.5	14.7	8.5	11.2	-	8.1	14.6	14.4	14.6	14.4	15.0	14.0	16.0	15.8	16.0	15.6	15.6	15.6	15.6	17.0	NA	17.2	17.3	17.1	16.9	16.9	17.5	17.9
crassus (SWP)	**2.9**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. sp.	19.0	**18.6**	-	13.3	12.9	13.1	7.3	9.3	-	6.2	13.6	13.4	14.0	13.8	14.4	13.6	16.8	16.6	16.8	16.0	15.8	15.8	16.6	17.4	NA	17.0	16.2	16.0	16.2	16.2	16.4	16.8
subcrassus	34.6	34.1	**31.4**	-	5.4	5.8	14.7	17.0	-	13.7	17.6	17.4	17.4	17.4	17.5	17.2	18.3	18.5	18.7	18.7	18.4	18.4	18.2	19.6	NA	18.8	17.5	17.5	17.7	17.5	17.9	17.9
pileatus (NA)	33.4	33.0	34.1	26.2	-	**0.4**	13.1	15.8	-	14.1	17.9	17.7	17.5	17.3	17.6	16.7	17.9	17.7	18.0	17.8	17.7	17.7	18.0	19.5	NA	18.6	17.4	17.4	17.4	17.2	17.8	17.4
pileatus (PAC)	34.2	33.4	33.3	26.8	**9.3**	-	13.1	15.8	-	14.1	18.1	18.0	17.8	17.6	17.8	17.0	18.0	17.8	18.0	17.8	17.8	17.8	17.9	19.5	NA	18.6	17.0	17.0	17.0	16.8	17.4	17.0
mucronatus	26.7	27.3	25.7	32.3	36.0	34.6	-	6.5	-	5.0	13.8	13.6	13.6	13.2	13.9	13.0	17.8	17.6	17.8	17.3	17.2	17.2	17.8	18.0	NA	17.6	15.2	15.0	15.2	15.0	15.6	16.0
subtenuis	29.6	29.4	28.1	37.1	36.6	36.1	18.6	-	-	7.1	15.1	14.9	15.5	15.3	16.1	15.9	19.1	18.9	19.1	18.1	17.7	17.7	17.9	18.2	NA	18.3	15.4	15.2	15.5	15.5	15.6	15.6
longiceps	24.3	24.8	21.8	32.4	37.0	35.7	23.1	23.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-
monachus	28.1	28.7	25.7	30.6	35.0	33.0	24.0	24.2	20.6	-	13.8	13.6	13.8	13.6	14.0	13.6	17.2	17.0	17.2	17.2	17.0	17.0	17.0	17.5	NA	17.4	14.6	14.4	14.6	14.6	14.8	15.2
langee	37.6	37.6	35.2	33.9	37.4	37.6	33.2	35.9	37.9	**37.7**	-	0.6	0.8	2.6	3.0	3.4	13.9	13.7	13.9	14.7	14.3	14.1	14.5	14.4	NA	13.2	11.9	12.1	11.9	12.3	12.3	12.5
parki	38.6	36.7	36.4	34.3	36.5	35.4	36.2	35.2	38.2	38.1	06.9	-	1.0	2.6	3.0	3.6	13.9	13.7	13.9	14.7	14.3	14.1	14.5	14.4	NA	13.3	11.9	12.1	11.9	12.3	12.1	12.5
P. sp.	35.7	36.9	34.0	33.1	36.9	37.2	36.3	35.1	35.7	34.0	18.8	19.8	-	**1.8**	2.2	2.6	13.9	13.7	13.9	14.9	14.5	14.3	14.7	14.4	NA	13.3	12.1	12.3	11.7	12.1	12.5	12.7
sewelli (NA)	38.0	39.1	35.8	33.9	39.0	38.7	37.1	34.6	34.9	35.6	25.0	24.3	**17.4**	-	**0.6**	1.8	14.1	13.9	14.1	14.5	14.1	13.9	14.7	13.7	NA	12.9	11.7	11.9	11.3	11.7	11.7	12.3
sewelli (PAC)	39.6	40.6	36.1	33.1	39.0	39.0	38.4	35.5	36.2	36.5	25.5	25.5	19.0	**2.3**	-	2.6	14.5	14.3	14.5	**15.4**	14.9	14.7	15.3	14.1	NA	13.3	12.5	12.7	12.1	12.5	12.8	13.1
attenuatus	34.8	35.9	32.6	32.8	36.7	36.7	32.4	34.3	34.9	33.3	22.7	24.2	16.3	11.7	13.5	-	14.1	13.9	14.1	14.5	14.5	14.3	14.7	13.6	NA	12.7	11.9	12.1	11.5	11.9	12.3	12.5
nasutus (CA)	37.6	38.9	34.1	38.3	40.5	39.4	34.7	34.5	34.1	31.7	31.2	32.0	28.4	30.4	31.4	27.9	-	**0.2**	0.4	3.2	3.0	3.2	3.6	7.4	NA	7.4	15.2	15.4	14.7	14.9	15.6	16.0
nasutus (Peru)	37.4	38.2	34.0	37.9	40.5	39.4	34.6	34.0	34.3	31.9	31.3	32.4	29.4	31.0	32.0	28.1	**1.6**	-	**0.2**	3.0	2.8	3.0	3.4	7.6	NA	7.6	15.2	15.4	14.7	14.9	15.6	16.0
nasutus (Sulu)	38.1	38.6	35.2	40.3	39.3	38.5	37.3	33.1	34.9	32.7	30.5	30.9	28.0	29.3	30.3	26.1	5.5	**5.3**	-	3.2	3.0	3.2	3.6	7.4	NA	7.4	15.4	15.6	14.9	15.1	15.8	16.2
nasutus (K/PH)	36.7	37.2	34.4	35.9	39.3	38.2	36.3	33.5	34.0	**32.1**	30.8	29.8	26.7	28.6	29.2	27.1	15.5	15.8	16.8	-	**0.6**	0.8	2.8	8.0	NA	7.6	15.6	15.8	15.2	15.4	16.0	16.4
nasutus (SWP)	35.8	37.3	33.5	36.9	38.7	37.6	38.9	37.0	34.2	32.4	32.6	31.9	30.6	30.5	30.5	28.8	17.0	16.6	18.0	**8.7**	-	0.2	3.2	8.2	NA	7.6	15.3	15.5	14.9	15.1	15.8	16.2
nasutus (NA)	36.8	37.4	34.1	37.5	39.0	37.9	38.5	36.5	34.4	33.2	33.1	31.8	31.0	31.2	31.2	30.5	16.7	16.7	18.2	9.4	**3.2**	-	3.4	8.4	NA	7.8	15.1	15.3	14.7	14.9	15.6	16.0
gigas	38.1	38.3	35.8	37.4	42.0	40.9	37.7	36.2	34.8	34.4	32.0	30.9	30.3	30.8	30.8	30.0	16.3	16.6	18.4	12.8	13.7	13.8	-	7.6	NA	8.2	15.3	15.5	15.1	15.1	15.8	16.2
rostrifr. (NWP)	38.2	38.8	36.4	34.4	40.4	37.4	37.4	35.1	35.1	32.5	30.8	31.5	28.6	29.9	30.2	27.4	24.3	24.1	24.2	21.2	20.2	21.6	23.4	-	**0.0**	2.8	13.5	13.7	13.5	13.5	13.7	**14.1**
rostrifrons (SEP)	39.0	39.5	36.6	32.5	38.7	38.1	38.0	37.3	34.8	35.0	31.8	32.4	28.7	31.9	32.2	30.1	26.2	26.3	27.1	22.2	20.9	21.3	24.4	**7.4**	-	-	-	-	-	-	-	-
cornutus	38.9	39.6	36.2	35.5	38.0	37.9	37.0	36.6	39.6	36.4	31.4	30.1	29.2	32.8	33.1	31.9	25.6	26.1	27.4	23.2	24.4	25.1	24.8	20.0	20.6	-	13.5	13.7	13.5	13.5	13.9	**14.3**
hyalinus 1	32.0	32.2	32.3	35.4	36.5	38.3	36.1	36.6	35.7	34.7	35.8	36.5	35.1	34.3	35.0	31.8	32.2	32.2	34.2	32.9	32.9	32.9	31.5	34.0	34.8	32.9	-	**0.2**	2.4	2.2	0.6	1.0
hyalinus 2	34.9	34.8	35.0	34.5	35.0	37.8	38.5	38.2	39.2	36.1	35.7	34.8	35.7	35.6	35.6	31.9	35.2	34.3	35.9	33.3	32.9	33.6	31.6	34.0	35.6	33.3	**7.4**	-	2.6	2.4	0.8	1.2
bungii	32.9	33.7	32.5	35.0	38.1	39.1	35.1	35.7	37.3	34.3	33.2	32.8	31.4	33.5	33.7	31.6	28.9	29.1	30.0	26.4	26.3	28.7	28.2	28.1	30.9	27.6	22.1	20.3	-	0.8	2.6	2.6
californicus	34.7	35.3	32.9	34.7	38.8	37.6	35.7	36.2	37.3	34.8	33.3	32.4	31.4	32.7	32.9	30.1	29.7	29.2	30.0	27.5	27.7	28.0	28.0	26.0	30.2	27.5	22.0	20.2	3.0	-	2.4	2.8
elongatus	35.1	35.6	35.5	36.5	35.6	33.9	33.6	37.1	39.2	32.3	35.2	35.8	35.8	36.1	37.7	35.7	35.7	35.1	36.1	35.5	34.8	34.1	34.7	34.3	37.7	32.5	25.4	23.2	21.4	21.5	-	0.8
inermis	35.0	35.4	31.4	34.0	37.9	36.9	31.0	36.1	34.2	28.9	33.9	34.0	33.6	33.7	34.3	32.5	31.1	31.3	32.8	26.6	27.3	28.6	28.9	29.4	31.1	29.9	25.5	25.6	21.7	19.7	25.3	-

Electronic Appendix 2. Uncorrected p-distance estimates between all included eucalanid taxa for 16S rRNA and ITS2 datasets. Values above the diagonal represent divergence at ITS2, values below the diagonal at 16S rRNA. Values in **bold** are distance estimates between new genetic lineages and their closest congeners. Data for *R. rostrifrons* (SEP) at ITS2 is listed as NA, due to identical sequence at this locus for the two *rostrifrons* lineages.

Electronic Appendix D: Figure 4

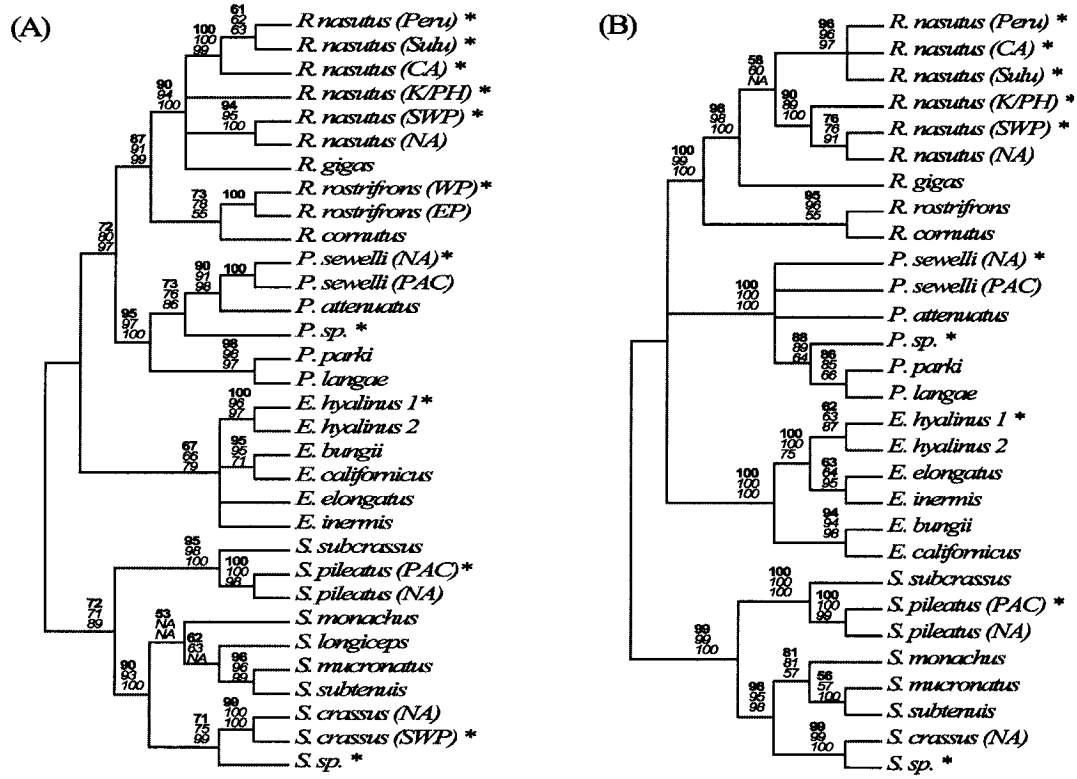

Figure 4. Results for phylogenetic analysis of (*a*) 16S rRNA, analysed separately, and (*b*) ITS2, analysed separately. Values above each node correspond, from top to bottom, to bootstrap support from ML and MP analyses, with final values corresponding to posterior probability support from the Bayesian analysis. Values in **bold** are ML values. * denotes new lineages.

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Chapter III

Genetic and Morphological Description of *Eucalanus spinifer* T. Scott, 1894 (Calanoida: Eucalanidae), a Circumglobal Sister Species of the Copepod *E. hyalinus* s.s. (Claus, 1866)

Abstract

A distinct genetic form of the eucalanid calanoid copepod *Eucalanus hyalinus* s. l. was identified in subtropical and temperate waters of the Pacific, Atlantic, and Indian Oceans. Results from mitochondrial (16S rRNA, COI) and nuclear (ITS2) genetic markers suggest that the two *E. hyalinus* forms are reproductively isolated. *Eucalanus spinifer* T. Scott is taken out of synonymy with *E. hyalinus* and is applied to the smaller of the two genetic forms, increasing the number of species in the genus *Eucalanus* to a total of six. Adult females of the sister species *E. hyalinus* s.s. and *E. spinifer* can be distinguished morphologically by the shape of the anterior portion of the head in lateral and ventral views, asymmetry in the length of antennule segments XXII and XXIII, length ratios of XXII/V and XXIII/V of the longer of the first antennules, the length of the caudal rami, and total body size, as measured by total length or prosome length. We illustrate and describe both adult females and males of *E. spinifer*, and discuss aspects of sexual dimorphism. The sister species are

sympatric throughout much of their circumglobal biogeographic distributions, but appear to have centers of abundance in slightly different water mass types.

Introduction

The family Eucalanidae has proven to be one of the more challenging calanoid families for taxonomic identification of species, due to the absence of highly distinctive sexually modified appendages (Fleminger, 1973; Bradford-Grieve, 1994). Improvements in our understanding of the diversity in the family have been slow (Claus, 1866; Giesbrecht, 1892; T. Scott, 1894; Johnson, 1938; Lang, 1965, 1967; Fleminger, 1973; Geletin, 1976). The family has grown from two to four genera (Geletin, 1976) and several species complexes have been recognized. Nevertheless, the recent work of Goetze (2003) has uncovered further genetic complexity that is not reflected in the number of species described from morphological characteristics.

The focus of this paper is a pair of cryptic species within the present taxonomic designation *Eucalanus hyalinus* sensu lato. Giesbrecht (1892) described *Eucalanus elongatus* var. *hyalinus* from the Mediterranean and in 1894, T. Scott briefly described *Eucalanus spinifer* from the Gulf of Guinea (Scott 1894). Giesbrecht subsequently considered *E. spinifer* to be a junior synonym of *E. elongatus* var. *hyalinus* (Giesbrecht and Schmeil, 1898). The *E. elongatus* complex initially contained several varieties, some of which were elevated to specific status with further subspecies (Johnson 1938). Confusion about the identity of *E. elongatus* and *E. hyalinus* continued until Lang (1965) clarified the distinct biogeographic distributions of

Eucalanus species in the Pacific and Indian Oceans. It is now clear that *Eucalanus elongatus* is restricted to the Indo-west Pacific and coastal waters of the Northern Indian Ocean (Lang, 1965, 1967), while *E. hyalinus* s. l. is distributed circumglobally in subtropical and temperate waters. Currently the genus *Eucalanus* contains five species: *E. californicus* Johnson, 1938; *E. bungii* Giesbrecht, 1892; *E. inermis* Giesbrecht, 1892; *E. elongatus* (Dana, 1849), and *E. hyalinus* (Claus, 1866).

Eucalanus hyalinus s. l. is a common member of subtropical and temperate zooplankton assemblages worldwide (e.g., (Vervoort 1963); (Lang 1967); (Fleminger 1967; Fleminger 1973); (Deevey and Brooks 1977); (De Decker 1984); (Bradford-Grieve 1994). Although rarely abundant, it is a commonly noted species due to its large size and unusual translucent appearance. *E. hyalinus* s. l. is found at epipelagic or upper mesopelagic depths (Paffenhofer and Mazzocchi 2003), and feeds on auto- and heterotrophic nano- and microplankton (Kleppel, Burkart et al. 1996), as *elongatus*). *E. hyalinus* s. l. is routinely found in the Humboldt Current (Escribano and Hidalgo, 2000), California Current (Rebstock, 2001, 2002), Benguela Current (De Decker, 1984; Richardson et al., 2001, as *elongatus*), and the Bay of Biscay (Poulet et al., 1996, as *elongatus*), where it is often taken to be an indicator of oceanic water masses. *E. hyalinus* s. l. is also characterized by unusually high water content (low carbon and lipid content), low caloric content, low metabolic and excretion rates, and a long tolerance to starvation relative to other common calanoid copepods, suggesting an uncommon life history (Morris and Hopkins, 1983; Flint et al., 1991).

While conducting a molecular systematic study of the family Eucalanidae, the presence of two very distinct genetic forms of *E. hyalinus* was detected (Goetze, 2003). The two lineages were genetically differentiated at 2 mitochondrial (COI, 16S rRNA) and 1 nuclear (ITS2) gene loci, suggesting long-term reproductive isolation between the two forms. Large-scale geographic sampling of the two lineages revealed that they overlap in large portions of their biogeographic range. Genetic differentiation between forms is maintained across all populations worldwide, even in regions where they co-occur (Goetze, in prep). Combined, this evidence strongly suggests that the forms represent two reproductively isolated species. As can be seen in the results detailed below, the cryptic species are also differentiable by a number of subtle morphological characters and size. It is clear that the larger form should be ascribed to *E. hyalinus* s.s., as defined by Giesbrecht (1892, as *elongatus* var. *hyalinus*). We investigated, in order of priority, the availability of older names for the smaller form, and reinstate an old name for the smaller species.

In this paper we present a new description of *Eucalanus spinifer* T. Scott, 1894 females and males, and show how they may be distinguished from *Eucalanus hyalinus* s.s (Claus, 1866) by both morphological and molecular characters.

Materials and Methods

Collection information for specimens examined in this study is given in Table 1 (a full list of locations included in the genetic description is available from the first author). Individuals examined for morphological characters were preserved either in

1.8% borate-buffered formaldehyde, or 95% non-denatured ethyl alcohol. Ethanol-preserved specimens were collected with 202-333μm mesh nets (1 m ring or 0.71 m bongo) towed obliquely from the surface to between 200 and ~1100m at 29 locations on 6 cruises. Formaldehyde preserved specimens were examined from 5 samples, all of which were archived in the Pelagic Invertebrates Collection (Scripps Institution of Oceanography). Adult males were very rare or absent in much of the material available to the authors. Additional specimens were made available by the Zoological Museum, University of Copenhagen, to investigate the status of *E. spinifer*.

All measured specimens were preserved and stored in 95% non-denatured ethanol. The only criteria for selecting specimens were that the structures to be measured were whole and undistorted. Specimens or dissected parts were mounted in glycerol or gum chloral for measurement, following removal of a section of the body for DNA sequencing. For example, tissue was removed for DNA sequencing from the urosome or posterior prosome segments for specimens measured in the head region. Individuals were genotyped to ensure accurate species identification before measurements were made (Figure 1) of the length of the whole body (TL), prosome (PL), cephalosome (CL), head width and height (HT, WDT), antennule segment lengths, caudal rami lengths (CRL, CRR), and examined for integumental pore patterns. DNA sequence data were not obtained from 4 out of the 162 individuals included, due to problems with gene amplification. Measurements of antennule asymmetry and length ratios are quite sensitive to measurement error, and limbs must be dissected off the animal in order to obtain sufficiently accurate measurements.

Measurements of head height and width are very sensitive to orientation, and individuals were consistently mounted such that the lateral margins of the head and brain were in a single plane of focus. Additional unstained, genotyped specimens were also dissected and mounted in gum chloral or glycerol for examination.

Specimens prepared for illustration were stained in chlorazol black or chlorazol black E (CBE) and examined whole in either lactic acid or glycerol. Dissected parts were permanently mounted in gum chloral for illustration (Pantin, 1964). Both ethanol and formaldehyde-preserved specimens were used for illustrations.

Fleminger (1973) developed a suite of high-magnification characters (integumental pores) within the family Eucalanidae to detect and describe morphologically cryptic, but reproductively isolated populations within the family. For examination of these integumental pores, specimens were first treated with 20-25% KOH for tissue digestion followed by staining in 1% CBE (dissolved in 70% ethyl alcohol), following the protocol of Fleminger (1973). The only modifications to this protocol were to increase the length of digestion for up to 12 hours and to increase the KOH concentration to a maximum of 25%, to ensure complete digestion of all internal tissues. Thirty seconds in CBE often resulted in an excellent level of staining for regions of the posterior prosome and urosome, but in a slightly understained cephalosone and first thoracic segment. Understained regions were difficult to score accurately for the presence of pores, and consequently pore sites on the maxillar somite were removed from the analysis. Observations were made only of tergal

(dorsal) sites. The numbering scheme for site positions is as designated in figure 7 of Fleminger (1973). Abbreviations used here for somites include: A1, Antennule; A2, Antenna; Mnd, Mandible; Mx1, Maxillule; Mx2, Maxilla; Mxpd, Maxilliped; P1 through P5, pedigerous somite 1 through 5 respectively; Abd1-2, first urosomite; and Abd3 through Abd5, urosomite 3 through anal segment respectively.

DNA was extracted from individual copepods using the QIAGEN DNeasy kit or a modified lysis buffer protocol (Lee and Frost, 2002). Primers and conditions used for PCR amplification of mitochondrial COI, 16S rRNA, and nuclear ITS2 are given in Table 2. Two to 5 μl of PCR product were used in DNA sequencing reactions, which were carried out on a MegBACE 500 or ABI 373 automated DNA sequencer. DNA sequences were aligned using ClustalW (Thompson et al., 1994), followed by manual editing as necessary in MacClade (Maddison and Maddison, 2000). Sequences were trimmed at either end to obtain an alignment with all sequences of equal length. Final alignments for COI (N=383 *spinifer*, 450 *hyalinus*), 16S rRNA (N=31 *spinifer*, 25 *hyalinus*), and ITS2 (N=3, 3) contained 348, 311, 506bp respectively. The average number of nucleotide differences between species was calculated in the software package DnaSP 4.0 (Rozas et al. 2003), and K2P mean distances were calculated in MEGA 3.0 (Kumar et al., 2004).

Results

Eucalanus spinifer *T. Scott, 1894*

Eucalanus spinifer T. Scott 1894, p. 29, pl.1, figs 15-23.

Genetic Description

Genetic description is based on partial DNA sequences of 3 gene loci, mitochondrial cytochrome oxidase I (COI, 348 bp), mitochondrial 16S ribosomal RNA (16S rRNA, 311 bp), and nuclear Internal Transcribed Spacer 2 (ITS2, 506 bp). The highest levels of genetic differentiation are observed at COI, followed by 16S rRNA and ITS2. Gene sequences were obtained from 383 *spinifer* specimens collected in the North Pacific, South Pacific, North Atlantic, and Indian Oceans (locations as in Figure 12).

Mitochondrial COI: *Eucalanus spinifer* is characterized by 12 fixed nucleotide differences not shared with *E. hyalinus* s.s. Table 3a lists base composition and the level of intraspecific polymorphism of all nucleotide sites fixed between species. There are 95 additional nucleotide sites monomorphic in *E. spinifer*, but polymorphic in *E. hyalinus* s.s., and 26 nucleotide sites polymorphic in *E. spinifer*, but monomorphic in *E. hyalinus* s.s. The average number of nucleotide differences between individuals of *E. spinifer* is 0.8 (N=383), and the average number of nucleotide differences between *E. spinifer* and *E. hyalinus* individuals is 36.1 (N=383,

450). Average Kimura 2-parameter (K2P) pairwise distances observed between individuals of *E. spinifer* is 0.2% (N=383), while that between *E. spinifer* and *E. hyalinus* individuals is 12.3% (N=383, 450). Site positions listed in Table 3a refer to locations in the *Eucalanus spinifer* COI sequence under GenBank accession #AY647918.

Mitochondrial 16S rRNA: *Eucalanus spinifer* is characterized by 20 fixed nucleotide differences not shared with *E. hyalinus* s.s. Table 3b lists base composition and level of intraspecific polymorphism of all nucleotide sites fixed between species. There is one species-specific indel at site # 69, with *E. spinifer* characterized by a gap. There are six additional nucleotide sites monomorphic in *E. spinifer*, but polymorphic in *E. hyalinus* s.s., and 11 nucleotide sites polymorphic in *E. spinifer*, but monomorphic in *E. hyalinus* s.s. The average number of nucleotide differences between individuals of *E. spinifer* is 2.6 (N=31), and the average number of nucleotide differences between *E. spinifer* and *E. hyalinus* individuals is 26.015 (N=31, 25). Average K2P pairwise distances observed between individuals of *E. spinifer* is 0.9% (N=31), while that between *E. spinifer* and *E. hyalinus* individuals is 9.0% (N=31, 25). Site positions listed in Table 3b refer to locations in the *Eucalanus spinifer* 16S rRNA sequence under GenBank accession #AY647923.

Nuclear ITS2: *E. spinifer* is differentiated from *E. hyalinus* s.s. by the presence of one fixed nucleotide difference at nucleotide site # 375 (notation as for nucleotide

position of GenBank accession # AY647921). No intraspecific polymorphism is observed at this gene locus within either *E. spinifer* or *E. hyalinus* s.s. (N=3,3).

Morphological description

Material examined

66 adult females from 18 locations; 4 adult males from 2 locations (Table 1). Illustrated adult male specimen (Figures 9-11) from Ace-Asia, St. 2. Female morphological description from specimen collected at SOSO, St. 5, tow 8. Type locality: Central subtropical Pacific, 24° 25.96'N 156° 53.0'W.

Female *(Figures 2-5)*

Total length 5.42 to 6.81 mm, mean = 6.09, sd = 0.37, (N=38, 9 samples, Southwest Pacific, Indian Ocean). Prosome length 4.85 to 6.17 mm, mean = 5.44, sd = 0.31, (N=43, 9 samples). Cephalosome length 3.63 to 4.51 mm, mean = 4.10, sd = 0.23 (Figure 6, Table 4). Anterior head drawn out into a pointed process extending beyond the base of the rostrum. In lateral view, ventral border of anterior head of *spinifer* convex with only a slight inflection near the anterior tip (Figure 7). In ventral view, the anterior head angle (see Figure 1) of *spinifer* relatively obtuse, between 96 and 101°, and head Height/Width ratio between 0.26 and 0.35 (Figure 7B, Table 4). Rostrum in form of large, bulbous (in lateral view), ventroposteriorly directed appendage bearing two long, non-articulated terminal filaments (Figure 2).

Cephalosome fused to pedigerous somite 1; boundary between pedigerous somites 4 and 5 visible (Figure 2 A), although somites fused laterally. Cephalosome elongate, occupying 0.73 – 0.77 of the prosome (mean = 0.75, N=31); dorsal and lateral margins undulating in the region of the antennal and mandibular somites. Dorsal and lateral surfaces of pedigerous somites 2-5 covered with hairs. In lateral view posterolateral corners of the prosome extend postero-dorsally into pointed process, and in dorsal view these pointed processes are directed posterolaterally. Urosome of 3 free somites (Figure 2 A-D); genital double-somite symmetrical, not swollen in dorsal view but swollen ventrally, genital operculum oval-shaped, placed posteriorly on somite, covering a pair of semicircular gonopores; in lateral view, pair of narrow elongate seminal receptacles located posterolaterally, oriented along dorsoventral axis, but directed very slightly anteriorly on the dorsal side (Figure 2C, F); anal somite completely fused with caudal rami. Caudal rami asymmetrical, and always longest on the left. Asymmetry in the caudal rami appears related to asymmetry in the first antennule (see below). *E. spinifer* consistently has a longer first antennule on the right side of the body, and longer caudal ramus on the left. Caudal rami without seta in position I, seta II (Figure 2C) located laterally, four strong terminal plumose setae in positions III-VI with seta V on left enlarged, seta VII small, situated slightly on ventral surface at base of seta VI and directed posteriorly.

Antennules (Figures 2A; 3A, B) asymmetrical, usually held at right angles to body; longest always on right extending 5 segments beyond the left, and more than 5 segments beyond the caudal rami, both usually 23-segmented. Ancestral segments X

and XI usually completely fused (specimen illustrated here is unusual in that it has these segments completely separated on right, left segment fused on ventroposterior surface). Asymmetry in antennules is the result of lengthening of segments. Proximal segments I-IV through X-XI are mildly longer on the right, with increasing length asymmetry between segments XII and XXII, followed by declining asymmetry in segments XXIII-XXV (Figure 8A). Differences in segment length between sides ranges between 4 and 87 μm (ratios range between 0.99 and 1.25), with a minimum difference at segment V, and a maximum at segment XXI (Table 5). Armature elements, referring to ancestral segments, as follows: I-IV-9 + 1$_a$esthetasc (minute), V-2 + aesthetasc, VI-2, VII-2 + aesthetasc, VIII-2, IX-2, X-XI 4 + aesthetasc, XII-2, XIII-2, XIV-2, XV-2, XVI-2 + aesthetasc, XVII-2, XVIII-2, XIX-2, XX-2, XXI-1 + aesthetasc, XXII-1, XXIII-1, XXIV-1 + 1, XXV-1 + 1, XXVI-1 + 1, XXVII-XXVIII-6 (1 minute). Aesthetascs not well developed being short, narrow and tapering. Medium-sized densely plumose setae on proximal segments directed slightly anterior of ventral. Enlarged, stiff, pseudoannulate, apparently sparsely setulose setae (Weatherby et al., 1994) on segments V and IX directed anterolaterally, on segments XI, XVII, XX, XXIII and XXVI directed laterally. Flattened, pseudoannulate, sparsely setulose seta on segment XXV in same plane as enlarged proximal setae, but similar seta on segment XXVI set at right angles to seta on segment XXV. Two terminal setae of flattened, pseudoannulate type. This specimen unusually lacks an aesthetasc on the right A1 segment XI. This aesthetasc is present on the left A1, and in additional specimens on both sides.

Antenna (Figure 3C-E) with endopod twice as long as exopod; coxa and basis separate, coxa with 1 seta directed into midline, basis with 2 setae originating on raised knob. Endopod segment 1 with 2 setae, segment 2 with 8+7 setae (specimen illustrated here unusually has 8+8 setae on right and 8+7 setae on left, boundary between ancestral segments II, III and IV seems to be evident on posterior surface), patches of spinules decorate distal outer borders of apparent ancestral segments on posterior surface and spinules extend slightly around onto anterior surface (Figure 3D, E). Exopod 8-segmented with a full complement of 12 ancestral setae, segment 1 composed of ancestral segments I and II and with 2 seta, segment 2 composed of ancestral segments III-IV and with 2 setae, segments 3-7 with 1 seta each, ancestral segment IX (with 1 seta) and segment X (with 3 setae) appears to be separate on posterior surface but are fused on anterior surface. All setae plumose.

Mandible (Figures 3 F, G; 4 A, B) gnathobases asymmetrical (apparently relating to interlocking positions of largest teeth). Gnathobases both with 1 anterior seta bordered by spinules and 8 teeth that increase in size posteriorly. Posterior large tooth on right gnathobase separated from adjacent tooth by large gap equal to basal width of adjacent tooth, third tooth closely adjacent to second tooth. On left side second tooth from posterior border situated almost equidistant between first and third teeth. Basis with 3 setae, proximal-most of which extends as far as proximal border of basis; endopod segment 1 with 2 setae, segment 2 with 4 large, strong setae and 1 very small seta that are directed into the mid line; exopod with 6 setae, 5 of which are very long and strong.

Maxillule (Figure 4 C) praecoxal arthrite with 4 posterior surface setae as well as 10 marginal spines and setae and an additional seta on the anterior surface; coxal endite and basal endites 1 and 2 with 3, 4, 5, setae respectively; basis and endopod separate; endopod segments fused with 4/5+10 setae; exopod with 5/6 setae; basal exite with 1 seta; coxal epipodite with 9 setae.

Maxilla (Figure 4D) praecoxa with an outer distal protrusion, endite 1 with 7 setae (1 very small), endites 2-4, endite 5 with 4 setae; coxal epipodite bearing long plumose seta; endopod segment 1 fused with basis and with 1 seta, endopod segments 2-4 with 1, 2, 3 setae respectively, one seta on segment 3 is very short and extending back towards basal endite 1. Inner surface of lobes 1-5 decorated with fine, long spinules.

Maxilliped (Figure 4E, F) syncoxa slightly longer than basis; syncoxal endites with groups of 1, 2, 3, 3 setae, oval patch of very small spinules at base of endite 4 on posterior surface and few hairs on the border near these setae; basis without decoration and with 3 medial setae; endopod segment 1 largely separate from basis, with 2 setae, free endopod segments 2-6 with 4, 4, 3, 3+1, 2+2 setae respectively.

Swimming legs (Figures 4 G-J, 5 A-C): segmentation and disposition of spines and setae as illustrated. *Leg 1* coxa with few inner distal spinules; basis with minute outer spinule and conspicuously produced inner border lined with long spinules, stout plumose seta arises from anterodistal inner corner and opposes outer swelling of endopod segment. Exopod segment 1 with large, posteriorly produced lobe, endopod

segment 1 outer swelling decorated with numerous long, very fine spinules. Legs 2-4 very similar.

Variability Some variability was observed among adult female individuals in the following characters: 1) the extent of fusion of ancestral segments X-XI of the antennule, 2) the number of setae on the maxilliped endopod segment 2, and 3) the asymmetrical number of setae on the endopod of the antenna between right and left sides of the body. The adult female illustrated here had unusually unfused antennule segments X-XI (partial fusion on left ventropsterior surface). All other adults examined for this character were fully fused on both sides. The illustrated female also had an unusual 4 setae present on maxilliped endopod segment 2, while 3 setae are typical. This setal number was observed to vary in both *E. spinifer* and *E. hyalinus*, with a small minority of specimens (4 out of 33) having 4 setae on this segment. This may be a sub-adult, CV character that is occasionally retained in the adult form. Finally, the female illustrated here was observed to have 8+8 setae on the right A2 endopod and 8+7 setae on the left. An endopod with 8+7 on both sides is the normal configuration.

Integumental pore pattern Sensillar pore sites for *E. spinifer* are as described for *E. hyalinus* (Fleminger, 1973), with the following standard number of dorsal sites on each somite: A1:10, A2: 4, Mnd: 6, Mx1: 6, Mx2: 10, Mxpd: 16, P1: 7, P2: 7, P3: 9, P4: 6, P5: 4, Abd 1-2: 3, Abd 3: 2, Abd 4: 2, Abd5: 1. No sensillar pores were observed to differ consistently in presence or position between *E. spinifer* and *E. hyalinus*. Therefore, no sites were identified that could be used as a species-specific

diagnostic character to differentiate the two species. This is similarly the case with sister species *E. californicus* and *E. bungii* (Fleminger, 1973). Intraspecific variability in the presence/absence of pores was observed at some sites in both species. The following sites varied within *E. spinifer* (N=9): MdTar1 + 2 (right pair), Th3Tar1 +2 (right pair), Th3Tb medial site, Th4Tal1 in one individual observed on right side rather than left, as is usual, Th5Tal1 varies in position, Th5Ta medial site, Abd 1+2Tal varies in position, Abd1+2Tb medial site, and the maxilliped somite was typically observed to have six pairs of sites, with an additional one or two pores in medial positions on a few specimens. Occasionally, individuals were also observed to have pore sites in addition to those listed above. Such sites included two specimens with pores anterior to A1a, one specimen with three pores below the 5 Th1Tb pores, one specimen with an extra pore left of Abd1+2Tal1, and one specimen with a second pore on the left side of Th4. Sites on Mx2 were not scored, as understaining resulted in specimens too difficult to accurately observe. Six additional pore sites were observed on the dorsal surface of the caudal rami (3 on each side), and five on the ventral surface, including one large pore in alignment with the asymmetrically large seta V on the longer, left caudal ramus.

Male (description relative to female, Figures 9-11)

Ilustrated specimen: Total length 5.08 mm, prosome length 4.14 mm, cephalosome length 3.18mm (Additional individuals, SW Pacific: TL: 4.66, 4.75, 4.58mm, PL: 3.76, 3.88, 3.80mm, CL: 2.88, 3.00, 3.04mm). Sexual dimorphism

present in the shape of the head and prosome; number of segments on the urosome; shape, setulation, and overall size of feeding appendages; Leg 5; absence of spinule patches on dorsal and lateral surface of posterior prosome; body size. Anterior head in dorsal view more rounded than in female, with broad triangular shape to anterior portion of the head (Figure 9C). In lateral view, the anterior head appears blunt and rounded, not drawn out into pointed process (Figure 9B). Rostrum and rostral filaments as in female. Two small hairs of the frontal organ prominent on ventral surface of head, anterior to the rostrum (Figure 9B).

Cephalosome fused to pedigerous somite 1 as in female, but with more pronounced undulation of lateral and dorsal margins in the region of antennal and mandibular somites. Pointed processes at posterolateral margin of prosome less pronounced in males than females, terminating obtusely (Figure 9C, E). Posterior process gives the appearance of being more laterally directed than in adult female. Pedigerous somites 2-5 on prosome lack patches of minute spinules on dorsal and lateral surface, which are present in adult females of both *E. spinifer* and *E. hyalinus* s.s.

Urosome of 4 free somites (Figures 9E, F), with second somite longer than 1st, 3rd or 4th; 5th (anal) segment fused to caudal rami. Gonopore slit positioned medio-laterally on the left side of the first urosomite (Figure 9E). Urosomite 1, anterior to gonopore appears to have rough surface extending to the segment boundary. Caudal rami asymmetrical, as in female, with the longer side on the left. Insufficient numbers of specimens were available to assess whether the pattern of asymmetry is consistently

with longer side on the left, as is found in adult females. Caudal rami armature as in female. Intestinal tract asymmetrically positioned right of saggital in the posterior prosome of all males examined.

Antennules (Figure 10 A, B): Symmetrical, consisting of 24 free segments; extending more than 5 segments beyond the caudal rami; not geniculate. Antennule segments XXII and XXIII, which are maximally asymmetrical in the adult female, exhibit ratios of 1.02 and 1.09 respectively in the adult male. Ancestral segment I not fused to II-IV, with suture more pronounced along posterior margin than anterior margin of antennule. Ancestral segments X and XI completely fused; segments XI-XII partially fused with suture highly visible, does not appear to be articulated. The suture line is curved, and not aligned on dorsal and ventral surfaces of the antennule. Specimen illustrated in figure 10 A, B with incomplete first antennules, extending only to segment XXIV on right antennule, and XXV on left antennule. Armature elements, referring to ancestral segments, as follows: I – 2 + aethetasc, II-IV - 6 + 5 aesthetascs (minute), V-2 + 2 aesthetascs, VI-2 + aesthetasc, VII-2 + 2 aesthetascs, VIII-2 + 2 aesthetascs, IX-2 + 2 aethetascs, X/XI- 4 + 4 aesthetascs (illustrated specimen unusually has only 1 seta on left segment XI, full complement on right) , XII- 2 + 2 aesthetascs, XIII-2 + 2 aesthetascs, XIV-2 + 2 aesthetascs, XV-2 + 2 aesthetascs, XVI-2 + 2 aesthetascs, XVII-2 + 2 aesthetascs, XVIII-2 + 2 aesthetascs, XIX-2 + 2 aesthetascs, XX-2 +2 aesthetascs, XXI-1 + 2 aesthetascs, XXII-1 + 2 aesthetascs, XXIII-1 + 2 aesthetascs, XXIV-1+1 posterior surface + 2 aesthetascs, XXV-1 + 1 posterior surface, XXVI-1 + 1 posterior surface, XXVII-XXVIII- 5 +

aesthetasc. Most setae and aethestascs incomplete on this specimen; description completed by examination of additional specimens. Also see the *E. hyalinus* s.s. left antennule in figure 13 A for illustration of an individual with more complete armature (no differences between species in meristic chararcters).

Antenna (Figure 10 C): Coxa and basis broad and flat relative to female, with no sexual dimorphism in armature. Endopod segment 1 broadens distally, with 2 setae, 1 very reduced. Boundary between ancestral segments II, III, IV visible on posterior surface. Ancestral segment II with 8 setae, as in female, but 1 seta is reduced nearly to a hair, and 2 setae are thin, short and tapering relative to the remaining 5 plumose setae. Seven setae on ancestral segments III-IV, with 1 very small seta on posterior surface (apparently absent in illustrated specimen). Patches of spinules on posterior surface of endopod segments II-IV present as in female. Exopod 7-segmented with ancestral segment IX and X fused (these segments separated in female), with 12 setae, 6 of which are highly reduced in the adult male. Three setae on segments I-II are highly reduced and not plumose. Similarly, 3 terminal setae all reduced and hairlike. Six strong setae on ancestral segments IV-VIII remain plumose.

Mandible (Figures 10 D, E): Mandibular basis significantly broader than in female, with 3 inner marginal setae reduced nearly to hairs. Endopod segment 1 with 2 setae, segment 2 with 5 setae as in female. Exopod with 6 strong plumose setae, 1 seta which is small in the adult female is strong and plumose in the adult male. Gnathobase highly reduced relative to female, with teeth barely discernable.

Anterodorsal seta covered with spinules present in only some individuals, highly reduced relative to female. Exoskeleton of gnathobase flaccid, lacking rigidity.

Maxilulle (Figure 10 F): Praecoxal arthrite setae and spines reduced relative to female, totaling only 9. Anterior surface seta absent, as well as 5 setae on posterior surface and margin; no spinules on proximal margin of arthrite. Coxal endite with 2 highly reduced, nearly hairlike setae, basal endite 1 and 2 with 3 and 4 setae respectively. Endopod with 11/12 setae, exopod with 6 setae. Basal exite with 1 seta; coxal epipodite with 9 setae, with the 2 proximal-most setae reduced relative to female. Overall size of limb reduced relative to female.

Maxilla (Figure 11 D): Reduced relative to female, with smaller overall size, flaccid exoskeleton. Praecoxal endite 1 with 6 setae (1 very small), lacking the proximal-most seta on female; endites 2, 3, 4 with 3 setae; endite 5 with 4 setae. Coxal epipodite with 1 seta; endopod with 7 setae, 1 on anterior surface (apparently absent in illustrated specimen). Spinules present on proximal margin of endite 1 only, absent in all other endites.

Maxilliped (Figure 11 E): Syncoxa with only 7 setae, 5 of which are highly reduced, 1 proximal and 1 distal seta plumose; distal spinule patch on posterior surface of syncoxa absent. Basis with 3 setae, as in female, but with 2 proximal setae reduced. Endopod segments 1, 2, 3, 4, 5, 6 with 2, 3, 4, 3, 3+1, and 4+1 (2 very small) setae respectively, 1 more than in the adult female. Distal-most seta on segment 4, and 2 distal-most setae on anterior surface of segment 5 modified into a broad, flat paddle-like structure for basal 1/3 of the seta, before changing into normal plumose setae in

the remaining upper portion. Six setae, located on endopod segment 1 (proximal seta), 2 (proximal setae), 3 (proximal 2 setae), 4 (proximal seta, illustrated), 5 (proximal seta, illustrated) are plumose basally and scale-textured distally, and may serve a sensory function. Setae on endopod segments1-4 inwardly directed. The 2 proximal-most, anterior setae on the 5th segment inwardly directed, and with the distal-most, anterior seta, and the posterior seta near the 4th and 5th segment boundary outwardly directed. Setae on endopod segments 5 and 6 outwardly directed. Orientations for all setae as observed in close relative *Pareucalanus attenuatus* (Ohtsuka and Huys, 2001, Figure 5C), although the broad, flat modified setae on segments 4 and 5 appear to be absent in *P. attenuatus.*

Leg 5 (Figure 11 A, B, C): Left limb 4-segmented, substantially longer than right limb. Seta present distally on segment 3 of both right and left legs. Tufts of setules on terminal/subterminal portion of apical segment, as well as on the medio-distal surface of the 3rd segment of the left exopod, both features commonly found in other calanoid genera (Ohtsuka and Huys, 2001). Right limb 4-segmented, extending beyond distal border of left basis, with seta near distal margin of segment 3. Final segment on right limb broken in illustrated male. Some characteristics of segment shape as illustrated in Figure 11B are due to orientation on the slide: compare the 2 individuals figured (Figure 11 A, B) for an accurate interpretation of limb shape.

Distribution (Figure 12)

Circumglobal, oligotrophic subtropical waters. Occurs in high abundance in subtropical gyres, and is largely replaced by *E. hyalinus* s.s in productive waters near subtropical fronts, and upwelling zones. Absent from tropical and equatorial waters.

Remarks

When we investigated, in order of priority, the availability of older names for the smaller form, we had to consider *Calanus erythrochilus* Leuckart, 1859 and *Eucalanus spinifer* T. Scott, 1894. *Calanus erythrochilus*, although probably a *Eucalanus*, is not considered to be available because no measurements were given, the figures are not detailed enough to determine the species, and as *C. erythrochilus* was taken near Nice, in the Mediterranean, it is considered probably to be a synonym of *E. hyalinus* s.s. *Eucalanus spinifer* T. Scott, 1894, on its small size alone, appears to be available for the species identified here. The illustrations in Scott's plate 1, figures 15-23, lack sufficient detail to determine accurately the shape of the anterior region of the head, and do not include measurements of both antennules, making an assessment of the degree of asymmetry in the antennule impossible. Scott described proportional lengths of ancestral antennule segments I-IV – XXVI along one antennule (not specifying which side), but unfortunately, the lengths given seem to correspond to the shorter left antennule (see Fig. 5), since this is the side he illustrated in Pl. 1, Fig 15. No information is presented regarding lengths of the caudal rami. However, a total body length of 5.5mm, as given for an adult female, could only correspond to the smaller of the two genetic forms examined here.

Subsequent examination of material from the Danish (Atlantide) Expedition to the Coasts of Tropical West Africa 1945-46 (Vervoort, 1963 as *E. elongatus hyalinus*), kindly made available by the Zoological Museum (University of Copenhagen, Denmark), confirmed that the smaller species is indeed found in the Gulf of Guinea.

A search for the type specimens corresponding to the original species descriptions of either Claus or T. Scott was unsuccessful, and it appears highly probable that the types for both *E. spinifer* and *E. hyalinus* are no longer extant. Claus' material for *Calanella hyalina* was not present in the Zoological Collection University of Marburg (where Claus was director from 1862 to1871); the Natural History Museum, Vienna or the Zoological Collection of the University of Vienna (Claus was in Vienna after Marburg); or the Goettinger Collection, Naturmuseum Senckenberg (SMF), Frankfurt (some Claus material, but no *E. hyalinus* type material). T. Scott's *E. spinifer* type material is also not held at the Natural History Museum, London, although their collections contain some T. Scott material from the Gulf of Guinea Expedition. It appears probable that the *E. spinifer* types are also no longer extant (G. Boxshall, pers. comm.).

The species *E. hyalinus* s.s. and *E. spinifer*, as designated here, form a sister species pair, and are each other's closest relatives in the family Eucalanidae (Goetze, 2003, as *E. hyalinus* 1 and 2).

Eucalanus hyalinus (Claus, 1866)

? Calanus erythrochilus Leuckart, 1859, pp 260, pl.6

Calanella hyalina Claus, 1866, p. 8

Eucalanus elongatus var. *hyalinus*, Giesbrecht, 1892, pp. 131, 133, 134, 150, 739, 740, pl. 11 figs. 2, 7, 12, 20, 25, 32, 36, pl. 35 figs. 1, 2, 13, 23, 24; Johnson, 1938, p. 170, figs. 3, 17, 19, 21, 24; Brodsky, 1950, p. 103, fig. 29

Eucalanus elongatus, Marques, 1947, p. 35; Marques 1956, p. 8; Marques, 1957, p. 11; Vervoort, 1957, p.32, Marques 1958, p. 223; Marques, 1959, p. 206; Marques, 1961, p. 51; Owre, 1962, p. 491.

Genetic Description

Genetic description is based on partial DNA sequence from the same gene loci as for *E. spinifer* above, and includes only information not contained in the *E. spinifer* description. A total of 450 specimens are included from the North and South Pacific, Indian Ocean, and North Atlantic.

Mitochondrial COI: The average number of nucleotide differences between individuals of *E. hyalinus* s. s. is 2.7 (N=450), and the average number of nucleotide differences between *E. spinifer* and *E. hyalinus* individuals is 36.1 (N=383, 450). Average K2P pairwise distances observed between individuals of *E. hyalinus* s. s. is 0.8% (N=450), while that between *E. spinifer* and *E. hyalinus* individuals is 12.3% (N=383, 450). Site positions listed in Table 3a refer to locations in the *Eucalanus hyalinus* COI sequence under GenBank accession #AY647919.

Mitochondrial 16S rRNA: The average number of nucleotide differences between individuals of *E. hyalinus* s. s. is 1.1 (N=25), and the average number of nucleotide differences between *E. spinifer* and *E. hyalinus* individuals is 26.0 (N=31, 25). Average K2P pairwise distances observed between individuals of *E. hyalinus* s. s. is 0.4% (N=25), while that between *E. spinifer* and *E. hyalinus* individuals is 9.0% (N=31, 25). As noted above, there is also one species-specific indel, which occurs at site # 69, with *E. hyalinus* characterized by a base A. Site positions listed in Table 3b refer to locations in the *Eucalanus hyalinus* 16S rRNA sequence under GenBank accession #AY647920.

Nuclear ITS2: As noted above, the presence of one fixed nucleotide difference at nucleotide site # 375 differentiates *E. hyalinus* s. s. from *E. spinifer*.

Morphological description

Materials examined

96 adult females from 21 locations; 3 adult males from 2 locations (Table 1). One adult male illustrated from SOSO, St. 72-18.

Female

Total length 6.17 to 7.50 mm, mean = 6.83, sd = 0.33, N=61 (13 samples, South Pacific, North Pacific, Indian Ocean). Prosome length 5.46 to 6.92 mm, mean = 6.15, sd = 0.30, N=71 (13 samples, as above). Cephalosome length 4.10 to 5.08 mm,

mean = 4.58, sd = 0.24 (Figure 6, Table 4). Total length as reported by Giesbrecht for Mediterranean Sea specimens is 5.9 to 7.1 mm (prosome 5.2-6.2 mm, urosome 0.7-0.9 mm), slightly smaller than specimens measured here from the North and South Pacific, and Indian Ocean. Cephalosome occupying between 0.72 to 0.78 of the prosome (mean = 0.75, N=71). In ventral view, head drawn into attenuated tip at anterior end, with height/width ratios between 0.34 and 0.47, and head angles between 81° and 91° (Figure 7A, Table 4). In lateral view, ventral border of anterior head convex, shifting to concave near the anterior tip, leading to pronounced point at terminus. Total length of the first antennule between 7.84 mm and 9.85 mm (segments I-XXV only), with individuals with a longer antennule (long side) also tending to exhibit longer caudal rami. Lengths of caudal rami for longer side range between 245 μm and 302 μm, and for the shorter side between 208 μm and 274 μm.

Adult females of *E. hyalinus* s.s. exhibit dimorphic asymmetry (*sensu* Ferrari, 1984). Asymmetry in the length of antennules, caudal rami, side with the longer seta V on the caudal ramus, and the position of the intestinal tract fluctuates in concert between right and left sides of the body. A longer right antennule is always found with a longer left caudal ramus, a longer seta V on the left CR, and an intestinal tract right of saggital. This condition was much more common among the specimens examined, than was the opposite, with a longer left antennule. Intriguingly, Giesbrecht (1892) illustrated a *hyalinus* individual with the asymmetry of a longer antennule on the left side.

Antennules Asymmetry in the antennule is most pronounced at segments XXI-XXIII, though all segments exhibit at least mild asymmetry (Figure 8A). Ratios of length asymmetry in XXII are between 1.36 and 1.59, and for XXIII between 1.30 and 1.54. Measurements are reported with the length from the longer antennule (usually right) divided by the length from the shorter side. Ratios of antennule segment lengths for XXII/V are between 3.06 and 3.63, and for XXIII/V between 2.55 and 3.06 (Table 4).

Variability A full description of *E. hyalinus* s.s can be found in Giesbrecht (1892). Giesbrecht (1892) figures dorsal habit of male and female, anterior head of female in ventral and lateral views, female urosome, antenna, mandibular palp, maxillule, maxilliped, leg 1 proximal endopod and segments and basis, leg 2 endopod and outer border of expopod, male leg 5. Included in the present description are observations of morphological variability in *E. hyalinus* s.s., based on genotyped specimens.

Integumental pore pattern As noted above, no sensillar pores were observed to consistently differ in presence or position between *spinifer* and *hyalinus*. The following sites varied within *E. hyalinus* s.s. (N=8): A2Tar1+2 (right pair), Th1Tbl1+2 and r1+2 in one specimen observed in vertical rather than horizontal orientation, Th4Tal1, Th5Tal1, Th5Ta medial site, Abd1+2Tal, Abd1+2Tb, and Abd3Tal, and the Maxilliped somite typically observed with 6 pairs of sites, with 1 specimen with an additional pair in a medial position. One unusual individual was observed to have an additional pore site at MdTb on the left side.

Male (Figure 13)

Illustrated specimen: TL: 4.83 mm, PL: 4.22 mm, CL: 3.24 mm. (Additional specimen: TL: 4.92 mm, PL: 4.00 mm, CL: 3.00 mm). Giesbrecht reports for Mediterranean specimens: TL: 3.9-4.8mm, (PL: 3.3-4.0mm, UL: 0.6-0.8mm). All meristic characters described for *E. spinifer* above are held in common with *E. hyalinus* s.s. We figure the left antennule (and segments XXV-XXVIII) primarily to illustrate an individual with more complete armature than the illustrated *E. spinifer* specimen. Figures of the adult male head in lateral view, urosome in lateral view, and P5 are included to enable comparisons between species, as these illustrations contain features that may assist others in identifying useful shape characteristics for species discrimination. It is currently unknown whether *E. hyalinus* s.s. adult males exhibit dimorphic asymmetry, as do adult females. The *E. hyalinus* adult male also lacks dorsal and lateral hairs on P2-P5 in comparison to the adult female of the same species.

Leg 5 Left leg segments 3 and 4 appear to be slimmer (Figures 13E, F) than in *E. spinifer*; this may be due to the angle at which the limb was drawn rather than species differences in segment shape. It is necessary to compare across multiple individuals to obtain an accurate view of leg segment shape. Possible species differences in segment length are included in the Discussion below.

Giesbrecht (1892) also describes the *hyalinus* adult male, but figures only the dorsal habit, and leg 5. Our observations of the antennules differ from his habitus

drawing in his Pl. 35, Figure 2. Giesbrecht illustrates 25 free segments, but describes only 24-free segments in the text (both including 1 fused segment at X-XI). There are 24-free segments, as illustrated here in Figure 13 A. Aesthetascs thinner and longer than in females. Most segments have 2 setae and 2 aesthetascs except for segments I, XXI-XXIII (1 seta and 2 aesthetascs), segment XXIV which has 2 setae and 1 aesthetasc, XXV and XXVI which have no aesthetascs, and segment XXVII-XXVIII which has 5 setae and 1 aesthetasc.

Distribution (Figure 12)

Circumglobal, subtropical and temperate waters. Common in eutrophic, cooler waters near upwelling zones and subtropical fronts. Absent from tropical and equatorial waters.

Remarks

Claus' original species description of *Eucalanus hyalinus*, under the name *Calanella hyalina* (Claus, 1866), from Nice, France, contains no figures and little descriptive text by which to determine which of the two forms he was examining. No type specimens were designated, and as noted above, it appears that Claus' material has been lost. Claus records the total body length of adult females as between 7 and 8 mm, a size range corresponding only to the larger of the two *E. hyalinus* s. l. forms in the present study (Table 4). Giesbrecht (1892), working with material from near the type locality, illustrated an individual with longer left antennule, a condition found

only in the larger genetic form (*E. hyalinus* s.s) in our specimens. Collections included in this study from near the type locality in the Ligurian Sea contained only specimens of the large *E. hyalinus* form, as verified by DNA sequence data (N=16). On this basis, we propose that the species name *Eucalanus hyalinus* s. s. be retained for the larger of the two forms. Given the current absence of any holotype material, we designate a Neotype specimen and a Type Locality of X, in the Mediterranean Sea.

Material examined by a number of workers cannot be reliably assigned to *E. hyalinus* s.s or *E. spinifer*. These studies include: (1) Fleminger (1973, Figure 14), which probably included adult females of both species, as evidenced by the range of total body lengths observed. (2) Geletin's (1976) figures and data do not indicate whether both species were included. However, both species occur in the study area. (3) Johnson's (1937, 1938) material probably largely included *E. hyalinus*. It is impossible to know with certainty, but from the biogeographic distributions of the two species, it appears likely to be predominantly *E. hyalinus* in the study region. (4) Lang (1967, Figure 1(1,2,3) and Figure 4). It is impossible to determine which species were included.

Discussion

Morphological distinction of E. spinifer and E. hyalinus

A distinct form of the species *Eucalanus hyalinus* s.l., originally discovered through DNA sequence data, was found here to be morphologically distinguishable from its sibling, sister species by a suite of subtle morphometric, shape and size

characters. Despite careful examination of both adult females and males, no meristic characters were found to differ between the two species. Furthermore, the two species were non-differentiable by their integumental pores signatures, a suite of characters developed by Fleminger (1973) within the family Eucalanidae to detect and describe morphologically cryptic, but reproductively isolated populations within the family. Nevertheless, there are a few good characters by which to distinguish the two genetically separate species. These characters relate to the degree of asymmetry in the female antennules, patterns of relative length of segments along female antennules, aspect ratios of the female anterior head in ventral view, and whole body and body region measurements of size.

Females

The extent of asymmetry in segment length along the antennules differs between *E. spinifer* and *E. hyalinus* and is the best character for species distinction (Figure 8A; Table 4). Although the antennules of both species are asymmetrical, *E. hyalinus* is characterized by higher levels of asymmetry. Both species are mildly asymmetrical at proximal segments I-IV through X-XI, increasing in asymmetry between XII and XXII, and declining in asymmetry in segments XXIII-XXV. The greatest species differences occur at the maximally asymmetrical segments XXII and XXIII. Segment XXII is the only antennule segment in which the species are strictly non-overlapping in the range of length ratios observed (Figure 8B, Table 4). This difference holds for specimens collected from a range of locations. Nevertheless, even

for antennule ratios that overlap mildly, the majority of specimens fall in the non-overlapping portion of the range, and these characters may prove useful for the identification of some specimens (eg, XXIII/V, Table 5). Individuals observed to have ratios of the longer over the shorter ancestral antennule segments XXII and XXIII of larger than 1.36 and 1.32 respectively, can be identified as *E. hyalinus* s.s. Similarly, individuals with a XXIII/V ratio (measured on the longer antennule) larger than 2.59, and XXII/V larger than 2.89 can also be identified as *E. hyalinus s.s.*.

The species can also be discriminated by the length ratios along only one antennule, comparing proximal and distal segments of the longer antennule (e.g. XXII/V, Figure 8C). Ratios including antennule segments XXII and XXIII are most useful for species discrimination, with *E. hyalinus* s.s. exhibiting higher length ratios of XXII/V and XXIII/V (Table 4, 5). Specimens with XXII/V ratios of smaller than 2.9 and larger than 3.0 can be identified as *E. spinifer* and *hyalinus* s.s., respectively. Because a large number of the specimens collected in routine plankton surveys are often damaged and missing distal segments of the antennule, use of a combination of the antennule characters presented here may be necessary to effectively identify specimens in many ecological studies.

The shape of the anterior head in lateral and ventral views may also be used to discriminate the two species, with *E. hyalinus* s.s. having a more attenuated, elongate anterior head than *E. spinifer*. In lateral view, the ventral border of anterior head of *E. spinifer* appears only slightly convex, with a mild inflection near the anterior tip. In *E. hyalinus*, this curvature shifts from convex to concave, resulting in a finer process at

the terminus of the anterior head. In ventral view, the anterior head angle of *E. spinifer* is relatively obtuse (96 - 101°), and head height/width ratio is between 0.2614 and 0.350 (Figure 7B, Table 4). This character distinguishes *E. spinifer* from *E. hyalinus* s.s for the majority of specimens, though the height/width ratios slightly overlap. The head angle, though non-overlapping between species in the specimens measured here, is a more difficult measurement to make.

The distribution of adult female body sizes of *E. spinifer* overlaps *E. hyalinus* s.s., but with smaller mean total length, prosome length, and cephalosome length (Figure 6, Table 4). Individuals with a prosome length smaller than 5.4 (TL: 6.1 mm) fall into the non-overlapping portion of the size range, and can be identified as *E. spinifer*. Similarly, individuals observed to have a prosome length longer than 6.2 mm (TL: 6.8mm) are identifiable as *E. hyalinus* s.s. Although size characters are known to vary widely as a function of the environment during development (e.g. Deevey, 1960), the specimens included here were collected at a range of locations, and across a range of dates and seasons. Species-specific differences in size are also observed in both the length of the longer caudal ramus, as well as in the total length of the longer antennule (Figure 14A). *E. spinifer* is the smaller of the two species in both measurements. There also appear to be specific differences in the length of the shorter caudal ramus (Figure 14B), although no differences are observed in the extent of asymmetry in the caudal rami of the two species.

The pattern of dimorphic asymmetry in adult females, originally noted by Giesbrecht (1892), appears to be restricted to *E. hyalinus* s.s.. *E. spinifer* consistently

has a longer first antennule on the right side of the body, and longer caudal ramus on the left. In contrast, the asymmetry in both antennules and caudal rami in *E. hyalinus* fluctuates between the right and left sides of the body, with the longer antennule always on the opposite side of the body from the longer caudal ramus. In concert with these asymmetries in the antennule and caudal rami, the position of the intestinal tract in the posterior region of the prosome is always found right of saggital in *E. spinifer* (same side as longer antennule), and shifts from right to left sides of the body in *E. hyalinus,* with the intestinal tract always on the side with longer antennule. Following results found here, any individuals observed with a longer left antennule in the adult female are specimens of *E. hyalinus* s.s.

Males

No meristic characters were observed to differ between *E. hyalinus* and *E. spinifer* in the adult males. Morphometric or shape characters may distinguish the two species, but sufficient material was not available to determine this with certainty. The following characters appeared to differ between the small number of specimens of *E. spinifer* and *E. hyalinus* examined, and may prove fruitful for future work on this issue: (1) The proportions of the third and fourth segments of the left leg in P5 appear to differ between the two species, with *E. hyalinus* s.s. having a proportionally longer segment 3 relative to segment 4 than is found in *E. spinifer.* Also, the length:width proportions of segments 3 and 4 in *E. hyalinus* appear to be greater than in *E. spinifer* (Figure 13). (2) The shape of the anterior-most portion of the head in lateral view in *E.*

spinifer is developed into a broad point, which is shifted slightly dorsal of medial. *E. hyalinus* has a more rounded anterior-most termination of the head, which is centrally located. (3) The shape of the rostrum in lateral view in *E. spinifer* appears to be more bulbous than in *E. hyalinus*. This difference was also noted in adult females, but seemed too difficult to characterize adequately as a useful species character. (4) The shape of the pointed processes on the posterior margin of the prosome in *E. spinifer* may be less pronounced and more rounded than in *E. hyalinus*. Further work is required, on a more extensive collection of specimens, to validate the utility of the male characters listed here.

Asymmetry and sexual dimorphism

Although asymmetries are not uncommon morphological traits of calanoid copepods, there are a number of features that are unusual about the observations reported here. Firstly, few adult female calanoids are known to have asymmetrical antennules, although this is a characteristic of adult males in the Epacteriscioidea, Pseudocyclopioidea. Arietelloidea and Diatomoidea. Species within the genus *Paramisophria* (Calanoida, Arietellidae) exhibit asymmetry in female antennules, as well as asymmetrical compression of the cephalothorax, lengths of the antenna endopod and exopod, distribution of integumental pores, and in the genital double-somite, all of which appear to be related to an unusual habit of swimming on the left side, just above the bottom (Fosshagen, 1968; Ohtsuka and Mitsuzumi, 1990). We are not aware of any epipelagic species outside the family Eucalanidae that are similarly

asymmetrical in the female antennule. Secondly, we observe a suite of characters that exhibit "unique concordance" and are dimorphically asymmetrical (*sensu* Ferrari, 1984; Ferrari and Hayek, 1990) within *E. hyalinus* s.s.. The first antennules, caudal rami, position of the intestinal tract in pedigerous somites 2-5, and position of the longer seta V on the caudal rami, all asymmetrical in the adult female, are observed to fluctuate between right and left sides of the body in different individuals. To our knowledge, this phenomenon remains quite unusual in the calanoids, and has only been reported in the recent literature for species in the genus *Pleuromamma* (Steuer, 1932; Ferrari, 1984; Ferrari and Hayek, 1990).

Ohtsuka and Huys (2001), in their thorough analysis of sexual dimorphism in calanoid copepods, describe a number of sexually dimorphic characters in a closely-related eucalanid species, *Pareucalanus attenuatus*. Many of the features they describe, including the doubling of aesthetascs in the adult male antennule between segments II-XXIV, presence of dorsal and lateral spinules on pedigerous somites 2-5 in the adult female, but absent in the adult male, shorter maxilliped with uniquely oriented plumose endopod setae in the adult male, and atrophy of the adult male gnathobase, are similarly observed in *Eucalanus spinifer* T. Scott. Sexually dimorphic features not described for *P. attenuatus*, which appear in *E. spinifer* include: modification of setae on maxilliped endopod segments 4-6 into a basally broad and flat portion, which changes into a plumose seta distally (with possible sensory setae on proximal segments). We also describe and illustrate sexual dimorphism in antennule

asymmetry (more extensive in female), the shape and size of the antenna, mandibular palp, maxillule, and maxilla, some of which also show dimorphism in armature.

Ecological and biogeographical differentiation between species

The sibling species *E. hyalinus* and *E. spinifer* are likely to be quite old, despite their morphological similarity. Although no lineage-specific molecular clock is available to estimate precise species ages, an application of locus-specific molecular clocks from decapod crustaceans results in estimates of the timing of divergence between *E. hyalinus* and *E. spinifer* of 10.5 my (COI, 1.66%/my, Schubart et al., 1998), 13.4 my (16S rRNA, 0.65%/my, Schubart et al., 1998), 12.4 my (COI, 1.4%/my, Knowlton and Weigt, 1998), and 21.7 my (16S rRNA, 0.38%/my, Cunningham et al., 1992). The speciation event likely took place in the Miocene, and the sibling species pair has undergone little evolution in phenotype since the divergence event. The maintenance of such morphological conservatism across a long evolutionary history has been found in a number of other marine and freshwater copepod groups, and is becoming the norm (eg, Bucklin et al., 1998; Rocha-Olivares et al., 2001; Lee and Frost, 2002; Dodson et al., 2003). The species pair may differ to greater extent in their ecological requirements than in their morphology.

Eucalanus spinifer and *E. hyalinus* s.s are sympatric throughout much of their biogeographic range, and are likely to co-occur in many of the regions where ecological studies of *E. hyalinus* s. l. have been conducted (eg, Price and Paffenhofer, 1986; Richardson et al., 2001). Both species are circumglobal in subtropical waters,

and occupy much of the distributional range of the original biogeographic description of *E. hyalinus* s.l. (Lang, 1965; Fleminger and Hulsemann, 1973). The two species appear to have different centers of abundance, with *E. spinifer* being the dominant epipelagic species in the center of the oligotrophic subtropical gyres (e.g. Tomczak & Godfrey, 1994), and *E. hyalinus* s.s. occurring in higher abundance (or exclusively) at the more productive periphery (e.g. SeaWiFS Project, 2004) of the subtropical gyres, and in the adjacent cooler waters at Subtropical Fronts and upwelling zones. In our samples, *E. hyalinus* s.s. was the dominant species in the California Current, regions south of the Subtropical Front in the South Pacific, north of the Gulf Stream extension/North Atlantic current, and within the Mediterranean Sea. *E. spinifer* dominated within the subtropical gyres of the North and South Pacific, subtropical-tropical waters of the Eastern Indian Ocean, and subtropical gyre waters of the North Atlantic. The two species co-occurred in roughly equivalent abundances in samples from the Kuroshio Current, and Agulhas Current and Cape region south of South Africa (Goetze, in prep). The ecological differentiation implied by these differences in distribution suggests that the dynamics of these two species may be driven by quite different oceanographic parameters. Nothing is currently known about possible differences in habitat depth, or seasonality in abundance or reproduction between these two species.

Conclusions

Morphologically cryptic, sibling species, which are identified through biochemical or molecular markers, may prove difficult to discriminate by conventional morphological criteria. Results presented here for sister species *E. spinifer* and *E. hyalinus* s.s. demonstrate that, at least in some cases, discriminating such species will be possible once their presence is known. This result provides some hope that the remaining, currently undescribed, genetic species within the family Eucalanidae (Goetze, 2003) will be distinguishable on a morphological basis. Patterns observed in the biogeographic distributions of the two sibling species suggest that they differ in their ecological requirements.

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Table 1. Collecting locations, dates, and depths for specimens included in morphological studies. Samples from regions labeled Indian Ocean, Southwest Pacific, South Pacific, and North Pacific are all ethanol-preserved. (---- denotes unknown depth)

Location	Cruise-Station #	Lat/Long	Date	Depth	species, sex examined
Indian Ocean	VANC10MV-01	35°3.12S, 23°43.8E	5/16/2003	0-566m	*hyalinus*, *spinifer*, females
	VANC10MV-02	35°4.02S, 24°30.0E	5/16/2003	0-920m	*hyalinus*, females
	VANC10MV-22	12°51.78S, 94°17.16E	6/6/2003	0-1100m	*spinifer*, females
	VANC10MV-24	13°12.72S, 104°39.51E	6/9/2003	0-1050m	*spinifer*, females
	VANC10MV-25	13°51.12S, 109°2.64E	6/10/2003	0-1070m	*spinifer*, females
Southwest Pacific	COOK14MV-13	23°51.88S, 175°02.58W	10/13/2001	0-900m	*spinifer*, females
	COOK14MV-16	25°47.38S, 175°18.03W	10/15/2001	0-950m	*spinifer*, females
	COOK14MV-17	26°52.37S, 175°38.88W	10/15/2001	0-950m	*spinifer*, females
	COOK14MV-18	27°55.70S, 175°58.04W	10/15/2001	0-950m	*spinifer*, females, males
	COOK14MV-19	29°05.15S, 176°09.08W	10/16/2001	0-950m	*spinifer*, females
	COOK14MV-24	30°23.85S, 178°12.26W	10/19/2001	0-950m	*spinifer*, *hyalinus*, females
	COOK14MV-26	27°45.66S, 176°48.90W	10/20/2001	0-950m	*spinifer*, *hyalinus*, females
	COOK14MV-28	24°23.15S, 175°13.32W	10/21/2001	0-950m	*spinifer*, females
South Pacific	DRFT07RR-08	34°02.38S, 140°03.48W	12/23/2001	0-950m	*hyalinus*, *spinifer*, females
	DRFT07RR-10	35°23.54S, 146°17.77W	12/24/2001	0-1050m	*hyalinus*, *spinifer*, females
	DRFT07RR-13	37°27.10S, 156°00.52W	12/26/2001	0-1000m	*hyalinus*, females
	DRFT07RR-14	38°31.69S, 161°14.33W	12/27/2001	0-1000m	*hyalinus*, females
	DRFT07RR-18	40°52.67S, 173°00.18W	12/28/200	0-1040m	*hyalinus*, females
	DRFT07RR-19	41°24.08S, 175°39.77W	12/30/2001	0-1000m	*hyalinus*, females
	DRFT07RR-20	41°58.17S, 178°33.97W	12/31/2001	0-950m	*hyalinus*, females
	DRFT07RR-21	42°57.13S, 176°15.67E	1/1/2002	0-480m	*hyalinus*, females
North Pacific	ACE-ASIA-02	28°12.42N, 162°8.4W	3/17/2001	--------	*spinifer*, females, male, illust. male
	ACE-ASIA-13	32°45.0N, 154°55.62E	3/28/2001	--------	*hyalinus*, *spinifer*, females
	CALCOFI 93.45	32°20.8N, 118°33.3W	3/29/2002	0-200m	*hyalinus*, females
	CALCOFI 93.50	32°10.8N, 118°53.6W	3/29/2002	0-200m	*hyalinus*, females
	CALCOFI 93.55	32°0.8N, 119°14.0W	3/29/2002	0-200m	*hyalinus*, females, males
	IMECOCAL 117.40	28°27.49N, 115°34.99W	7/22/2002	0-200m	*hyalinus*, females
	IMECOCAL 117.50	28°7.2N, 116°13.8W	7/21/2002	0-200m	*hyalinus*, females
	IMECOCAL 117.55	27°57.45N, 116°33.67W	7/21/2002	0-200m	*hyalinus*, females
	IMECOCAL 117.65	27°37.55N, 117°12.65W	7/20/2002	0-200m	*hyalinus*, females

Table 1, continued.

Location	Cruise-Station #	Lat/Long	Date	Depth	species, sex examined
Formalin	SOSO st. 1, tow 1	21°31.96N, 157°27.0W	7/30/1982	0-212m	*spinifer*, females
	SOSO st. 5, tow 8	24°3.0N, 156°33.0W	8/2/1982	0-212m	*spinifer*, females, illustrated female
	SOSO, st.72-18	33°25.96N, 117°54W	8/29/1982	0-212m	*hyalinus*, male, illustrated male
	CALCOFI 93.50	32°10.8N, 118°53.6W	4/11/1964	0-200m	*hyalinus*, females
Type locality, spinifer:	Atlantidae, st. 82	5°27N, 0°07E	1/29/1946	0-100m	*spinifer*, females

Table 2. Gene loci useful in species identification of *Eucalanus spinifer* and *Eucalanus hyalinus*, PCR primers [5'-3'], and PCR amplification conditions.

Gene locus	PCR Primers	PCR conditions
COI	COI_RNI [GTAGT(AGCT)GTAAC(AT)GCTCATGC] COI_VH [TAAACTTCAGGGTGACCAAAAAATCA]	50°C annealing temperature, 35-40 cycles
16S rRNA	16SAR [CGCCTGTTTATCAAAAACAT] 16SCB [ATTCAACATCGAGGTCACAA]	48°C or 50°C annealing temperature, 35-40 cycles
ITS2	ITS3F [GCATCGATGAAGAACGCAGC] ITS10R [TACGGGCCTATCACCCTCTACG]	52° C annealing temperature, 35-40 cycles

Table 3. Nucleotide site, base composition, and level of intraspecific polymorphism for sites fixed between *E. spinifer* and *E. hyalinus* s. s. Sites that are polymorphic at the intraspecific level, but do not share any mutations across species, are included. Data results from (A) 348 bp fragment of mitochondrial COI, with site positions noted as in Genbank accession # AY647918. (B) 311 bp fragment of mitochondrial 16S rRNA, with site positions as in Genbank accession # AY647923.

(A)

Site #	*hyalinus* s.s.	*spinifer*	polymorphism
47	C/T	A/G	both species polymorphic, no shared mutations
50	A/G	C	*hyalinus* polymorphic
53	T/C	G	*hyalinus* polymorphic
56	T	G/A	*spinifer* polymorphic
92	G/A	C	*hyalinus* polymorphic
110	A/G	C	*hyalinus* polymorphic
185	C	T	both species fixed
197	C/T	A/G	both species polymorphic, no shared mutations
200	C	T	both species fixed
288	G	A	both species fixed
308	T/C	A	*hyalinus* polymorphic
326	T/C	G	*hyalinus* polymorphic

(B)

Site #	*hyalinus* s.s.	*spinifer*	polymorphism
27	C	T	both species fixed
30	C	T	both species fixed
38	G	T	both species fixed
39	G	A	both species fixed
43	A	G	both species fixed
65	T	C	both species fixed
67	T	C	both species fixed
69	A	-	indel, fixed
73	C	T	both species fixed
80	C	T	both species fixed
97	C	T	both species fixed
114	T	G/A	*spinifer* polymorphic
121	C	T	both species fixed
225	T	C	both species fixed
234	T	A	both species fixed
251	C	G/A	*spinifer* polymorphic
253	A	G	both species fixed
283	T	C	both species fixed
290	A	G	both species fixed
300	T	C	both species fixed
309	T	C	both species fixed

Table 4. Key characters to differentiate *Eucalanus hyalinus* s.s. from *E. spinifer*. All A1 segments are ancestral segment numbers, following Huys and Boxshall (1991). For A1 asymmetry, "long/short" means measured on whichever side is longer (typically right), divided by whichever side is shorter. (A) characters independent of body size, (B) characters dependent on body size.

Character	*Eucalanus spinifer*	*Eucalanus hyalinus*
A. Size independent		
1) A1 asymmetry, ratio A22 long/A22 short	1.18 – 1.35	1.36 – 1.59
2) A1 asymmetry, ratio A23 long/A23 short	1.08 – 1.32	1.30 – 1.54
3) A1, longer side, ratio A22/A5	2.17 – 2.88	3.06 – 3.63
4) A1, longer side, ratio A23/A5	2.08 – 2.59	2.55 – 3.06
5) Anterior head, ratio Ht/Wdth	0.26 - 0.35	0.34 – 0.47
6) Anterior head angle	96-101°	81-91°
B. Size dependent		
7) Total Length, adult female (mm)	5.4 – 6.81	6.17 – 7.50
8) Prosome Length, adult female (mm)	4.85 – 6.17	5.46 – 6.92
A) Cephalosome Length, adult female (mm)	3.63 – 4.51	4.10 – 5.08
9) A22 length, longer side (μm)	368 – 472	519 – 670
11) Caudal Rami longer side, length, adult female (μm)	189 – 241	245 – 302

Table 5. Antennule segment lengths and ratios for adult females of (A) *E. spinifer*, and (B) *E. hyalinus* s.s. Lengths listed as mean length in micrometers, with ranges in brackets. The ratio is the longer divided by the shorter antennule. *E. spinifer,* N=30; *E. hyalinus* s.s., N=32.

(A) *E. spinifer*

A1	I-IV	V	VI	VII	VIII	IX	X-XI	XII
Right:	546 (672-406)	164 (184-146)	159 (179-142)	181 (201-156)	187 (216-167)	189 (212-167)	289 (321-250)	269 (307-245)
Left:	550 (627-461)	160 (179-142)	155 (179-142)	177 (212-160)	180 (212-162)	181 (208-162)	278 (311-245)	248 (278-222)
Ratio:	0.99	1.03	1.03	1.02	1.04	1.04	1.04	1.09
	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX
Right	298 (330-264)	356 (406-330)	427 (462-377)	427 (471-387)	450 (491-406)	448 (491-406)	457 (500-410)	428 (485-377)
Left:	270 (302-245)	331 (358-302)	376 (415-343)	370 (406-343)	381 (425-349)	373 (407-343)	380 (417-340)	350 (382-316)
Ratio:	1.10	1.08	1.14	1.16	1.18	1.20	1.20	1.22
	XXI	XXII	XXIII	XXIV	XXV			
Right:	456 (515-406)	423 (480-368)	380 (431-330)	274 (321-236)	188 (208-170)			
Left:	369 (412-330)	337 (377-302)	314 (382-284)	241 (270-217)	179 (193-157)			
Ratio:	1.24	1.25	1.21	1.14	1.06			

Table 5, continued.

(B) *E. hyalinus* s.s.

A1	I-IV	V	VI	VII	VIII	IX	X-XI	XII
Longer:	628 (725-500)	186 (212-170)	182 (208-160)	209 (236-189)	217 (250-189)	220 (255-198)	343 (392-288)	331 (365-293)
Shorter:	606 (670-533)	174 (193-151)	169 (193-156)	193 (217-176)	199 (222-179)	199 (217-172)	311 (353-269)	283 (314-264)
Ratio:	1.04	1.07	1.08	1.09	1.09	1.11	1.10	1.17

	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX
Longer:	370 (416-297)	450 (520-396)	540 (604-467)	548 (604-462)	578 (632-509)	572 (623-500)	599 (647-538)	564 (623-519)
Shorter:	314 (340-283)	383 (417-349)	429 (461-377)	434 (475-391)	452 (500-410)	442 (500-396)	453 (505-406)	420 (471-377)
Ratio:	1.18	1.18	1.26	1.26	1.28	1.29	1.32	1.34

	XXI	XXII	XXIII	XXIV	XXV
Longer:	627 (708-547)	591 (676-519)	519 (585-453)	381 (434-316)	221 (245-198)
Shorter:	448 (505-406)	406 (461-363)	367 (406-330)	299 (377-255)	203 (227-189)
Ratio:	1.40	1.46	1.41	1.27	1.09

Figure 1

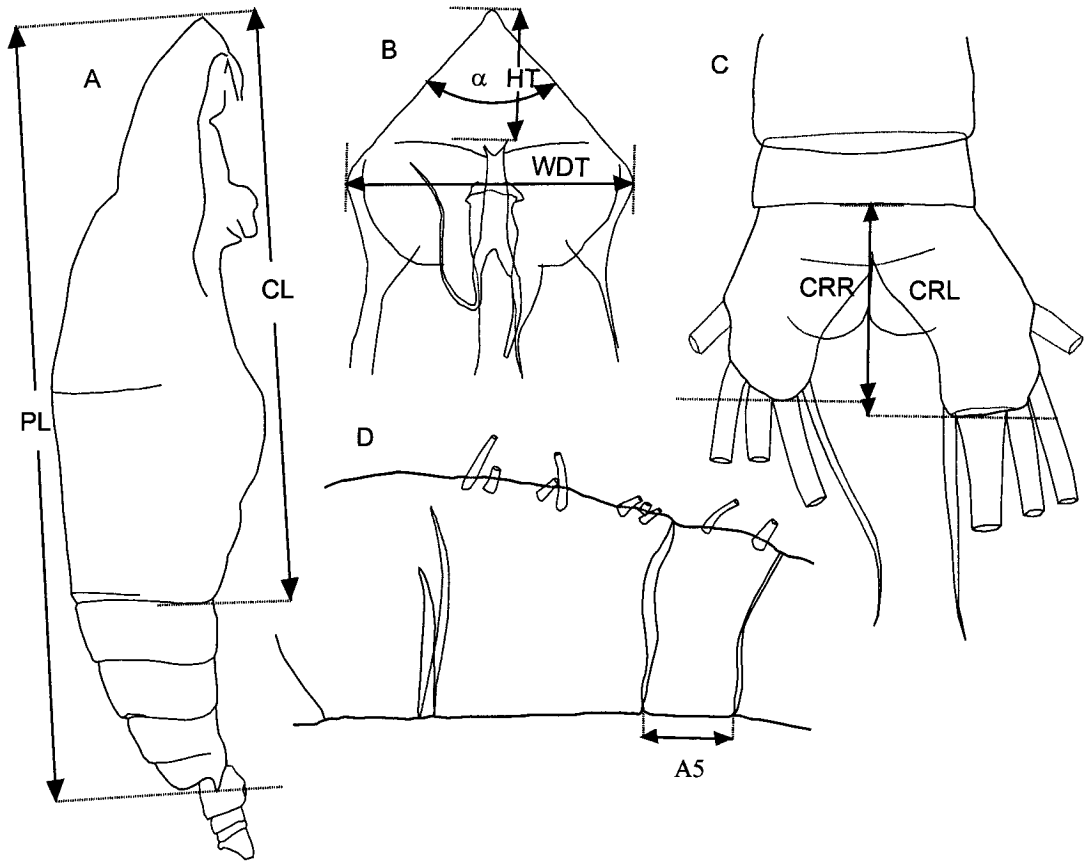

Fig. 1. Method of length measurement; (A) prosome length and cephalosome length, measured in right lateral view; (B) head height, width, and angle (α) measured in ventral view; (C) caudal rami length on right and left sides, measured in ventral view; (D) antennule segment length, as illustrated on segment V (male specimen), measured on posterior margin of antennule. Figure in A modified from Fleminger (1973)

Fig. 2. *Eucalanus spinifer* T. Scott adult female from SOSO St. 5, tow 8. (A) habitus, dorsal view; (B) habitus, lateral view; (C) posterior prosome and urosome, dorsal view; (D) posterior prosome and urosome, lateral view; (E) labrum, lateral view; (F) urosome, ventral view.

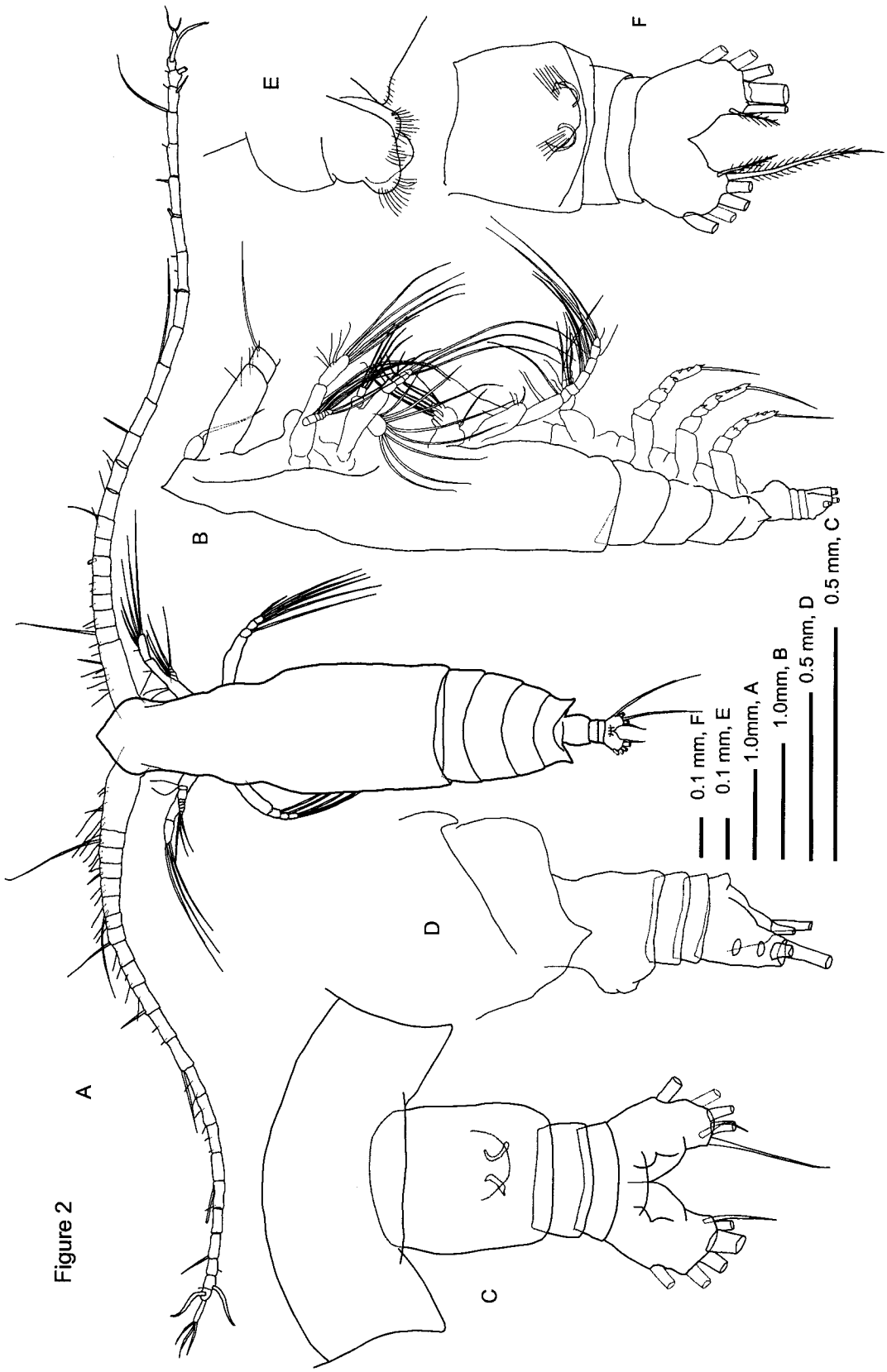

Figure 2

Fig. 3. *Eucalanus spinifer* T. Scott adult female from SOSO St. 5, tow 8. (A) left antennule; (B) right antennule; (C) right antenna, posterior surface; (D) antenna, posterior surface; (E) antenna, anterior surface; (F) mandibular gnathobase; (G) mandibular palp; (H) setal array on distal part of left antennule, fused segments XXVII-XXVIII.

Figure 3

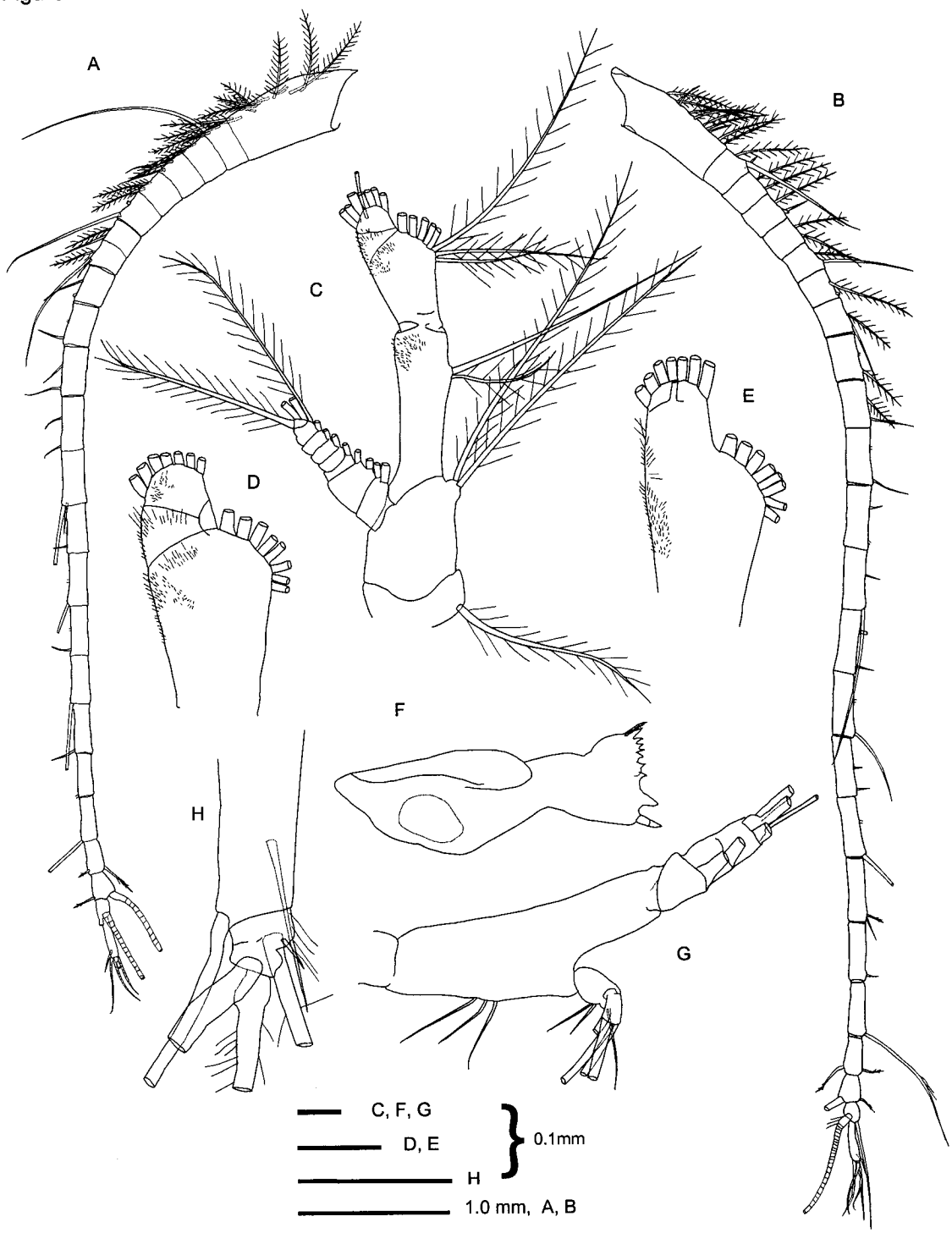

Fig. 4. *Eucalanus spinifer* T. Scott adult female from SOSO St. 5, tow 8. (A) mandibular gnathobases, (non-SOSO specimen from VANC10MV-22); (B) mandibular gnathobases, right above, left below; (C) maxillule, right, anterior surface; (D) maxilla, left, posterior surface; (E) maxilliped, right, posterior surface; (F) patch of spinules on antero-distal surface of syncoxa; (G) leg 1, anterior surface, left; (H) first endopod segment of leg 1; (I) first exopod segment of leg 1; (J) large seta on proximal border of endopod segment 1.

Figure 4

Figure 5

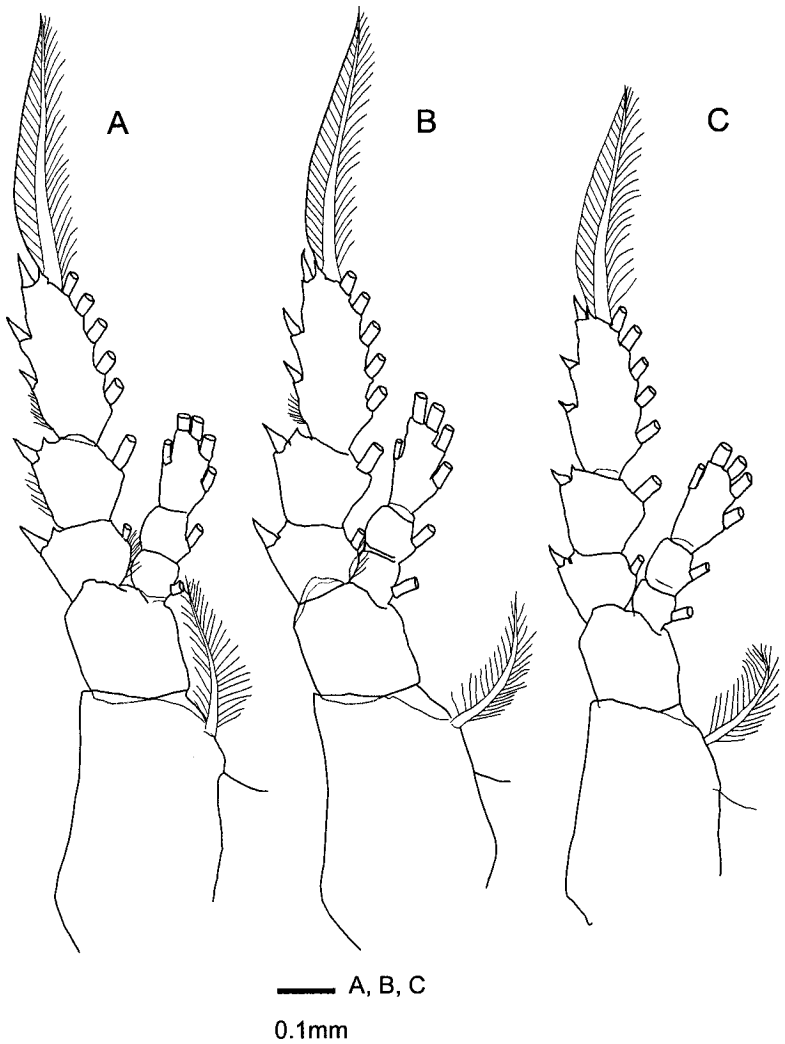

Fig. 5. *Eucalanus spinifer* T. Scott adult female from SOSO St. 5, tow 8. (A) leg 2, posterior surface, left; (B) leg 3, posterior surface, left; (C) leg 4, posterior surface, left.

Figure 6

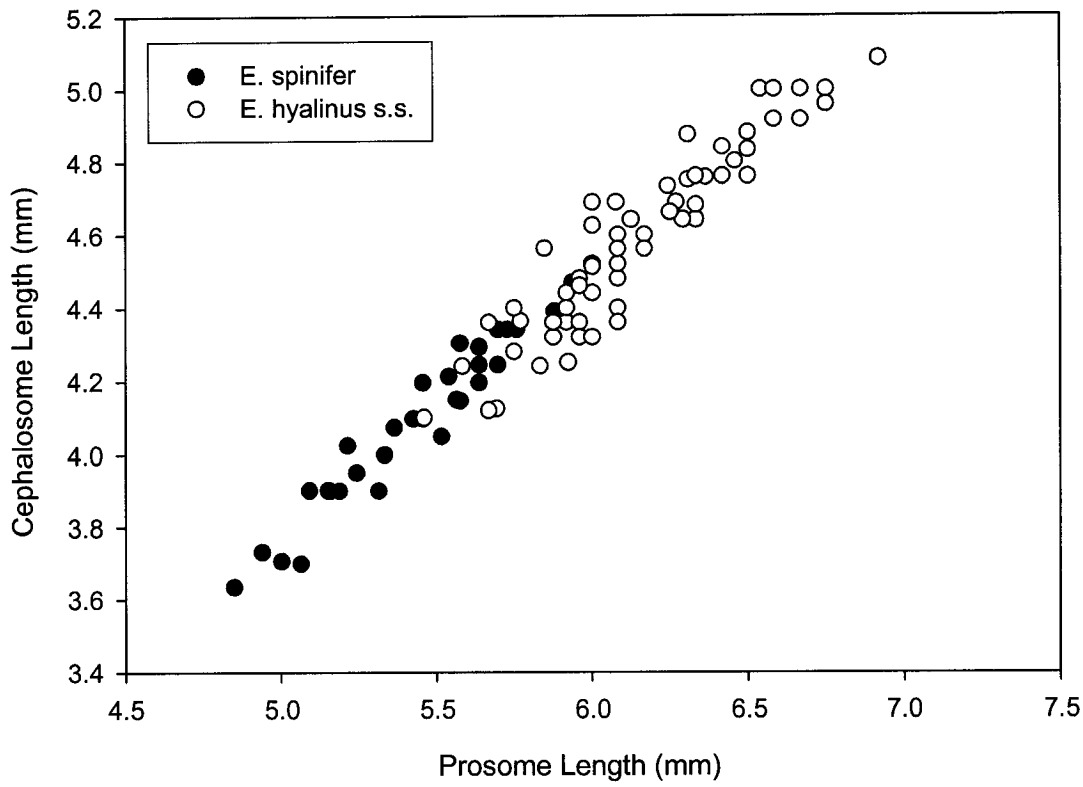

Fig. 6. Cephalosome length (mm) versus prosome length (mm) of *E. hyalinus* s.s. and *E. spinifer*. Seventy-one (3 not sequenced) and 31 (1 not sequenced) individuals included of *E. hyalinus* and *E. spinifer*, respectively. Specimens collected at 20 locations, North and South Pacific, Indian Ocean.

Fig. 7. Species differences in the shape of the anterior portion of the head in *E. hyalinus* s.s. and *E. spinifer*. (A) head shape in 3 individuals of each species; (B) ratio of height/width versus width (μm) of the anterior head in both species.

Fig. 7.

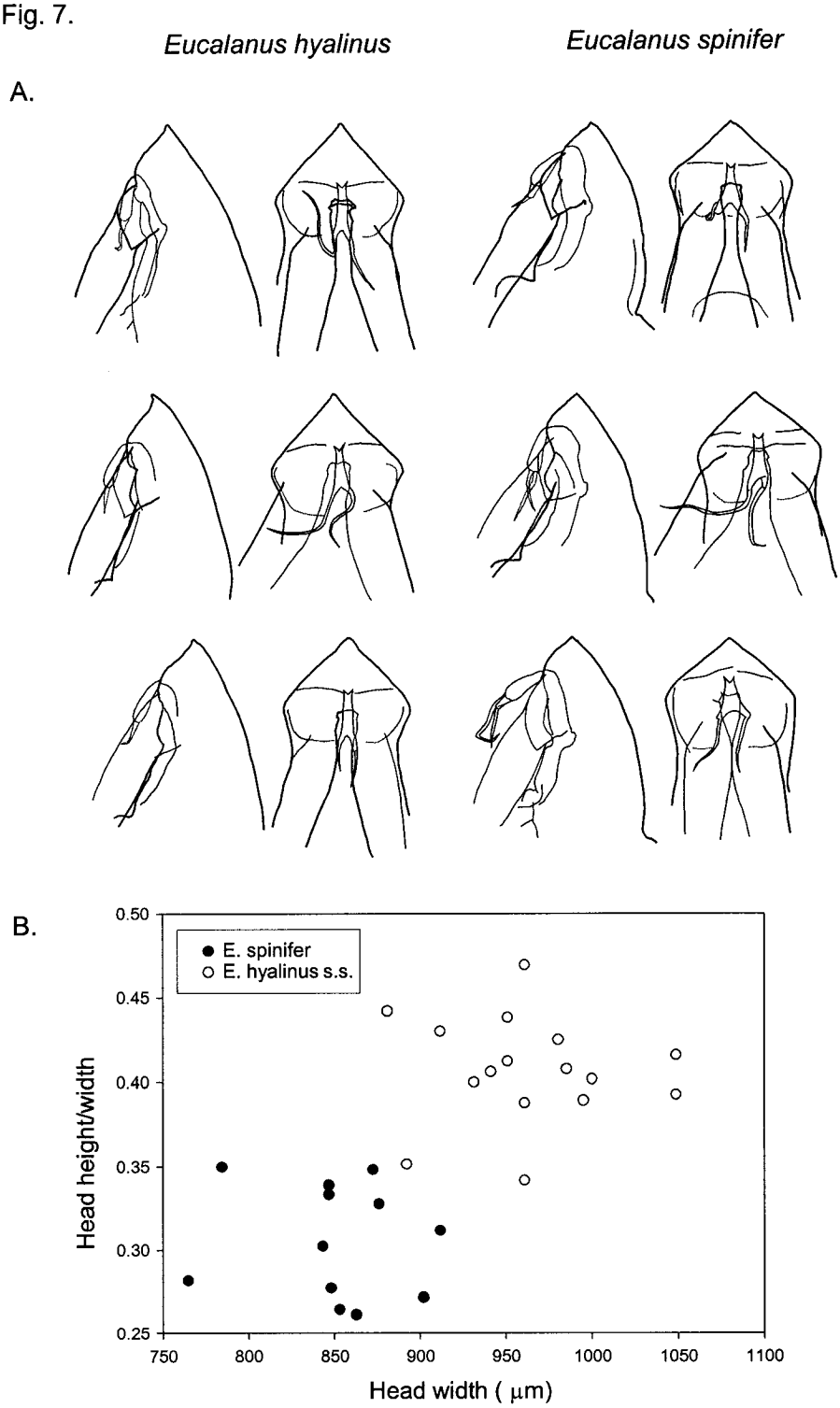

Fig. 8. Asymmetry in the first antennule of the adult female. (A) asymmetry in segment length along the antennule (mean ± 95% C.L). The greatest species differences in asymmetry occur at segments XXI to XXIV. All *spinifer* females have a longer right antennule; three out of 31 *hyalinus* females have a longer left antennule. All ratios plotted with the longer over the shorter side. Segment number is ancestral segment number, following Boxshall and Huys (1991), with segments I-IV and X-XI fused. *E. spinifer* specimens from South Pacific and Indian Ocean (N=29), *E. hyalinus* individuals from South and North Pacific, as well as Indian Ocean (N=31). (B) length asymmetry in segment XXII versus segment length (μm) (C) length evolution along the longer antennule; ratio XXII/V versus segment length (μm).

Fig 8.

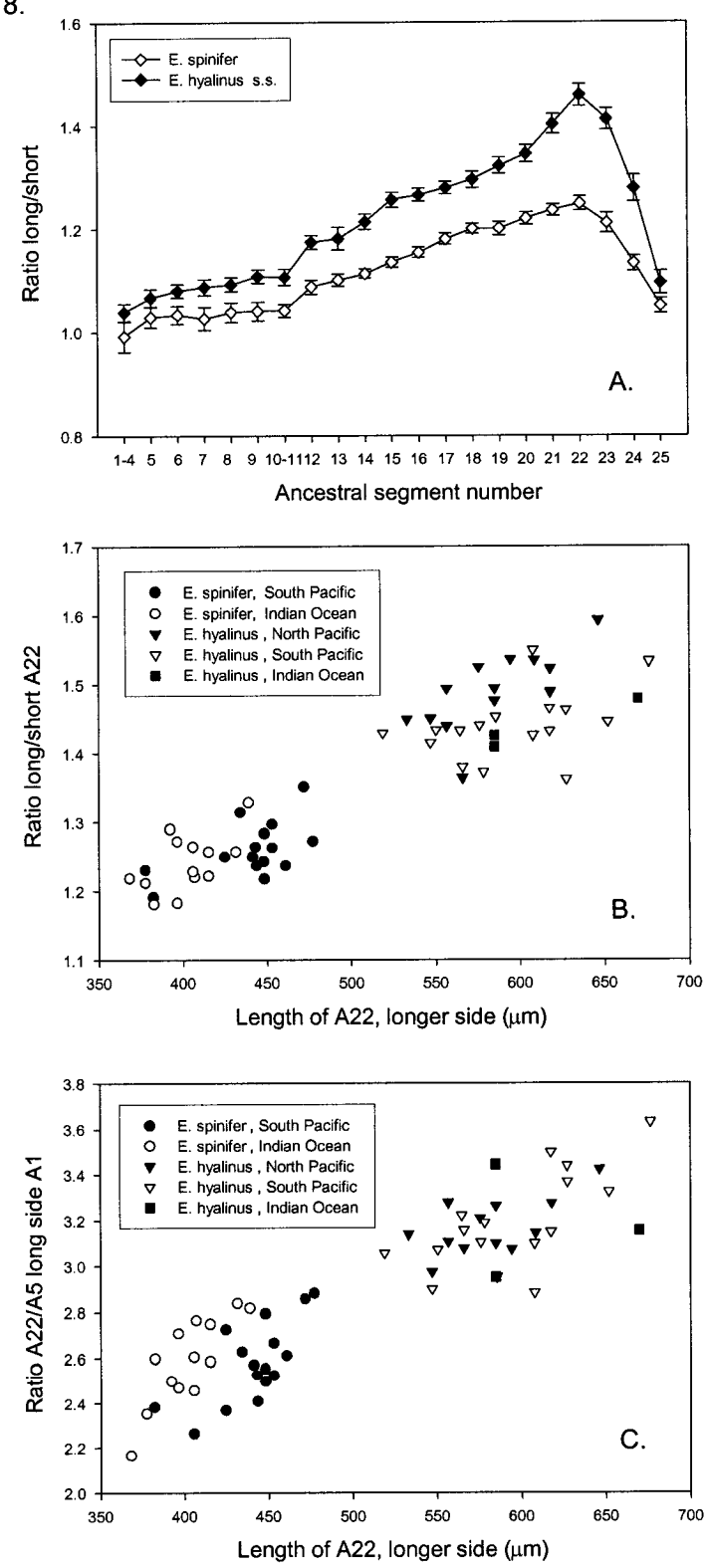

Fig. 9. *Eucalanus spinifer* T. Scott adult male from Ace-Asia, St. 2. (A) Head, ventral view; (B) Head, lateral view; (C) Habitus, dorsal view; (D) Habitus, lateral view; (E) Urosome, dorsal view; (F) Urosome, lateral view.

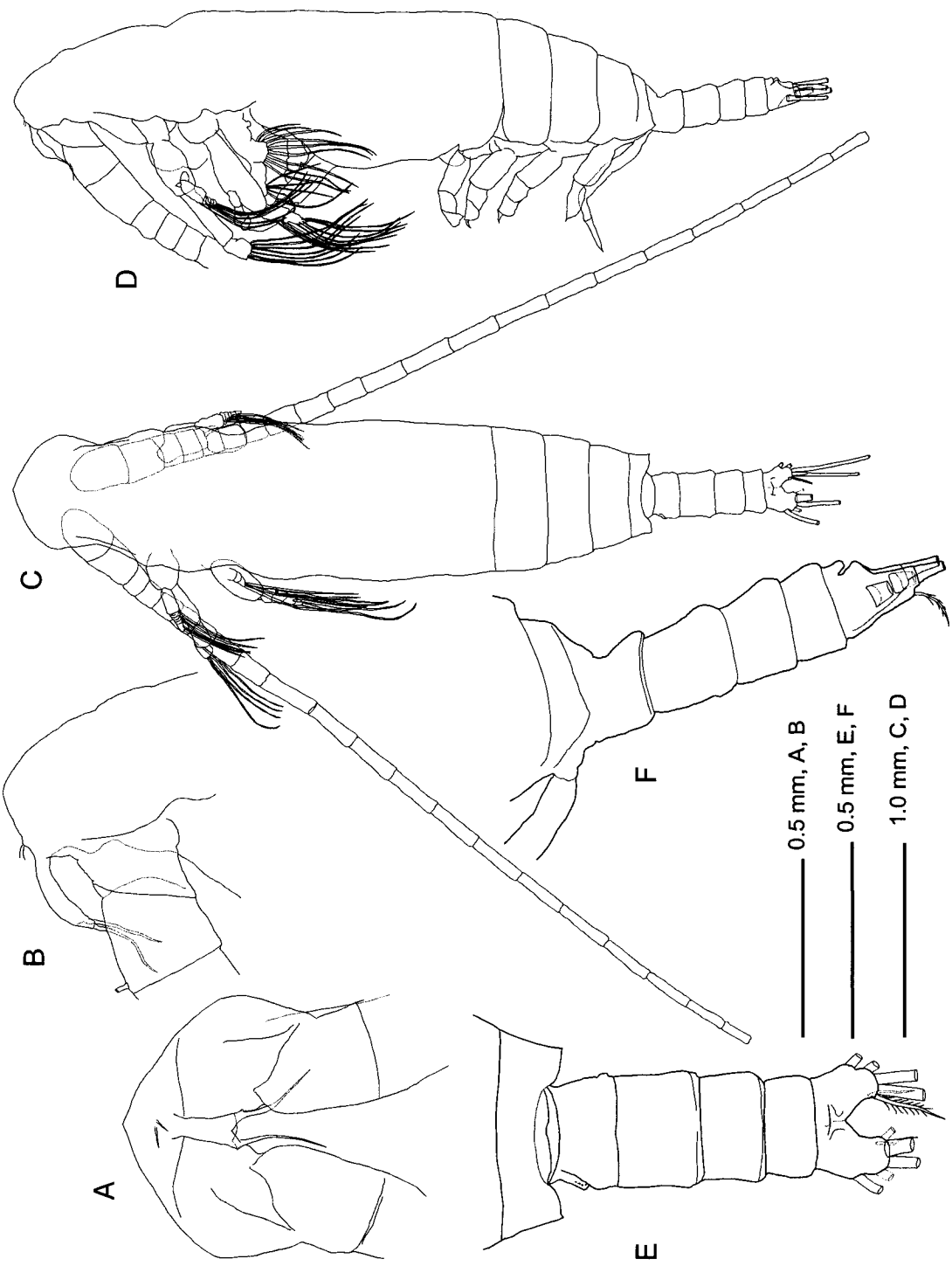

Figure 9

Fig. 10. *Eucalanus spinifer* T. Scott adult male from Ace-Asia, St. 2. (A) Left antennule; (B) Right antennule; (C) antenna, left, posterior surface; (D) mandibular palp, left, anterior surface; (E) mandibular gnathobase, right, specimen from 27° 55.7 S, 175° 58.0 W; (F) maxillule, left, posterior surface.

Figure 10

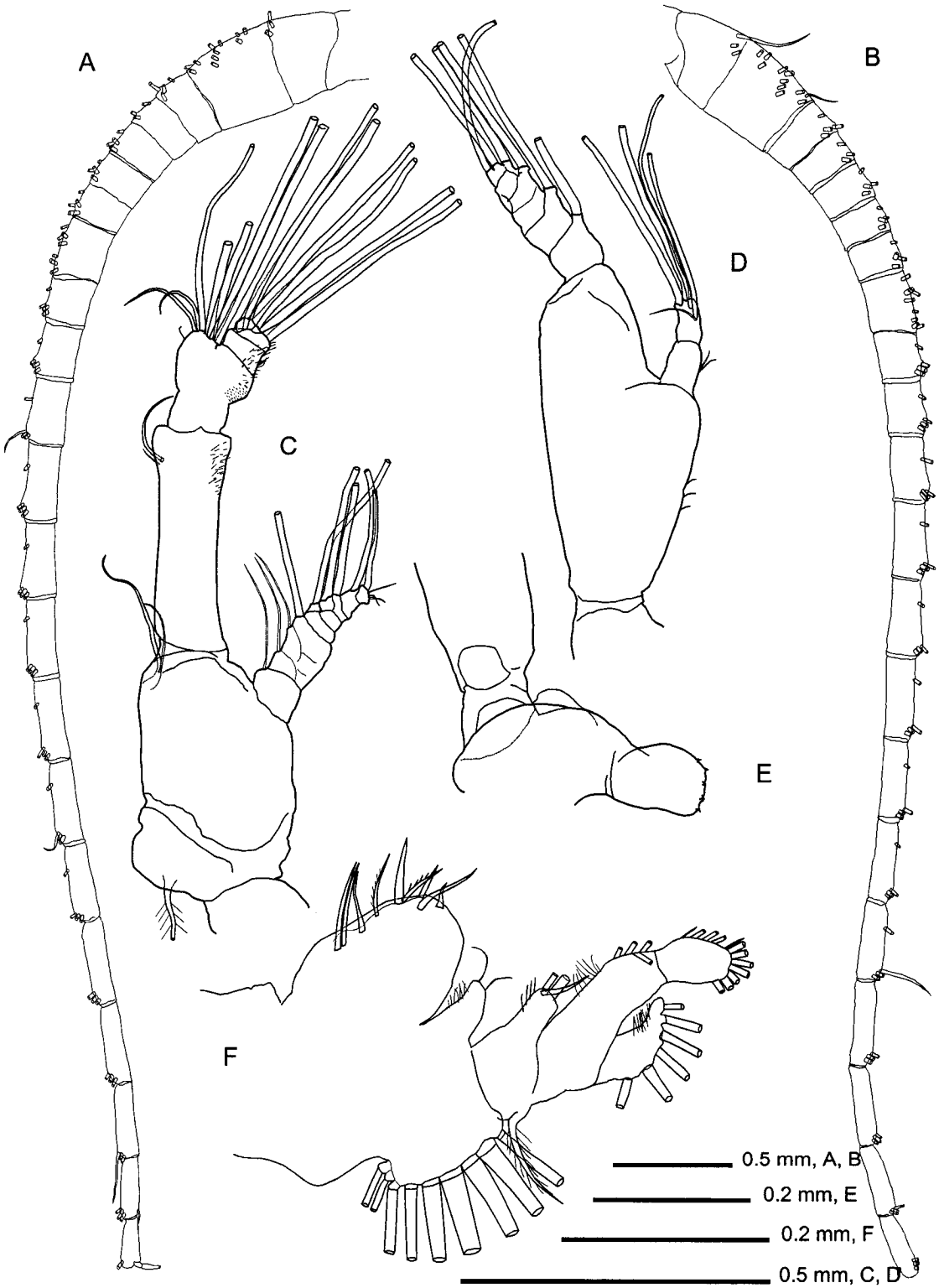

Figure 11

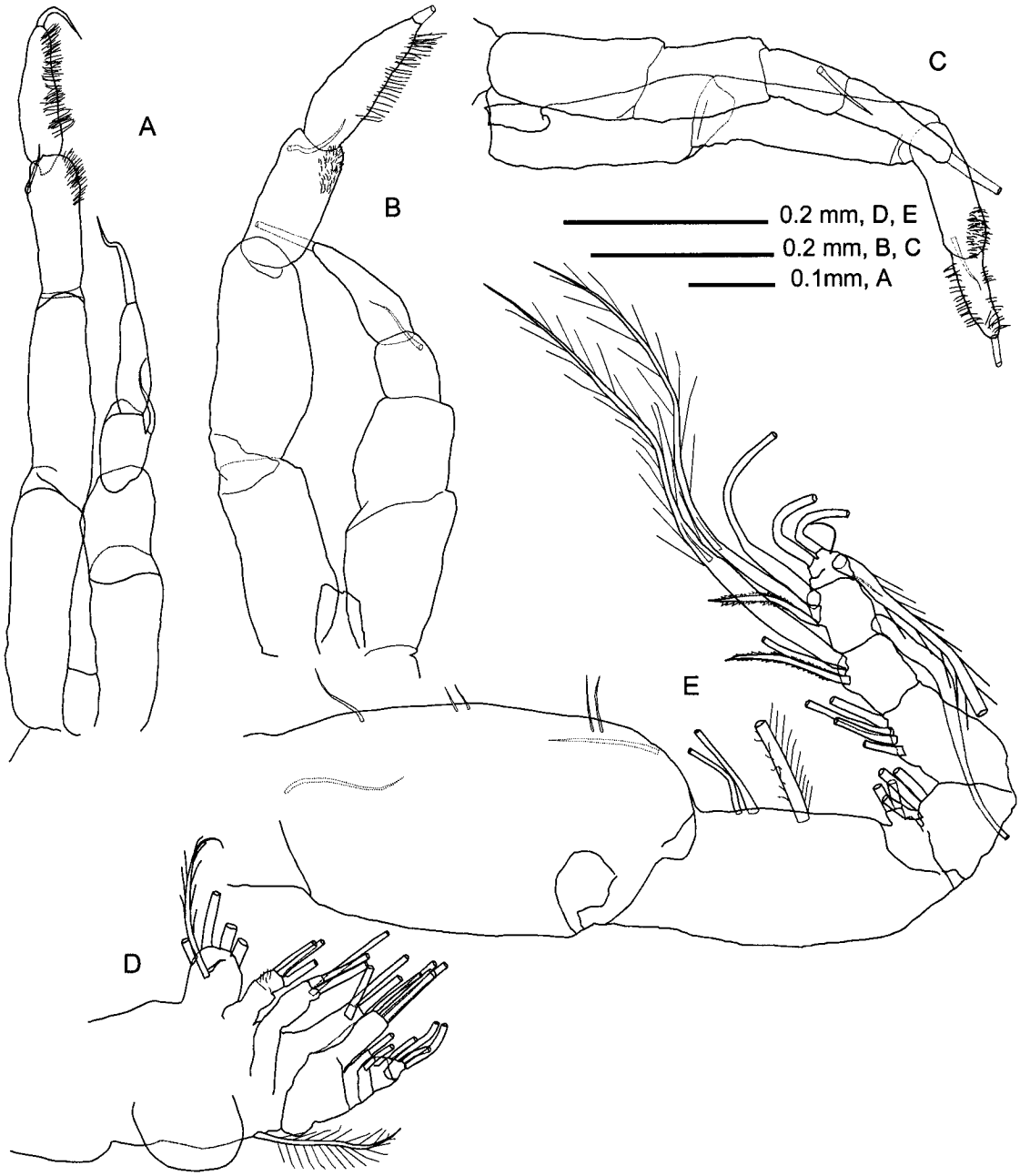

Fig. 11. *Eucalanus spinifer* T. Scott adult male from Ace-Asia, St. 2. (A) P5, anterior view (specimen from COOK14MV-18); (B) P5, anterior view; (C) P5, lateral view; (D) maxilla, right; (E) maxilliped, right.

Fig 12

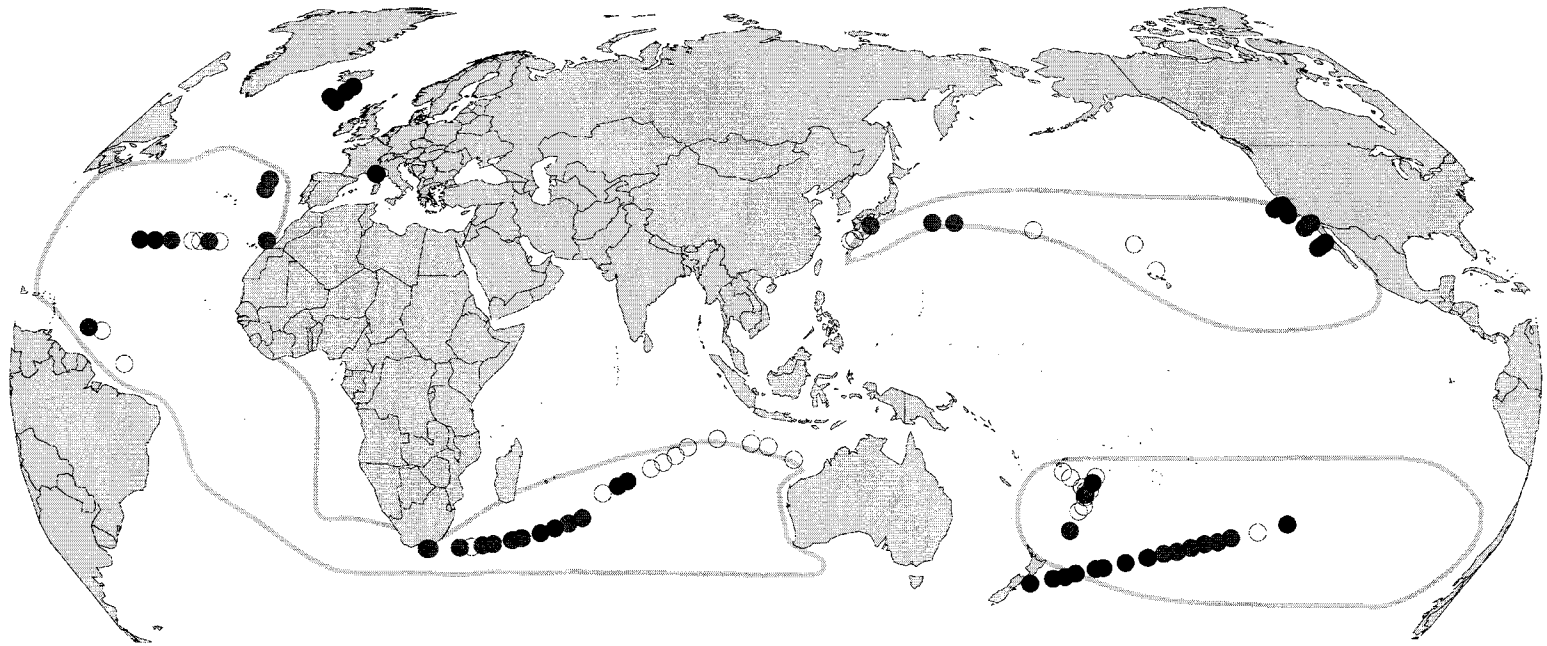

Fig. 12. Biogeographic distributions of *E. hyalinus* s.s and *E. spinifer*. Solid black circles correspond to *E. hyalinus* s.s., open circles to *E. spinifer*, and grey circles to plankton tows in which the species co-occurred. Original distribution of *E. hyalinus* s.l. outlined, modified from Fleminger and Hulsemann (1973).

Figure 13

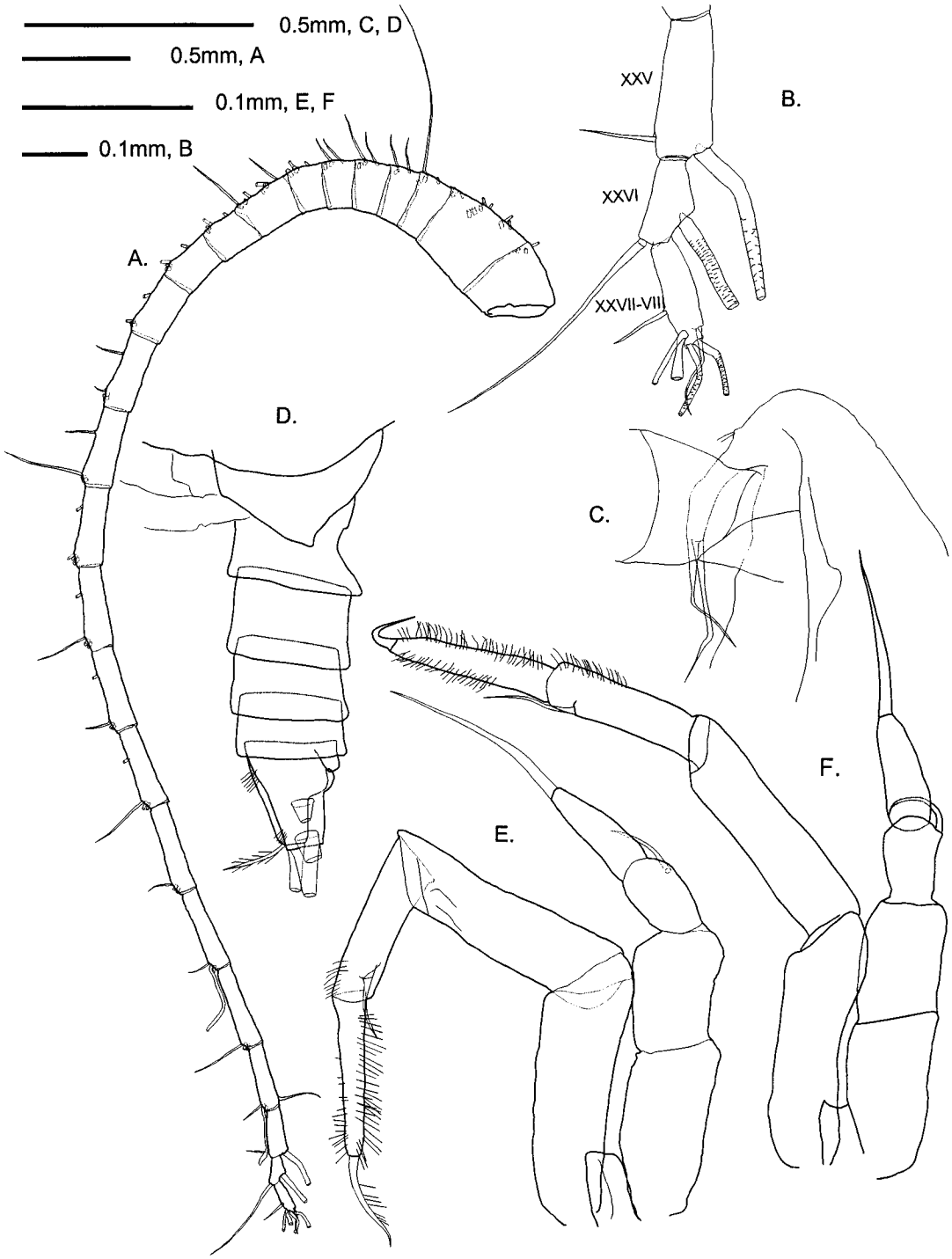

Fig. 13. *Eucalanus hyalinus* s. s. (Claus, 1866) adult male from SOSO St. 72-18. (A) left antennule; (B) left antennule ancestral segments XXV-XXVII-III; (C) head, lateral view; (D) urosome, lateral view; (E) P5, anterior view; (F) P5, anterior view, specimen from CalCOFI 93.55.

Fig. 14

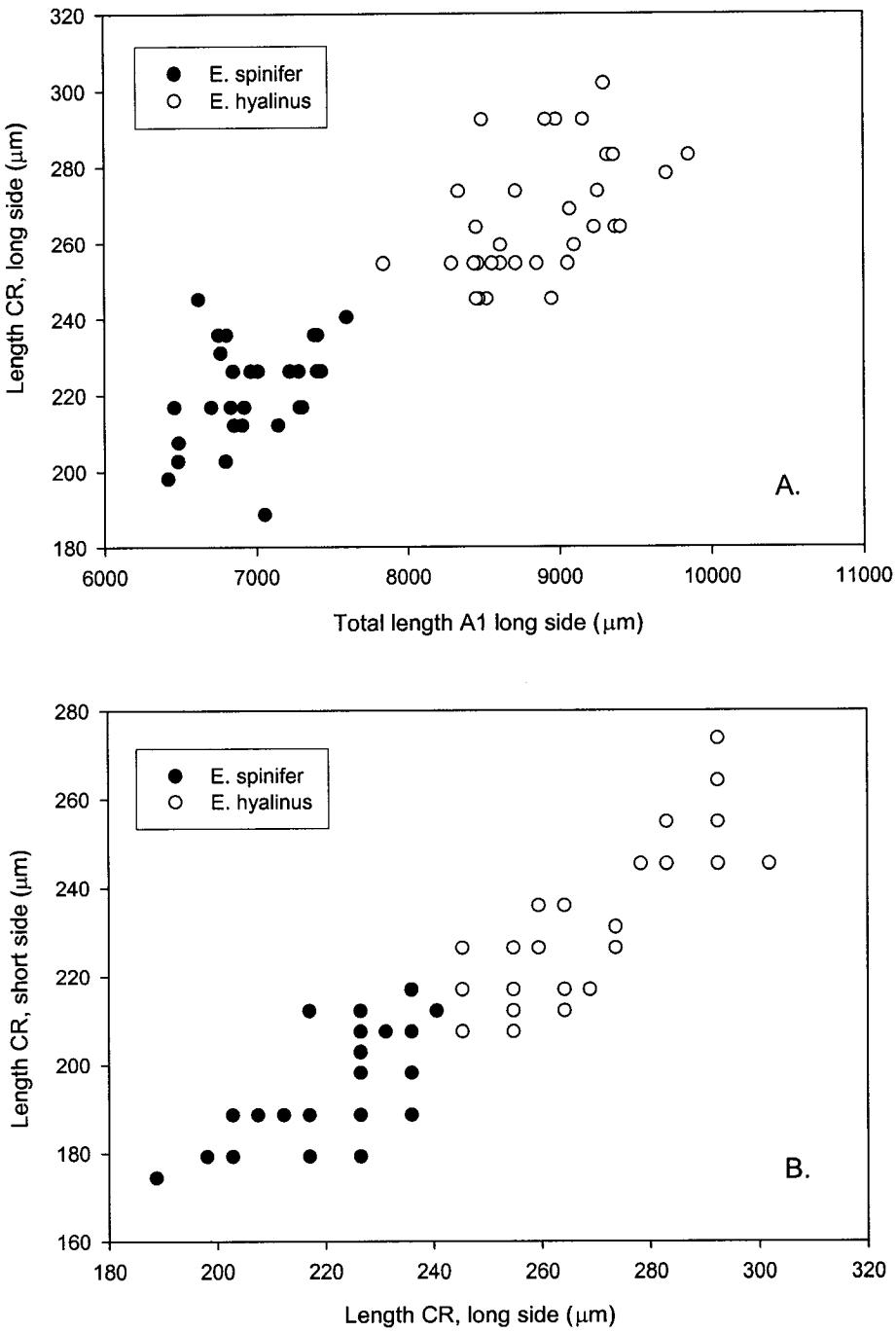

Fig. 14. Caudal rami length in *E. hyalinus* s.s. and *E. spinifer*. (A) longer side caudal ramus versus the total length of the longer antennule (µm, segments I-XXV only), (B) caudal ramus, shorter versus longer sides.

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Chapter IV

Global Population Genetic Structure and Biogeography of the Oceanic Copepods *Eucalanus hyalinus* and *E. spinifer*

Abstract

Although theory dictates that limited gene flow between populations is a necessary precursor to speciation under allopatric and parapatric models, it is currently unclear how genetic differentiation between conspecific populations can arise in open ocean plankton species. I examined two recently distinguished sympatric, circumglobal sister species, *Eucalanus hyalinus* and *Eucalanus spinifer*, for population genetic structure throughout their global biogeographic ranges. Here I show that oceanic zooplankton species can be highly genetically structured on macrogeographic spatial scales, despite experiencing extensive gene flow within features of the large-scale ocean circulation. Mitochondrial DNA analyses of 450 and 383 individuals of *E. hyalinus* and *E. spinifer*, respectively, revealed that habitat discontinuities at the boundaries of subtropical gyres in the North and South Pacific, as well as continental land masses, acted as effective barriers to gene flow for both species. However, the impact of specific barriers on population genetic structure varied between the sister species, despite their close phylogenetic relationship and similar circumglobal biogeogeographic distributions. The sister species differed in their oceanographic distributions, with *E. spinifer* dominating oligotrophic waters of the subtropical gyres

and *E. hyalinus* more abundant along central water mass boundaries and in frontal zones and upwelling systems. This species-specific difference in the oceanographic habitat is an important factor determining the historical and contemporary patterns of dispersal of the two species. I suggest that species-specific ecological differences are likely to be a primary determinant of population genetic structure of open ocean plankton.

Introduction

Limited gene flow among populations is a necessary precursor to speciation. Under allopatric and parapatric speciation models, populations must achieve either limited numbers of individuals moving between populations or a limited geographic distance over which individuals effectively disperse (Mayr 1942; Endler 1977). The marine pelagic environment appears to lack strong isolating barriers that could cause vicariant allopatric or peripatric speciation (e.g., Palumbi, 1992; Palumbi et. al. 1997). Parapatric speciation, in which reproductive isolation evolves through geographically restricted gene flow and selection, may be an important mode of speciation in the open sea. However, under the parapatric model, speciation only proceeds to completion when adjacent populations exchange individuals, and is deterred by long distance dispersal (Endler 1977; Gavrilets et al. 2000). Here I address whether either strong barriers to gene flow or migration over a restricted spatial scale, as required for the speciation process, are observable in open ocean plankton populations.

Population genetic studies of marine zooplankton provide one avenue for addressing this question and could, in principle, provide evidence for oceanographic or

geological features that act as effective barriers to gene flow. Most genetic studies of oceanic marine plankton species, however, have not detected strong barriers to dispersal between conspecific populations. Studies addressing barriers to gene flow in open ocean unicellular planktonic Foraminifera have documented the presence of identical genotypes in disparate portions of the global ocean, which has suggested recent or ongoing genetic exchange between geographically very distant populations (Darling et al. 2000; Norris and de Vargas 2000; Norris 2000). *Turborotalia quinqueloba*, *Globigerina bulloides*, *Neogloboquadrina pachyderma* were all reported to share small subunit (SSU) haplotypes between populations in Arctic and Antarctic waters (Darling et al. 2000; but see Darling et al. 2004), and *Orbulina universa*, *Globigerinella siphonifera* II, and *Globorotalia truncatulinoides* have also been found to share SSU or ITS genotypes in widely disparate regions of their global distribution (De Vargas et al. 1999; de Vargas et al. 2004). Examination of results for metazoan planktonic copepods *Calanus finmarchicus* (Bucklin et al. 2000; Bucklin and Kocher 1996) and *Nannocalanus minor* (Bucklin et al. 1996), the euphausiids *Meganyctiphanes norvegica* (Bucklin et al. 1997), *Euphausia superba* (Zane and Patarnello 2000; Zane et al. 1998; Fevholden and Scheppenheim 1989), and *Euphausia crystallorophias* (Jarman et al. 2002), as well as the squid *Moroteuthis ingens* (Sands et al. 2003) also suggests many examples of geographically very distant populations (>1,000 km) which exhibit little or no genetic differentiation.

Given that dramatic reductions in gene flow between populations, as required for parapatric and allopatric speciation, appear rare in the oceanic pelagos, how can we

explain the evolution of a diverse pelagic marine calanoid copepod fauna (ca. 1800 described marine species, Mauchline 1998)? There are a number of possible explanations for the paradox. These include: 1) sympatric or micro-allopatric speciation may be a common mode of speciation in the open sea (Palumbi 1992; Hellberg 1998; Dieckmann and Doebeli 1999; Carlon and Budd 2002; Doebeli and Dieckmann 2000, 2003), 2) speciation may occur during periods of transient geographic isolation, over shorter time periods than is currently expected (Palumbi 1994), 3) speciation in allo- and parapatry may have occurred more readily in historical oceans than in our current ocean, (a 'ghosts of barriers past' hypothesis), or 4) studies of gene flow in pelagic zooplankton species may have underestimated the presence and importance of barriers to gene flow.

The work presented here is designed to address point four, the issue of barriers. Before attributing speciation in the open sea primarily to other modes of speciation due to our inability to envision how allo- or parapatric speciation may occur, a thorough examination of the evidence for an absence of barriers to gene flow is necessary. Barriers to gene flow may not have been recognized previously due to a) insufficient coverage of all geographic scales over which barriers to gene flow may be expected to occur (Benzie 2000) , b) confounding of mesoscale genetic variability with differentiation on larger spatial scales (Jarman et al. 2002; Jarman and Nicol 2002), c) use of genetic markers with insufficient variability to resolve differentiation on the appropriate timescales, or d) taxonomic undersampling (species studied to date may not be representative of other planktonic fauna).

What types of oceanographic or geological features might be expected to act as effective barriers to gene flow in planktonic organisms? Although some of the barriers examined for coastal, benthic marine species are potential candidates for planktonic organisms, not all environmental barriers will be held in common. The features that appear likely to reduce or eliminate gene flow between conspecific plankton populations include: continental land masses; equatorial and tropical waters dividing subtropical and temperate populations in Northern and Southern hemispheres (disjunct oceanographic habitat, Brinton 1962; McGowan 1972; Stepien and Rosenblatt 1996; Burridge 2002 and references therein); strong frontal zones at water mass boundaries and surface currents (Wei and Kennet 1988); highly oligotrophic areas of the open ocean, which support too little biomass for large-bodied particle feeders (Park 1994); regions of strong upwelling, which may act as thermal barriers for stenotropic organisms (Fleminger 1986; Apte and Gardner 2002; Waters and Roy 2004), and finally, short-term changes in habitat area or connectivity due to climate fluctuations on glacial-interglacial or shorter timescales (Brinton 1962; Fleminger 1967; Fleminger 1975). Different barriers are likely to be important for different fauna, depending on the specifics of their biogeographic distributions and ecological requirements.

A second major aspect of understanding the importance of barriers to gene flow in open ocean systems is in understanding their efficacy for different members of codistributed fauna. Marine holoplankton species live in the water column throughout their entire life history and, by definition, lack the ability to swim against ocean

currents. Realized dispersal for such species will then be the result of an interaction between organismal life history and behavior with oceanographic advection and diffusion on relevant temporal and spatial scales (e.g., Sinclair 1988; Wroblewski 1982; Eiane et al. 1998). If physical oceanographic processes are the primary factor determining dispersal, then planktonic species with similar biogeographic distributions can be expected to have shared historical and contemporary patterns of gene flow between populations. If, on the other hand, intrinsic factors such as thresholds for environmental tolerance or vertical migration behavior act as critical ecological modifiers controlling realized dispersal or a species response to historical environmental change, then species can be expected to have unique population genetic structures (Kyle and Boulding 2000; McMillen-Jackson and Bert 2003; Marko 2004; Hurtado et al. 2004).

In order to investigate the importance of barriers to gene flow in population differentiation of open ocean organisms, I examine the population genetic structure of a pair of circumglobal, sympatric sister species of oceanic marine copepods, *Eucalanus hyalinus* s.s. (Claus, 1866) and *Eucalanus spinifer* T. Scott, 1894, on spatial scales ranging from mesoscale to global. The *Eucalanus* sister species are large (5.4-7.5 mm total length) members of the mesozooplankton in temperate, subtropical, and tropical waters worldwide. Both species are antitropical in distribution in the Pacific Ocean, occurring in central waters of both the North and South Pacific (Fig. 1, Lang 1965, 1967; Fleminger and Hulsemann 1973). Both species spend their entire life history in the epipelagic and upper mesopelagic portions

of the water column (Roe 1972, as *E. elongatus*; Deevey and Brooks 1977; Paffenhöfer and Mazzocchi 2003), though it is currently unknown whether they differ in seasonal or geographic patterns of habitat depth. Until recently *Eucalanus spinifer* was a cryptic species within *Eucalanus hyalinus* s. l. (Goetze and Bradford-Grieve, submitted) and as a result little is known about its natural history or ecological requirements. Animals within the inclusive designation *Eucalanus hyalinus* s.l. are reported to be particle feeders on auto- and heterotrophic nano- and microplankton (Turner 1991; Kleppel et al. 1996, as *E. elongatus*), with generation times on the order of 28-35 days (Paffenhofer 1991). The biogeographic distributions of both species are typical of other oceanic marine zooplankton and could be considered canonical examples of the 'central water mass' distribution in the Pacific (McGowan 1971; Reid et al. 1978). Using mtDNA sequence data from 833 individuals, I characterize the population genetic structure both within and across disjunct populations of each species throughout their global biogeographic range. The objective is to address the following questions: 1) What is the spatial scale of population genetic structure in oceanic zooplankton? 2) What oceanographic or geological features act as effective barriers to gene flow for open ocean fauna? and 3) Are the presence and efficacy of barriers to gene flow congruent for a circumglobal, sympatric, sister species pair?

Materials and Methods

Specimen and oceanographic data collection. – A total of 450 *E. hyalinus* s.s. and 383 *E. spinifer* individuals were collected from 106 locations in the North Pacific,

South Pacific, North Atlantic, and Indian Ocean basins (Fig. 1). Specimens collected on 5 major cruises in 2001-2003 (Fig. 1) were obtained by towing 202 or 333 μm mesh plankton nets (1m diameter ring or 0.71m bongo) obliquely between 400 to 1100m and the surface. The remaining specimens were kindly provided by other scientists, and were obtained using a variety of sampling techniques across a range of sampling depths. A full list of collecting localities is available from the author. Bulk plankton material was preserved in 95% non-denatured ethyl alcohol, changed to new alcohol within 12 hours of collection, and stored in ethyl alcohol at -20°C for later sample sorting. Eucalanid specimens were identified as *E. hyalinus* s.l. by the presence of pointed processes on the posterolateral margins of the prosome (visible in both dorsal and lateral views). A companion paper to this work describes morphological characters for discrimination of adult females of *E. hyalinus* s.s. and *E. spinifer* (Goetze and Bradford-Grieve submitted).

Plankton tows on five cruises were accompanied by oceanographic data from the same (or nearby) sampling locations. Conductivity, temperature, and depth (CTD) casts were completed at 44 stations, providing vertical profiles of water temperature, salinity, density (Seabird 911 *plus*), and in situ fluorescence (Chelsea, Seapoint fluorometers). Dissolved oxygen was also measured on a Seabird SBE 43 probe, but only on cruise VANC10MV. Discrete seawater samples were also collected from 6 depths in the upper 200 m of each cast with 10 L Niskin bottles on a rosette, and filtered onto Whatman glass fiber (GFF) filters. Filters were extracted in 90% acetone and analyzed on a Turner Designs fluorometer to obtain chlorophyll a and

phaeopigment concentrations (Strickland and Parsons 1972). CTD sensor data were processed according to standard Seabird recommendations for each instrument (Seabird 2003) and only data from the downcast were included in the 1 m depth binned final data file.

DNA extraction, PCR, and sequencing. – DNA was extracted from individual adult female copepods following either a lysis buffer protocol (Lee and Frost 2002) or the QIAGEN DNeasy tissue extraction kit. The only modifications to the QIAGEN manufacturer's protocol included limiting the 55° C lysis incubation step to 1-1.25 hour maximum, reducing the quantity of elution buffer to 100µl, and increasing the duration of the elution incubation step to >10 min. Primers used for routine PCR amplification of mitochondrial cytochrome oxidase I (COI) were COI_VH [5' – TAAACTTCAGGGTGACCAAAAAATCA – 3'], and COI_RNI [5' - GTAGT(AGCT)GTAAC(AT)GCTCATGC – 3'] (Folmer et al. 1994; Goetze 2003). For polymerase chain reaction (PCR), each 25µl reaction contained 0.75µl of Sigma RedTaq under recommended conditions. The PCR protocol included a 30 s hot start, followed by 40 cycles of 95° C for 30 s, 50° C annealing temperature for 30 s, and a 72° C extension step for 1 m, followed by a 4-5 m extension step at 72°C. Amplification products were purified and cycle sequenced directly on a MegBACE 500 or ABI 373 automated DNA sequencer. Prior work established that PCR amplification products obtained using these same primers on genomic DNA matched copies expressed in total RNA for *E. hyalinus* s.s. (Goetze 2003).

Genetic data analysis. – DNA sequences were checked for ambiguity prior to alignment, and either trimmed or excluded from the analysis if ambiguities were present within the final alignment region of 375 bp for *E. spinifer* and 349 bp for *E. hyalinus* s.s. A model of molecular evolution for the final sequence alignment of each species was selected by AIC as implemented in Modeltest (Posada and Crandall 1998) for use in distance calculations between haplotypes. For *Eucalanus hyalinus* s.s., the Tamura Nei model was selected as the optimal model, with a gamma rate heterogeneity correction parameter of 0.9344. For this species, the model used to calculate the distance matrix between haplotypes had a substantial impact on final Φ_{ST} estimates. Although the particular model of evolution used had little impact on results for *E. spinifer*, the best fit model, Tamura Nei with a gamma parameter for rate heterogeneity of 0.1574, was used in all distance calculations. Neighbor-joining phylogenetic trees of COI haplotypes within each species were generated in PAUP* 4.0b 10 (Swofford 2002), using models of molecular evolution selected above for distance calculations. NJ bootstrap support for nodes separating N. Atlantic and N. Pacific clades was estimated in MEGA (Kumar et al. 2004), and consensus parsimony trees were completed in PAUP* 4.0b 10.

Nucleotide diversity, sums of squared haplotype frequencies, and Tajima's D were calculated in Arlequin 2.001 (Schneider et al. 2000). Haplotype diversities were calculated from sums of squared haplotype frequencies following Nei (1987, eq. 8.4). Significance of Tajima's D was assessed by comparison to a simulated distribution of random samples, generated under the assumption of selective neutrality and

population equilibrium. Segregating sites were identified using the software program DnaSP 4.0 (Rozas et al. 2003). I completed an analysis of molecular variance (AMOVA) to test for population structure of both *Eucalanus* species in Arlequin 2.001. Statistical significance of the fixation indices Φ_{ST}, Φ_{SC}, Φ_{CT} (calculated following Weir and Cockerham 1984) was estimated by 1023 permutations of individuals across populations and groups (Excoffier et al. 1992). An AMOVA was conducted on the global dataset for each species, including all populations, as well as on a regionally pooled dataset, which combined all individuals sampled within each hemisphere and ocean basin. Ten and 16 individuals of *E. hyalinus* and *E. spinifer*, respectively, which were not included in the global analysis for each species due to small sample size, were included in the regionally pooled AMOVA analysis. Fisher's exact test was also used to test for significance of population structure in the regional dataset. Throughout the paper I use the word 'samples' rather than 'populations' to refer to the collection of individuals from a particular locality.

One factor complicating the data analysis, which I expect to be common to other studies of marine plankton, was the high incidence of unequal (or small) sample sizes. A standard sampling pattern was employed, including a standard depth of tow and approximate volume of water filtered, which, due to heterogeneities in animal concentrations across oceanographic regions, resulted in uneven numbers of specimens between samples. To counteract the problem of small sample sizes, specimens were pooled across tows within oceanographic regions to obtain sample sizes of a minimum of 15 individuals where possible. Distinct oceanographic regions

were identified by shifts in large-scale oceanographic properties of the upper water column (e.g., temperature, salinity, or in vivo fluorescence).

In order to examine the population genetic structure of both species for the presence of a pattern of isolation by distance within major oceanographic regions, I plotted pairwise Φ_{ST} estimates against geographic distance between samples. A Mantel's test was used to test for partial correlations between genetic and geographic matrices, using the program IBD (Bohonak 2002). Geographic distances between samples were calculated according to a great circle route between sampling locations. This analysis was completed only on the South Pacific samples of *E. hyalinus*, and the Indian Ocean samples of *E. spinifer*, because these regions contained sufficient sample sizes and continuous geographic sampling coverage, enabling a comparison of the genetic-geographic distance relationship along a continuous axis of distance.

Finally, estimates of migration between populations were completed in MDIV (Nielsen and Wakeley 2001), with 5,500,000 steps in the MCMC chain, of which the first 500,000 were removed as a burn in. Maximal M and T values were set at 10 in both cases, and final estimates were far from these boundaries.

Oceanographic data analysis. – Summary statistics were calculated for each CTD cast to characterize aspects of the distribution of chlorophyll a, a phytoplankton pigment used here as a proxy for phytoplankton concentration, and stratification and thermal structure of the upper water column. The parameters calculated included the depth of the chlorophyll a maximum, chlorophyll a concentration at the maximum,

water temperature at the chlorophyll a maximum, integrated chlorophyll a over the upper 100, 150 and 200m of water column, breadth of the chlorophyll a peak, as measured by the depth at which chlorophyll concentrations were 75%, 50% and 33% of maximum values, the value of the Brunt-Väisala (N) frequency maximum (a measure of water column stratification), depth of the N maximum, and the depth of the 15, 16 and 17° C isotherms. In vivo fluorescence data for each cruise were corrected for baseline offset by computing the average offset of the fluorometer at depth (between 700 and either 800 or 1000m) across 3-6 deep casts per cruise. Vertical profiles of in vivo fluorescence data were converted to chlorophyll a concentration using a cruise-specific regression of extracted chlorophyll a concentrations, as measured by fluorometry, against in vivo fluorescence from the same water depth. Calculations were then made on the converted chlorophyll a profiles. Discriminant analysis, completed using the software program SYSTAT 10.0 (SPSS 2000), was used to identify which of the water column parameters described above best discriminate the oceanographic distributions of *E. hyalinus* and *E. spinifer*. Parameters included in the discriminant analysis were the depth of the chlorophyll maximum, chlorophyll a concentration at the maximum, water temperature at the chlorophyll maximum, integrated chlorophyll in the upper 150m, breadth of the cholorophyll maximum (50% max. value), N maximum value and depth of maximum, and the depth of the 16° isotherm. Statistical significance for differences in means between species was assessed by t-tests (Zar 1999).

Results

Genetic diversity

The sister species differed substantially in their levels of intraspecific genetic diversity, despite the initial expectation for similarity across the two species. As expected, given large population sizes and a global biogeographic range, *Eucalanus hyalinus* s.s. exhibited high levels of genetic polymorphism. Out of a total of 450 individuals sequenced, 239 unique COI haplotypes were observed (Table 1, Fig. 2). Haplotype diversities were high within sampling localities, and ranged between 0.34 and 0.97 (median = 0.92, Table 1). Most haplotypes were sampled only once within each sampling locality. Nucleotide diversities within each sample were also high in *E. hyalinus*, and ranged between 0.0026 and 0.1247 (median = 0.0109, π/site, Table 1). At the nucleotide level, genetic polymorphism was quite pronounced in *E. hyalinus*, with 99 segregating sites in the global dataset, 73 of which were parsimony informative. The average number of nucleotide differences between *E. hyalinus* individuals was 2.71.

In contrast, *Eucalanus spinifer*, despite having a comparably large, global, biogeographic range, harbored considerably less genetic diversity than its sister species. Only 60 unique haplotypes were observed in a global dataset of 383 individuals (Table 2, Fig. 3). Haplotype diversities within samples ranged from 0.00 to 0.81 (median = 0.51), significantly lower than *E. hyalinus* ($P<10^{-7}$, Mann-Whitney U; Table 2). Similarly, nucleotide diversities within sampling localities were approximately an order of magnitude lower than in *E. hyalinus* ($P<10^{-7}$, Mann-

Whitney U), and ranged from 0.000 to a maximum of 0.004 (only slightly larger than the *E. hyalinus* minimum; π/site, Table 1, 2). Samples were typically dominated by a few common haplotypes, with the remainder consisting of rare haplotypes (Table 2, Figure 5). The sequence alignment contained only 46 segregating sites, 17 of which were parsimony informative. The average number of nucleotide differences between *E. spinifer* individuals was 0.79, considerably lower than for *E. hyalinus* (2.71).

Large-scale geographic patterns of population structure

Both *Eucalanus* species were characterized by strong genetic structuring at the largest, global, spatial scales. Despite this commonality, the pattern of population structure differed substantially between the sister species. Phylogeographic structure can be observed in the distribution of haplotype clades in *Eucalanus hyalinus* (Fig. 2). The North Atlantic *E. hyalinus* population consisted entirely of a clade of haplotypes endemic to this ocean basin (North Atlantic clade in Fig. 2); no haplotypes were shared between the North Atlantic and other populations worldwide. This North Atlantic clade is differentiated by three fixed nucleotide substitutions from other haplotypes worldwide, and was supported by 50% NJ bootstrap support. One haplotype within this clade, H161, occurred in high frequency in the Mediterranean Sea and in subtropical waters of the North Atlantic, while other haplotypes within the North Atlantic clade dominated in the northern North Atlantic (Fig. 4). A second endemic haplotype clade, the North Pacific clade, dominated in samples on both eastern and western sides of the North Pacific (Fig. 4). A substantial amount of

genetic diversity was observed within the clade (27 haplotypes, Fig. 2) despite its restricted geographic distribution, suggesting a long residence time within the North Pacific. The North Pacific clade differed by 2 fixed nucleotide substitutions from other haplotypes worldwide. The second haplotype clade in the North Pacific, the N. + S. Pacific + Indian O. clade, contained representatives from both the North and South Pacific as well as the Indian Ocean. Although only one haplotype within this clade was directly shared between Pacific regions, a number of genetically close haplotypes spanned the geographic break between the three regions (Fig. 2). Finally, three major haplotype clades, South Pacific and Indian Ocean (SPI) clades 1, 2, and 3 (Fig. 2), were broadly shared between the Indian Ocean and South Pacific. Haplotype H2 was the most common haplotype in both South Pacific and Indian Ocean populations, although it never occurred in greater than 40% frequency. Samples in both regions were dominated by rare haplotypes from SPI clade 1 (Fig. 4).

These geographic patterns of haplotype distribution also resulted in highly significant population structure in *E. hyalinus*, as reflected by the AMOVA. Samples within each hemisphere and ocean basin were grouped into four regions in the analysis, as these groupings fall along natural discontinuities in the biogeographic range of the species. Subdivision between regions was highly significant (Φ_{CT} =0.356, $p<0.001$), with 35.6% of the variance in the dataset contained in the between region component (Va). Variance between samples within a region (Vb) was low (0.87% of variance), while most of the genetic variance was contained in the within population component (63.6%, Vc). The 'within population component' refers to the genetic

diversity contained within individuals from a collecting location (a sample), which does not, in this case, correspond to a true biological population. Due to an absence of strong genetic structure at the within-region level, individuals within regions were pooled for calculation of pairwise Φ_{ST} values between regions. Pairwise Φ_{ST} values between regions ranged from a maximum of 0.663 between the North Atlantic and Indian Ocean populations, to a minimum of 0.0016 between the Indian Ocean and the South Pacific (Fig. 4). All regional comparisons except the South Pacific and Indian Ocean were highly statistically significant, as expected given the presence of geographically restricted haplotype clades. Samples sizes for regional comparisons of the South Pacific and Indian Ocean were high, and the lack of significant genetic structure was due to high levels of genetic similarity in both haplotype composition and frequency between these two regions. The large-scale patterns of genetic structure, observed in the regional Φ_{ST} results, were also notable in the global comparison of all samples (Table 3), where pairwise comparisons for samples in different regions yielded high estimates for all but South Pacific and Indian Ocean comparisons (eg, $\Phi_{ST} = 0.826$ to 0.587 for North Atlantic samples compared to samples in other regions). A Fisher's exact test of the regionally pooled dataset was also highly significant ($p<0.00001$), confirming the presence of substantial genetic structure between regions.

Global genetic patterns for *Eucalanus spinifer* also demonstrated significant structure at large spatial scales. However, the underlying pattern of genetic diversity and results for specific population comparisons differed considerably from its sister

species *E. hyalinus*. The *E. spinifer* COI neighbor-joining tree lacked substantial clade structure, and only a weak pattern was apparent whereby genetically similar haplotypes were found in the same major oceanographic regions (Fig. 3). One not highly differentiated clade is dominated by haplotypes found in the South Pacific, with other portions of the NJ tree containing predominantly Indian Ocean haplotypes (Fig. 3).

Despite the absence of phylogeographic patterns, high regional genetic structure was also observed in the AMOVA for *E. spinifer*. Again, samples within each hemisphere and ocean basin were grouped into regions in the analysis. Subdivision between regions was highly significant (Φ_{CT} =0.197, p<0.001), though slightly less pronounced than in the *E. hyalinus* dataset, with 19.7% of the variance in the dataset contained in the between region component. Variance between samples within a region was low (4.14% of variance), while most of the genetic variance was again contained in the within population component (76.2%). Individuals collected within a hemisphere and ocean basin were similarly grouped for regional pairwise Φ_{ST} estimates, due to an absence of strong genetic structure between samples within a region. A Fisher's exact test of the regionally pooled dataset was highly significant (p<0.00001), providing additional support for genetic subdivision between regions. As in *E. hyalinus*, many, but not all, of the regional pairwise comparisons were statistically significant. However, the population most highly differentiated in *E. hyalinus*, the North Atlantic, was the least genetically differentiated in *E. spinifer*, with a pairwise Φ_{ST} of 0.00 compared to the Indian Ocean (Fig. 5). All other regional

population comparisons were significant, with a maximal pairwise Φ_{ST} estimate of 0.252 between the North and South Pacific (Fig. 5). The genetic pattern driving high Φ_{ST} estimates between regions was the presence of private, or endemic haplotypes, which occurred in high frequency in the North and South Pacific. Haplotype H18 was restricted in distribution to the South Pacific, and constituted 25-60% of the haplotypes in samples from the subtropical gyre of the South Pacific (Fig.5). Similarly, haplotype H10 appeared to be restricted to the western Pacific, and was the dominant haplotype in the Kuroshio Current (Fig. 5). Both Indian Ocean and North Atlantic populations had the circumglobal haplotype H1 as their most common haplotype, in addition to the presence of rare haplotypes in 0 - 40% of each sample.

Genetic patchiness at mesoscales

Results from the global AMOVA and within-region isolation-by-distance (IBD) analyses demonstrate that the dominant signal of genetic structure in these plankton species occurs on the macrogeographic spatial scale of hemispheres and ocean basins. Samples collected within a subtropical gyre system, and even more broadly, across distinct oceanographic regions within a hemisphere and ocean basin exhibit extensive genetic similarity. This result can be observed in both species and occurs across vast geographic regions (to 9,100 km, Fig. 6). On spatial scales of 200 - 9,100 km, no pattern of isolation by distance is observed in either the South Pacific samples of *E. hyalinus*, nor in the Indian Ocean samples of *E. spinifer* (Fig. 6 A, B), despite sampling coverage over an entire ocean basin and a number of distinct large-

scale oceanographic features (in the *spinifer* case, Fig. 7). Sampling coverage on the Indian Ocean cruise (Fig. 7) transited across the Agulhas Current, the western Indian Ocean subgyre, the eastern portion of the subtropical gyre, and across tropical and near coastal waters northwest of Australia. Despite this substantial oceanographic heterogeneity and large geographic distance, no genetic differentiation is observed between plankton samples along the cruise leg. Slopes for both the *E. hyalinus* South Pacific and *E. spinifer* Indian Ocean datasets were not different from zero in either ordinary least-squares or reduced major axis regression (OLS, P = 0.99, South Pacific, *E. hyalinus*, P = 0.22, Indian Ocean, *E. spinifer*). Mantel's test was also non-significant in both cases (South Pacific, *E. hyalinus*, P = 0.445, Indian Ocean, *E. spinifer*, P = 0.32).

A second pattern of genetic heterogeneity on smaller spatial scales was also observed within the regional dataset. Significant pairwise Φ_{ST} estimates between a number of samples collected in the same oceanographic region were observed in the results for *E. hyalinus*, ranging up to a maximum of 0.107 between populations 1 and 4 (930 km apart, Table 3, Fig. 6). A pairwise Φ_{ST} value of 0.107 is quite high for a marine organism, particularly a planktonic one, and is noticeably an outlier in the dataset. There are four additional significant comparisons for *E. hyalinus* within the South Pacific (Table 3). Significant pairwise Φ_{ST} values within region comparisons can be observed in the Indian Ocean, North Pacific, and North Atlantic samples of *E. hyalinus* (Table 3). This pattern of microgeographic heterogeneity is less pronounced for *E. spinifer* from the Indian Ocean, in concordance with the observation of less

genetic diversity in the species as a whole. There is, however, one significant comparison between populations 14 and 18, with a pairwise Φ_{ST} value of 0.016 (Table 3, Fig. 6B). Additional, significant comparisons occur within the North and South Pacific populations of *E. spinifer* (Table 3). However, none of the within region Φ_{ST} values for either *E. hyalinus* or *E. spinifer* are statistically significant if corrected for multiple testing by the Bonferroni criterion (pairwise $\alpha = 0.00017$, for 300 comparisons of *hyalinus*, pairwise $\alpha = 0.0002$ for 231 comparisons of *spinifer*; between region comparisons remain significant even at this α level).

Effective population size, demography and selection

Despite similarity in geographic range size, the sister species differed by roughly an order of magnitude in genetic diversity (Table 1, 2). We can assume that the site specific mutation rate for COI is likely similar for both sister species. This implies that, given a ratio of $\theta(\pi_1, spinifer)/\ \theta(\pi_2, hyalinus)$ of 0.1796, *E. spinifer* had a genetic effective population size (N_e) roughly 20% that of *E. hyalinus*. This result is independent of the method used to estimate θ, as similar results were obtained using θ(S) and θ(k). Estimates of genetic effective population sizes for regional comparisons between the 2 species yielded similar results. The N_e of the *E. spinifer* population in the North Atlantic was 11.4% of the *E. hyalinus* population in the same ocean basin; comparable estimates for the Indian Ocean, North Pacific, and South Pacific yielded 23%, 21.5%, and 23.6%, respectively. Direct calculations of N_e from θ estimates depend on an assumed generation time and mutation rate. Given an average

generation time of 31.5 days (Paffenhofer 1991), and assuming a substitution rate of $0.604 * 10^{-9}$ (per sequence, per site, per generation; Knowlton and Weigt 1998), estimates of N_e for *E. hyalinus* regional populations range from $9.5 * 10^8$ (N. Atlantic) to $4.08 * 10^9$ (North Pacific). Results for *E. spinifer* range from $1.08 * 10^8$ (N. Atlantic) to $8.76 * 10^8$ (N. Pacific). The generation time has little effect on the results, within estimates of 28-35 days. On the other hand, no lineage-specific molecular clocks are available for closely related organisms (a marine decapod shrimp is used here), and the substitution rate included could contain considerable error. Upward shifts in the substitution rates of an order of magnitude would result in a comparable downward shift in estimates of N_e.

Both *Eucalanus* species had predominantly negative values of Tajima's D, though only a subset of samples had values that were statistically significant (Table 1, 2). In *E. spinifer*, all but one of the significantly negative values occurred in the Indian Ocean samples, indicating that a historical population expansion or selection event may have occurred only within this ocean basin (Table 2). However, it does not appear from the π values that the Indian Ocean population could have been expanding from historically low population sizes, as the genetic effective population sizes estimated from these samples were not lower than for other samples worldwide. In *E. hyalinus*, the majority of samples also had negative Tajima's D values (Table 1). Significant values occurred in samples in the South Pacific, Indian Ocean, and Mediterranean Sea. These populations may be expanding, or the COI gene may be

under selection in these geographic regions. Additional data from nuclear gene loci would be necessary to discriminate between demographic and selection hypotheses.

Species-specific biogeography

Results indicate that although *E. spinifer* and *E. hyalinus* overlap broadly in biogeographic range, there are subtle differences in the types of water masses in which they commonly occur. Of the 106 plankton samples included in this study, 32 of them contained specimens of only *E. spinifer*, 43 contained only *E. hyalinus*, and 31 samples contained members of both species. Both species are antitropical in distribution, and co-occur in subtropical and temperate waters worldwide (Fig. 1). The distribution of the two species along oceanographic transects in the South Pacific and Indian Ocean (Figs. 7, 8) demonstrated that although the species overlap, they tended to occur in abundance in different hydrographic regions.

In the Indian Ocean, the sister species co-occurred in the Agulhas Current (western boundary current), with *E. spinifer* slightly more common in the western subgyre centered over the Mozambique basin during the initial ~1,800 km of the transect (Fig. 7). A shoaling of isotherms at 2,000 - 3,500 km indicates the recirculation of ~20 Sverdrups (10^6 m^3/sec) to the northwest at the eastern edge of the western Indian Ocean subgyre (Wyrtki 1973; Stramma and Lutjeharms 1997; Reid 2003). Associated with this large-scale oceanographic feature is an increase in concentration of chlorophyll a at the deep maximum layer (Fig. 7). *Eucalanus hyalinus* s.s. dominated within this region, before disappearing within the oligotrophic

waters in the eastern portion of the subtropical gyre, to the north and east of the Madagascar Ridge. Six plankton tows within the central Indian Ocean (between 3,600-6,200 km on Fig. 7) contained very little zooplankton biomass, and only six *E. hyalinus* and three *E. spinifer* specimens were collected in this region (despite a combined total of ~10.5 hours of towing time). Both species occur in this area, but in very low abundance. Only *E. spinifer* was present in tropical waters in the final ~2000 km of the transect (Fig. 7). This region was characterized by fairly high concentrations of chlorophyll a at depth (72 – 94 m), which overlaid a pronounced oxygen minimum zone centered at ~780-800 m depth (casts at VANC10MV-20 and -23 extend to 1000m, Fig. 7).

In the South Pacific, *E. spinifer* dominated in the warm, salty, subtropical gyre waters of the northeastern ~2800 km of the transect (Fig. 8). Chlorophyll a maxima were deep in the gyre, with a maximum observed depth of 182 m at the northeastern-most station (Fig. 8). Along this cruise leg, *E. hyalinus* was absent or rare within the gyre, though it co-occurred with *E. spinifer* along the margins of the gyre in regions close to the subtropical convergence. *E. hyalinus* increased in abundance at the subtropical convergence, indicated by the presence of shoaling isotherms between 4,000-5,000 km along the transect leg. Farther to the SW, the cruise track crossed back into subtropical waters, and stations were located just north of the Campbell Plateau, in all likelihood just north of the subtropical frontal zone (Vinogradov and Flint 1988, e.g. Fig. I.3). Chlorophyll a maxima were shallow (33 – 55 m) at near-coastal stations. Only *E. hyalinus* occurred at stations south and west of the crossing

of the subtropical convergence. Samples included in the *E. hyalinus* South Pacific IBD analysis (Fig. 6A) were collected in this southwest portion of the cruise leg.

Discriminant analysis of the summary data from 44 hydrographic casts also indicated a few oceanographic characteristics that appeared important in the oceanographic distributions of the two species. Stations at which only *E. hyalinus* or *E. spinifer* were collected were not highly differentiated in canonical variable space (Fig. 9). However, the analysis did indicate that three parameters, water temperature at the chlorophyll a maximum, depth of the Brunt-Väisala frequency maximum, and the breadth of the chlorophyll a peak at 50% of maximum, were the best predictor variables by which to discriminate oceanographic distributions of the two species. These parameters were identified regardless of whether the stations were categorized by presence/absence of the two species (*hyalinus* only, *spinifer* only, both species present) or including an indication of species relative abundances (> 60% *hyalinus*, >60% *spinifer*, other). The dominant variable in both analyses was the temperature at the chlorophyll a maximum. A t-test for differences in mean temperature at the chlorophyll maximum between species was highly significant in both the presence/absence ($t = -3.82$, $df = 22$, $p<0.001$), and relative abundance-based analyses ($t = -4.72$, $df = 36$, $p< 0.0001$). *E. hyalinus* tends to occur at stations with cooler temperatures at the chlorophyll maximum (mean = 16.9°C) than *E. spinifer* (mean = 21.9°C, Fig. 10). This pattern likely reflects the larger-scale distribution of the two species, with *E. hyalinus* occurring in waters that are cooler throughout the upper water column (not simply at the chlorophyll a maximum). Analyses by relative

abundance and presence/absence were able to correctly classify 82% and 59% of the stations, respectively, in a jackknifed classification matrix.

Discussion

Oceanic marine zooplankton species with global biogeographic ranges are thought to disperse very effectively on ocean currents due to their holoplanktonic life habit (e.g., Boltovskoy et al. 2002). However, given the large spatial scope of their distributions, widely separated populations are unlikely to be mixed effectively on ecological timescales. Prior to this study, it was unknown whether populations throughout the global biogeographic range of oceanic zooplankton species are genetically linked on evolutionary timescales. Results for both *Eucalanus hyalinus* s.s and *E. spinifer* demonstrate that populations are highly differentiated on the macrogeographic spatial scales of hemispheres and ocean basins, despite relative genetic homogeneity within subtropical gyre systems. However, despite the common pattern of large and significant genetic structure at the global spatial scale, the two species differ in the extent to which specific barriers to gene flow have impacted their population genetic structure. The observation of unique population histories between a pair of phylogenetically close and biogeographically similar species implies that species-specific ecological differences likely play an important role in determining contemporary and historical patterns of dispersal.

Genetic Structure of Oceanic Marine Zooplankton Populations

The dominant spatial pattern of genetic differentiation observed in both *Eucalanus hyalinus* s.s and *E. spinifer* was of high and significant genetic structure among subtropical gyre systems, coupled with genetic homogeneity within these same oceanographic features. The extent of the genetic differentiation between *E. hyalinus* and *E. spinifer* populations centered in different gyres was high for marine organisms, and for planktonic species in particular, though it is perhaps not unexpected given the large spatial scales involved (Hedgecock 1994). To the limited extent that they have been analyzed, large-scale phylogeographic patterns are uncommonly observed in marine zooplanktonic organisms, and have only before been reported in a planktonic chaetognath in coastal European waters (Peijnenburg et al. 2004) and in a closely related eucalanid copepod, *Rhincalanus nasutus* (Goetze 2003) (excluding obligate estuarine species). The pairwise Φ_{ST} estimates observed in *E. hyalinus*, in particular, were also high in comparison with many studies of high dispersal marine organisms. Studies of marine benthic species with a long pelagic larval stage often find an absence of genetic differentiation over large (1,000s of km) geographic spatial scales (sometimes the entire species biogeographic range; e.g., Hellberg 1996; Wares et al. 2001; Flowers et al. 2002; Uthicke and Benzie 2003), or evidence of slight genetic structure (eg, Benzie and Williams 1997; Williams and Benzie 1998), and rarely identify high levels of genetic differentiation (Sotka et al. 2004). Similarly, in the studies of oceanic plankton species available for comparison to results presented here, relatively low levels of genetic differentiation have been observed (eg, Bucklin et al. 2000; Bucklin et al. 2000; Zane and Patarnello 2000; Jarman et al. 2002), and in many

cases, due to limited sampling, it is unclear whether significant results simply reflect genetic heterogeneity on smaller spatial scales (see below). Only the euphausiid, *Meganyctiphanes norvegica*, appears to demonstrate analogous levels of genetic structure on large spatial scales (Zane et al. 2000).

The boundaries of subtropical gyre waters and continental land masses were the two features observed to act as effective barriers to gene flow in the *Eucalanus* sister species pair. One important aspect of the observations presented here is that continental land masses acted as barriers to gene flow in some, but not all cases. It is also notable that the locations of the intraspecific population genetic breaks observed in both species correspond to known biogeographic boundaries for planktonic organisms (McGowan 1971; McGowan 1972). This observation, does not, however, support Avise's hypothesis that intraspecific phylogeographic breaks will occur concordantly with species distributional boundaries (Avise 1992; Avise 2000), because in this case the species' distributions also terminate at the biogeographic boundary. This habitat discontinuity, reflected in the disjunct distributions of many central water plankton species (eg, Reid et al. 1978), appears to act as an effective barrier to gene flow for conspecific populations of both *E. hyalinus* and *E. spinifer* in Northern and Southern subtropical gyres. This result may be due either to physical retention of individuals within the gyre, or due to differing selection pressures between oceanographic water masses, which may reduce the probability of dispersal between regions.

The observation of broad genetic homogeneity within central gyres suggests that the spatial scale of an interbreeding, panmictic population for these *Eucalanus* species is the scale of the gyre system itself. Subtropical gyres are physically retentive recirculation features of the large-scale ocean circulation, and it is not surprising that individuals within such a retentive feature would constitute a biological population. If we examine the potential transport distance of individuals entrained in the highest velocity surface currents of a western boundary current, including maximum surface velocities of the Kuroshio Current at ~1.0 m/sec (Tomczak and Godfrey 1994) and a generation time of 35 days (Paffenhofer 1991), we find that a copepod that lacks vertical migration behavior could be transported ~3000km across the North Pacific. Although this represents the maximum possible transport within a copepod lifetime, and most individuals probably move only on the order of 10's to 100's of kilometers (because only a small fraction of the population will be entrained in maximal current velocities), successful navigation of a 3000 km trip by very few individuals per generation would be sufficient to genetically homogenize large spatial areas. It appears likely that this will be a general population genetic pattern for planktonic taxa that are able to maintain viable populations in open ocean, oligotrophic waters. The data presented here do not support the inference of multiple, intraspecific populations within each gyre, despite the occurrence of some significant pairwise Φ_{ST} comparisons within these regions.

The data presented here also contain the commonly found pattern of small, but statistically significant, genetic heterogeneity on small spatial scales. This feature,

termed 'chaotic genetic patchiness' by Johnson and Black (1982; 1984), is an expected feature of the spatial genetic structure of organisms with a planktonic phase of the life history (Hedgecock 1994). This pattern can arise due to small-scale spatial or temporal variability in reproduction, or by differential mortality during early life history stages (Hedgecock 1994; Flowers et al. 2002). Although these processes may be important on ecological timescales, they are ephemeral, and will not contribute to the accrual of genetic differences between populations on evolutionary timescales. Some of the pairwise Φ_{ST} values observed between samples within a subtropical gyre in the *Eucalanus* results were relatively high (0.107, Table 3, Fig. 6), but were not consistent in space, and did not appear to be related to any oceanographic features (e.g., frontal zones). This suggests that the pattern was driven by small-scale temporal and spatial genetic variability (confounded in this dataset) due to patchiness in the genetic composition of the zooplankton. Recognition of the prevalence of this pattern is limited in the zooplankton population genetics literature, although it is widely appreciated as an important process determining the genetic composition of recruits in marine benthic invertebrate and pelagic vertebrate populations (e.g., Li and Hedgecock 1998; Planes and Lenfant 2002; McPherson, Stephenson et al. 2003). The observation of no genetic differentiation across oceanographic frontal zones in either the Indian Ocean or South Pacific demonstrates that such features were not effective at limiting gene flow for organisms that can maintain populations on both sides of the front.

Species-specific Differences in Genetic Structure

The sister species pair, *Eucalanus hyalinus* s.s. and *E. spinifer,* do not differ in their reproductive modes, basic life history strategies, or in the general characteristics of their biogeographic ranges. As a result, the sister species were expected to show equivalent patterns of population genetic structure throughout their range. Yet results summarized in figures 4 and 5 do not support this expectation, as major features of the global population genetic structure were observed to differ across the species pair. The most dramatic differences occurred between populations in the North Atlantic and Indian Ocean, and between the South Pacific and Indian Ocean (Fig. 4, 5). In *E. hyalinus* s.s., the North Atlantic population is genetically isolated from other populations throughout the species' global range, and is constituted entirely of a clade of haplotypes that is endemic to that ocean basin (Figs. 2, 4). This population may be undergoing the initial stages of allopatric speciation. In contrast, the *E. spinifer* North Atlantic population was genetically non-differentiable from the Indian Ocean population, despite a geographic separation of 8950 km between the closest samples in each ocean basin (Fig. 5, Table 3). In the second example of large-scale differences in global population genetic structure, *E. hyalinus* s.s. showed no genetic differentiation between populations in the South Pacific and Indian Ocean basins, while *E. spinifer* is characterized by substantial genetic structure across the same ocean basins. So, why do the two species differ in their global population genetic structure?

Differences in genetic structure between closely related species can have a number of possible explanations. These alternatives include: 1) selection acting on the

genetic marker of interest, 2) species differences in the impact of historical events on population genetic structure, or 3) species-specific ecological differences, and their interactions with the oceanographic environment, which result in differing levels of equilibrial gene flow between populations.

Selection on the mitochondrial marker COI does not appear to be a likely explanation for the species-specific differences in population genetic structure. Although the Tajima's D test results do suggest that selection may be occurring in some populations (this may instead reflect expanding populations, Tables 1, 2), a complicated, species-specific, geographically varying selection regime would have to be invoked to explain the patterns observed. Although possible, such a scenario does not appear to be the most parsimonious explanation for the observations. Data from additional, unlinked nuclear loci would be necessary to fully distinguish selection hypotheses from other alternatives (Hare 2001; Hare et al. 2002; Brumfield et al. 2003).

An alternative explanation is that the species differ in the observable impact of historical events on population genetic structure. Distinguishing the effects of contemporary and historical forces on the population genetic structure of species is challenging (Wakeley 1996; Nielsen and Wakeley 2001; Knowles 2004; Palsboll et al. 2004), and the same results can often be explained by forces operating on different timescales. It is clear, however, that the differentiation between the *E. hyalinus* North Atlantic and Indian Ocean populations must reflect a historical population divergence event, as the current equilibrial levels of gene flow are zero migrants exchanged

between these populations (no shared haplotypes). The absence of genetic structure in the *E. spinifer* populations of these same regions could be the result of either ongoing high levels of gene flow between populations, or due to a recent colonization or range expansion event with insufficient time elapsed for genetic differentiation to accrue between populations. This second hypothesis would require the absence of *E. spinifer* in one of these major ocean basins prior to a hypothetical range expansion event. Although one cannot say with certainty whether or not such a situation existed, subtropical gyres, which form the primary habitat *E. spinifer*, have probably been present in both ocean basins for many millions of years (Barron and Peterson 1991). Glacial-interglacial cycles are predicted to have had small effects on even the sea surface temperature of subtropical gyre waters (Plaumann et al. 2003). Therefore, it appears more probable that the *E. spinifer* North Atlantic and Indian Ocean populations are connected via contemporary gene flow.

In the second case, of the South Pacific and Indian Ocean populations, *E. spinifer* is the species observed to have substantial genetic structure. However, in this case, a number of haplotypes are shared between regions, including the globally most common haplotype H1. This result can be explained by two models: 1) a recent divergence event between populations and an absence of ongoing gene flow, or 2) an older divergence event with ongoing, low levels of contemporary genetic exchange between populations. In order to discriminate between these two models, I used a Markov Chain Monte Carlo (MCMC) method to estimate the migration rate between populations under a coalescent model, as implemented in the program MDIV (Nielsen

and Wakeley 2001; Palsboll et al. 2004). Results indicate that the populations undergo continuing migration between regions at an average rate of 1.16 individuals (females) exchanged each generation (maximum likelihood estimate). The 95% Bayesian credibility interval is given by (0.18, 3.38), suggesting a minimum estimate of one individual exchanged between populations every fifth generation. In contrast, the *E. hyalinus* population comparisons between the South Pacific and Indian Ocean suggest that high levels of ongoing genetic exchange occur between these two regions. The sister species, therefore, appear to have widely differing equilibrial levels of gene flow between the South Pacific and Indian Ocean basins. What parameters might control species differences in equilibrial gene flow between populations in the same oceanographic regions?

Aspects of species-specific ecology, and their interactions with the oceanographic environment, may dictate the patterns of genetic connectivity between zooplanktonic populations worldwide. It is widely recognized that aspects of the life history or behavior of zooplanktonic organisms, such as diel vertical migration behavior (Wroblewski 1982), ontogenetic migration (Peterson et al. 1979; Conover 1988), the localization of reproduction relative to patterns of physical transport (Verheye et al. 1991), and dormancy in deep, quiescent portions of the water column (or in highly retentive deep basins) (Sameoto and Herman 1990; Osgood and Checkley 1997; Osgood and Checkley 1997; Johnson and Checkley 2004) can enhance physical retention of individuals in an oceanographic area over ecological timescales. Some of these behaviors have been demonstrated to be very effective at

maintaining populations in even highly non-retentive physical systems (e.g., upwelling systems, Peterson 1998; Batchelder et al. 2002). By analogy, we can expect that species-specific aspects of the behavior, physiology, and life history of planktonic organisms will be a dominant factor moderating their realized dispersal on evolutionary timescales.

The sister species *E. hyalinus* s.s and *E. spinifer* may differ by a number of ecological parameters. Given the observation that even distantly related co-occurring omnivorous copepods overlap broadly in food utilization (Mullin 1966; Turner 1991), it appears unlikely that these close congeners differ substantially in their food particle capturing abilities. A total absence of species-specific information regarding habitat depth and dormancy for these species precludes an assessment of their potential importance in determining dispersal for *E. hyalinus* and *E. spinifer*. However, results from the oceanographic transects (Fig. 7, 8) as well as the discriminant analysis (Fig. 9) suggest that the two species differ somewhat in the types of oceanographic habitats in which they commonly occur, despite broad sympatry over large portions of their biogeographic ranges. *E. spinifer* tends to occur in central subtropical gyre waters, where chlorophyll a maxima are deep, water temperatures at the maxima are relatively warm, the upper water column is highly stratified, and primary productivity is driven by pico- and nano- sized prokaryotic and eukaryotic plankton and is primarily fueled by regenerated nutrients. *E. hyalinus*, on the other hand, occurs primarily along the margins of subtropical central waters, near frontal zones, and in upwelling systems, where stratification of the upper water column is less pronounced, upwelled nutrients

probably augment primary production, chlorophyll a maxima are nearer the surface, and water temperatures at the maxima are cooler. This distributional difference may be linked to species differences in thermal tolerance or may simply reflect other aspects of successful life history closure in the two oceanographic environments.

This micro-biogeographic differentiation may result in critical differences in dispersal potential between populations of the two species. Individuals of *E. spinifer*, due to a slightly more northerly distribution in the Southern hemisphere, would be less easily entrained and transported in the Antarctic Circumpolar Current (ACC). The ACC is the circumpolar current in the southern hemisphere, and it transports large water volumes (100 Sv, 3 fronts combined) eastward around Antarctica (Orsi et al. 1995). One important aspect of this current is that the core current velocities (50 cm/s, Subantarctic Front), though strongest at the surface, decay slowly as a function of depth, and do not completely diminish until the bottom (2 cm/sec at 3500db, Meinen et al. 2003). For a copepod population, this feature will result in eastward transport in the ACC regardless of the habitat depth. In contrast to *E. spinifer*, *E. hyalinus* s.s., with a distribution more tightly linked to frontal zones along the southern margin of the subtropical gyre, will be more easily transported eastward in the ACC. Substantial mixing is known to occur between subtropical waters and the ACC, particularly in areas of confluence of the Subantarctic and Subtropical Fronts (e.g., Crozet Basin, Park et al. 1993). On evolutionary timescales, such transport would result in more effective dispersal between *E. hyalinus* populations in the southern hemisphere, and

would manifest itself in an absence of genetic structure among these populations, exactly as is observed.

Understanding the potential role of habitat preferences in genetic connectivity between *E. hyalinus* and *E. spinifer* populations in the North Atlantic and Indian Ocean is more challenging given the unfortunate absence of sampling coverage in the southern Atlantic. Nevertheless, if we consider what the predictions for dispersal would be, given the micro-biogeographic differences described above, we find that population genetic observations largely match expectations. The inclusive taxon, *Eucalanus hyalinus* s.l., was previously known to have a continuous distribution across tropical latitudes in the Atlantic Ocean (Vervoort 1963; Lang 1965), unlike its Pacific distribution. Given the habitat differences described above, it appears likely that *E. spinifer* dominates in warm waters of the central gyres and across the tropics (*spinifer* type locality: Gulf of Guinea, Scott 1894), with *E. hyalinus* more abundant in cooler, more enriched waters in subtropical and temperate latitudes. A continuous distribution across the tropical Atlantic could result in a genetically homogeneous population in the Atlantic for *E. spinifer*, while a genetic discontinuity would be expected between northern and southern hemisphere populations of *E. hyalinus*. Connectivity between *E. spinifer* populations in the Indian and Atlantic Oceans could easily occur via the warm-water eddy shedding retroflection of the Agulhas Current (this would predict asymmetrical transport). Such a transport pathway has been proposed to link Indian Ocean and Atlantic populations of loggerhead (Bowen et al. 1994) and green sea turtles (Roberts et al. 2004), bigeye tuna (Chow et al. 2000),

swordfish (Chow et al. 1997), blue marlin (Buonaccorsi et al. 2001), and sea urchins (Lessios et al. 2001). Estimates of water leakage from the Agulhas Current into the South Atlantic range from 2.8 to 15 Sverdrups (Peterson and Stramma 1991), which could transport substantial numbers of subtropical planktonic organisms between ocean basins. The habitat preference interpretation of dispersal presented here makes the testable prediction that the South Atlantic population of *E. hyalinus* s.s. will be genetically similar, if not indistinguishable, from the South Pacific and Indian Ocean populations and not share haplotypes with the North Atlantic population, and that the South Atlantic *E. spinifer* population will be indistinguishable from the North Atlantic and Indian Ocean populations.

The substantial differences in genetic effective population size between the sister species may also suggest important differences in their ecology that impact their genetic characteristics (π, Table 1, 2). Although the genetic effective population size of a species is known to vary as a function of fluctuations in population size, skewed sex ratio, the extent of generational overlap (Gaggiotti and Vetter 1999), and recent selective sweeps, one explanation for the difference is simply a substantial reduction in the census population size of *E. spinifer* relative to *E. hyalinus*. This might be expected of a central water species, which occurs in lower densities throughout its range due to low food availability for a large-bodied particle feeder in an oligotrophic gyre system. Other factors appear unlikely due to the close phylogenetic relationship of the two species, and the fact that *E. spinifer*, the species with lower N_e, inhabits the

more stable environment, and is therefore unlikely to undergo larger fluctuations in population size, as would be required to explain the pattern observed.

Comparisons across multiple, codistributed plankton species will be necessary to determine whether there are key ecological features that consistently determine dispersal between conspecific populations on evolutionary timescales. The appropriate data to identify these parameters have not yet been collected, as global studies examining intraspecific genetic variation and interpopulation gene flow are logistically difficult to acquire for planktonic species. However, ongoing studies in the closely related eucalanid *Rhincalanus nasutus* support the view that species-specific ecological characteristics will be a determining factor in the population genetic structure of oceanic plankton species (E. Goetze, unpubl. results). Although *R. nasutus* cannot be considered a true replicate comparison to the *Eucalanus* species, due to substantial differences in the level of genetic differentiation observed, a comparison is nonetheless informative. In *R. nasutus*, also previously thought to be a circumglobal oceanic copepod species (but cosmopolitan, rather than central water mass), the genetic pattern is one of highly divergent genetic lineages centered in disjunct upwelling zones around the world, which do not appear to be genetically linked through recent or ongoing dispersal (Goetze 2003). Upwelling zones with non-retentive circulation form the primary habitat of *R. nasutus* (Lang 1965; Castro, Bernal et al. 1993), and advective losses from the population are expected to be high. The absence of effective dispersal among populations suggests that individuals fail to survive transport across open ocean environments. One hypothesis that could explain

this result is that high metabolic demands, as observed even in deep water populations of this species (Ohman et al. 1998), may result in low tolerance to starvation, and reduced survivorship across oligotrophic, open ocean waters. *R. nasutus* may also lack the ability to capture food particles in the smallest size classes. These species-specific ecological traits would then play an important role in determining survivorship in the oligotrophic open ocean, and dispersal ability between upwelling zones.

Future work should target phylogenetically closely-related, codistributed plankton species known to differ by particular ecological characteristics in order to test the importance of key life history, behavioral, or physiological differences in determining realized dispersal between conspecific populations of planktonic organisms. Such an approach will enable us to identify the key characteristics, analogous to the length of the pelagic larval phase for marine benthic invertebrates that are primary determinants of population genetic structure. Such data will simultaneously uncover any shared phylogeographic patterns across taxa, which might suggest an important role for historical vicariant events in the open ocean. Key species-specific biological characteristics to examine would appear to be: 1) thermal tolerance (Fleminger 1986), 2) vertical distributions and consequent influence of vertical current shear, 3) tolerance to starvation (reduced metabolic rate), and the ability to capture food particles across a broad range of particle sizes (~2-200μm), 4) the presence, timing, and duration of a dormant phase of the life history, 5) reproductive rate, and 6) mortality schedule.

Implications for Speciation in the Open Ocean

Results presented here demonstrate that barriers to gene flow are an important feature in the development of genetic differentiation between conspecific populations of open ocean zooplanktonic organisms. One of the most effective barriers to gene flow observed in both species of *Eucalanus* was the habitat discontinuity at the central water mass boundaries in the Pacific Ocean. Subtropical gyres appear to serve as 'habitat islands' for these open ocean populations, with individuals ineffective at dispersing between these major hydrographic regions. These large-scale barriers to dispersal, including both continental land masses and large-scale features of the ocean circulation, likely play an important role in enabling allopatric speciation to occur in the open ocean environment. No evidence was observed for dispersal over geographically restricted spatial scales, as would be required for parapatric speciation to proceed to completion.

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Table 1. Samples for global AMOVA analysis and summary statistics of mtDNA genetic diversity for *Eucalanus hyalinus* s.s. Listed are: Number of individuals sampled (N), Number of plankton tows over which specimens were pooled (T), Number of haplotypes observed in the sample (H), Fraction of haplotypes that are unique to each sample (H/N), Haplotype diversity (h), Nucleotide diversity ($\pi \pm$ sd), Nucleotide diversity per site (π/site), Tajima's D, and the ocean basin in which specimens were collected. Bold values of Tajima's D indicate statistical significant at $\alpha = 0.05$.

Sample ID	N	T	H	H/N	h	$\pi \pm$ sd	π/site	Tajima's D	Ocean basin
1. DRFT-21	15	1	10	0.67	0.83	1.743 ± 1.07	0.00499	**-2.09**	South Pacific
2. DRFT-20	16	1	13	0.81	0.92	3.205 ± 1.75	0.00918	**-1.83**	South Pacific
3. DRFT-19	31	1	30	0.97	0.98	4.340 ± 2.21	0.01243	**-1.84**	South Pacific
4. DRFT-18	13	1	10	0.77	0.89	3.277 ± 1.80	0.00939	-1.42	South Pacific
5. DRFT-1516	23	2	20	0.87	0.95	4.811 ± 2.44	0.01379	**-2.03**	South Pacific
6. DRFT-14	22	1	16	0.73	0.89	3.095 ± 1.67	0.00887	**-1.58**	South Pacific
7. DRFT-13	19	1	18	0.95	0.97	3.810 ± 2.01	0.01092	-1.38	South Pacific
8. DRFT-12	10	1	9	0.90	0.93	3.389 ± 1.89	0.00971	-1.29	South Pacific
9. DRFT-91011	20	3	19	0.95	0.97	4.315 ± 2.23	0.12364	**-1.68**	South Pacific
10. DRFT-GYRE	19	3	14	0.74	0.89	4.006 ± 2.09	0.01148	**-1.80**	South Pacific
11. VANC-AG	15	5	11	0.73	0.87	2.533 ± 1.44	0.00726	**-1.87**	Indian Ocean
12. VANC-567	26	3	22	0.85	0.97	4.435 ± 2.26	0.01271	**-1.95**	Indian Ocean
13. VANC-8	17	1	15	0.88	0.96	2.837 ± 1.57	0.00813	**-1.62**	Indian Ocean
14. VANC-9	16	1	13	0.81	0.92	2.864 ± 1.59	0.00821	-0.86	Indian Ocean
15. VANC-10	10	1	9	0.90	0.93	4.241 ± 2.30	0.01215	-1.30	Indian Ocean
16. VANC-GYRE	14	4	12	0.86	0.93	3.593 ± 1.94	0.01029	**-1.73**	Indian Ocean
17. MED_SEA	16		4	0.25	0.34	0.908 ± 0.66	0.00260	**-2.06**	Mediterranean Sea
18. NAT-01	19	5	6	0.32	0.69	0.995 ± 0.70	0.00285	-0.97	North Atlantic
19. NAT-02	9		8	0.89	0.92	1.481 ± 0.98	0.00424	-0.91	North Atlantic
20. AA	17	3	13	0.76	0.93	4.716 ± 2.43	0.01351	-0.16	North Pacific
21. CAL_IN	15	2	10	0.67	0.88	4.417 ± 2.31	0.14670	-0.55	North Pacific
22. CAL_OFF	24	2	16	0.67	0.93	5.018 ± 2.53	0.09380	-0.81	North Pacific
23. IM_IN	21	4	13	0.62	0.90	5.048 ± 2.55	0.12470	-0.48	North Pacific
24. IM_OFF	20	3	13	0.65	0.86	4.413 ± 2.27	0.01265	-1.21	North Pacific
25. CENCA_OREG	12		10	0.83	0.93	5.212 ± 2.71	0.01494	-0.50	North Pacific

Table 2. Samples for global AMOVA analysis and summary statistics of mtDNA genetic diversity for *Eucalanus spinifer*. Listed are: Number of individuals sampled (N), Number of plankton tows over which specimens were pooled (T), Number of haplotypes observed in the sample (H), Fraction of haplotypes that are unique to each sample (H/N), Haplotype diversity (h), Nucleotide diversity ($\pi \pm$ sd), Nucleotide diversity per site (π/site), Tajima's D, and the ocean basin in which specimens were collected. Bold values of Tajima's D indicate statistical significant at $\alpha = 0.05$.

Sample ID	N	T	H	H/N	h	$\pi \pm$ sd	π/site	Tajima's D	Ocean basin
1. AA-01	20	1	4	0.20	0.28	0.3153 ± 0.336	0.00084	**-1.72**	North Pacific
2. AA-02	10	2	3	0.30	0.48	0.9226 ± 0.693	0.00246	-0.66	North Pacific
3. NW_PAC	10	5	5	0.50	0.63	1.4673 ± 0.968	0.00391	-1.49	North Pacific
4. COOK-23	15	2	6	0.40	0.76	1.2247 ± 0.823	0.00327	-0.78	South Pacific
5. COOK-6	12	1	6	0.50	0.81	1.4982 ± 0.969	0.00400	-0.38	South Pacific
6. COOK-12	25	1	5	0.20	0.62	0.8190 ± 0.607	0.00218	-0.67	South Pacific
7. COOK-1718	25	2	3	0.12	0.52	0.5717 ± 0.480	0.00153	0.12	South Pacific
8. COOK-2223	19	2	2	0.11	0.44	0.4631 ± 0.424	0.00124	1.10	South Pacific
9. COOK-293032	12	3	5	0.42	0.70	1.4478 ± 0.945	0.00386	-1.17	South Pacific
10. DRFT-8	21	1	5	0.24	0.64	0.9114 ± 0.657	0.00243	-0.58	South Pacific
11. DRFT-910	7	2	2	0.29	0.53	0.5801 ± 0.526	0.00155	1.34	South Pacific
12. DRFT-1112	15	2	7	0.47	0.77	1.2104 ± 0.816	0.00323	-0.82	South Pacific
13. VANC-1	24	1	8	0.33	0.50	1.1616 ± 0.776	0.00310	**-2.28**	Indian Ocean
14. VANC-2_5	23	4	6	0.24	0.45	0.6355 ± 0.515	0.00170	**-1.93**	Indian Ocean
15. VANC-GYRE	13	3	2	0.15	0.15	0.3207 ± 0.347	0.00086	-1.47	Indian Ocean
16. VANC-20	17	1	4	0.24	0.32	0.5005 ± 0.448	0.00134	**-1.84**	Indian Ocean
17. VANC-23	16	1	5	0.31	0.44	0.6498 ± 0.530	0.00173	**-1.93**	Indian Ocean
18. VANC-2425	20	2	9	0.45	0.64	1.4942 ± 0.940	0.00399	**-2.27**	Indian Ocean
19. VANC-27	15	1	7	0.47	0.63	0.9914 ± 0.708	0.00264	**-2.04**	Indian Ocean
20. VANC-MAD	10	6	2	0.20	0.19	0.4169 ± 0.413	0.00111	-1.40	Indian Ocean,
21. NATL-1	15	1	1	0.07	0.00	0.0000 ± 0.000	0.00000	-1.00	North Atlantic
22. NATL-2	17	5	3	0.18	0.22	0.2468 ± 0.294	0.00066	-1.50	North Atlantic

Table 3. Pairwise Φ_{ST} estimates between samples of *Eucalanus spinifer* (below diagonal) and *Eucalanus hyalinus* (above diagonal). Bold numbers indicate significant values ($\alpha = 0.05$). Sample numbers as in Tables 1 and 2.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	****	-0.003	0.022	**0.107**	0.006	0.024	0.021	0.035	0.022	0.008	0.014	0.021	0.011	**0.064**	0.026	0.022	**0.820**	**0.826**	**0.791**	**0.357**	**0.407**	**0.379**	**0.286**	**0.451**	**0.211**
2	**0.499**	****	0.008	0.031	-0.017	-0.017	-0.007	-0.013	-0.005	-0.014	-0.015	-0.008	-0.020	0.018	0.012	-0.011	**0.727**	**0.746**	**0.690**	**0.289**	**0.331**	**0.328**	**0.235**	**0.389**	**0.149**
3	**0.497**	-0.029	****	0.010	0.003	**0.033**	**0.040**	0.003	-0.008	0.001	0.012	0.003	0.021	-0.024	-0.019	-0.006	**0.611**	**0.637**	**0.587**	**0.237**	**0.268**	**0.276**	**0.198**	**0.323**	**0.127**
4	**0.294**	**0.343**	**0.350**	****	0.001	0.045	**0.051**	-0.004	0.034	-0.002	**0.073**	0.004	**0.057**	0.026	0.022	0.024	**0.690**	**0.713**	**0.644**	**0.212**	**0.248**	**0.252**	**0.178**	**0.305**	**0.132**
5	**0.217**	**0.360**	**0.360**	**0.082**	****	0.003	0.004	-0.016	-0.006	-0.026	-0.011	-0.002	-0.002	0.006	0.005	-0.015	**0.622**	**0.648**	**0.587**	**0.244**	**0.272**	**0.288**	**0.194**	**0.335**	**0.124**
6	**0.325**	**0.480**	**0.492**	-0.014	**0.097**	****	0.013	-0.031	0.009	0.009	0.014	-0.014	-0.010	0.040	0.028	0.008	**0.710**	**0.728**	**0.680**	**0.295**	**0.338**	**0.332**	**0.243**	**0.393**	**0.168**
7	**0.472**	**0.575**	**0.573**	0.002	**0.186**	-0.014	****	0.011	**0.027**	0.007	0.027	0.021	0.013	0.048	0.024	0.022	**0.692**	**0.713**	**0.654**	**0.267**	**0.307**	**0.312**	**0.206**	**0.372**	**0.111**
8	**0.189**	**0.488**	**0.491**	0.039	**0.098**	0.038	**0.138**	****	0.001	-0.015	0.024	-0.020	0.009	0.026	0.004	0.004	**0.738**	**0.755**	**0.686**	**0.238**	**0.284**	**0.285**	**0.195**	**0.341**	**0.131**
9	**0.259**	**0.332**	**0.336**	-0.051	0.024	-0.029	0.007	0.016	****	-0.001	-0.010	-0.002	-0.003	-0.011	-0.014	-0.035	**0.663**	**0.686**	**0.625**	**0.250**	**0.283**	**0.289**	**0.202**	**0.340**	**0.122**
10	**0.225**	**0.423**	**0.440**	-0.008	0.071	-0.005	0.040	-0.012	-0.015	****	0.002	0.000	0.000	0.013	-0.007	-0.007	**0.668**	**0.688**	**0.628**	**0.254**	**0.286**	**0.297**	**0.201**	**0.348**	**0.128**
11	**0.298**	**0.446**	**0.415**	-0.068	0.023	-0.072	-0.009	-0.077	-0.084	-0.085	****	0.001	-0.005	0.018	0.025	-0.010	**0.769**	**0.782**	**0.733**	**0.338**	**0.380**	**0.364**	**0.276**	**0.432**	**0.188**
12	**0.193**	**0.365**	**0.385**	-0.007	0.048	0.008	0.052	-0.010	-0.015	-0.039	-0.079	****	-0.016	0.001	-0.008	-0.010	**0.618**	**0.645**	**0.589**	**0.246**	**0.277**	**0.289**	**0.201**	**0.337**	**0.121**
13	-0.006	**0.284**	**0.324**	**0.192**	**0.135**	**0.233**	**0.331**	**0.098**	**0.159**	**0.147**	**0.109**	**0.116**	****	**0.036**	-0.009	-0.003	**0.743**	**0.760**	**0.709**	**0.311**	**0.354**	**0.345**	**0.251**	**0.406**	**0.166**
14	-0.004	**0.414**	**0.434**	**0.251**	**0.188**	**0.288**	**0.410**	**0.144**	**0.216**	**0.196**	**0.195**	**0.165**	0.000	****	-0.001	-0.009	**0.734**	**0.753**	**0.700**	**0.274**	**0.315**	**0.303**	**0.227**	**0.368**	**0.151**
15	0.001	**0.463**	**0.450**	**0.254**	**0.191**	**0.300**	**0.454**	**0.178**	**0.213**	**0.200**	**0.277**	**0.161**	-0.013	-0.009	****	0.003	**0.701**	**0.725**	**0.645**	**0.226**	**0.264**	**0.265**	**0.184**	**0.319**	**0.106**
16	0.002	**0.436**	**0.447**	**0.254**	**0.187**	**0.294**	**0.431**	**0.159**	**0.219**	**0.187**	**0.227**	**0.141**	-0.008	-0.013	-0.004	****	**0.711**	**0.732**	**0.667**	**0.260**	**0.297**	**0.298**	**0.208**	**0.353**	**0.121**
17	0.004	**0.398**	**0.414**	**0.236**	**0.175**	**0.275**	**0.406**	**0.142**	**0.191**	**0.183**	0.187	**0.147**	-0.013	-0.002	-0.005	0.001	****	**0.192**	**0.104**	**0.599**	**0.624**	**0.562**	**0.584**	**0.606**	**0.645**
18	0.015	**0.278**	**0.315**	**0.182**	**0.132**	**0.219**	**0.311**	**0.097**	**0.141**	**0.145**	0.089	**0.113**	-0.009	**0.016**	-0.002	0.006	-0.004	****	-0.013	**0.630**	**0.653**	**0.593**	**0.613**	**0.635**	**0.673**
19	**0.009**	**0.339**	**0.363**	**0.206**	**0.149**	**0.250**	**0.367**	**0.120**	**0.169**	**0.163**	**0.133**	**0.120**	-0.003	-0.014	-0.006	-0.021	-0.010	0.003	****	**0.556**	**0.578**	0.529	**0.545**	**0.568**	**0.588**
20	0.008	**0.424**	**0.412**	**0.230**	**0.162**	**0.281**	**0.434**	**0.165**	**0.181**	**0.181**	**0.239**	0.138	-0.019	-0.004	0.004	-0.003	-0.008	-0.021	-0.033	****	-0.044	-0.016	0.008	-0.009	0.060
21	-0.016	**0.585**	**0.545**	**0.316**	**0.247**	**0.349**	**0.526**	**0.248**	**0.279**	**0.242**	**0.492**	**0.206**	-0.017	-0.010	0.011	-0.008	-0.005	0.000	-0.002	0.043	****	-0.036	0.013	-0.035	0.091
22	-0.001	**0.508**	**0.500**	**0.291**	**0.226**	**0.325**	**0.480**	**0.198**	**0.254**	**0.223**	**0.324**	**0.189**	-0.006	0.002	0.003	0.000	0.001	0.009	**0.005**	0.013	-0.008	****	0.016	-0.023	**0.114**
23	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	0.045	0.028
24	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	**0.159**
25	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****

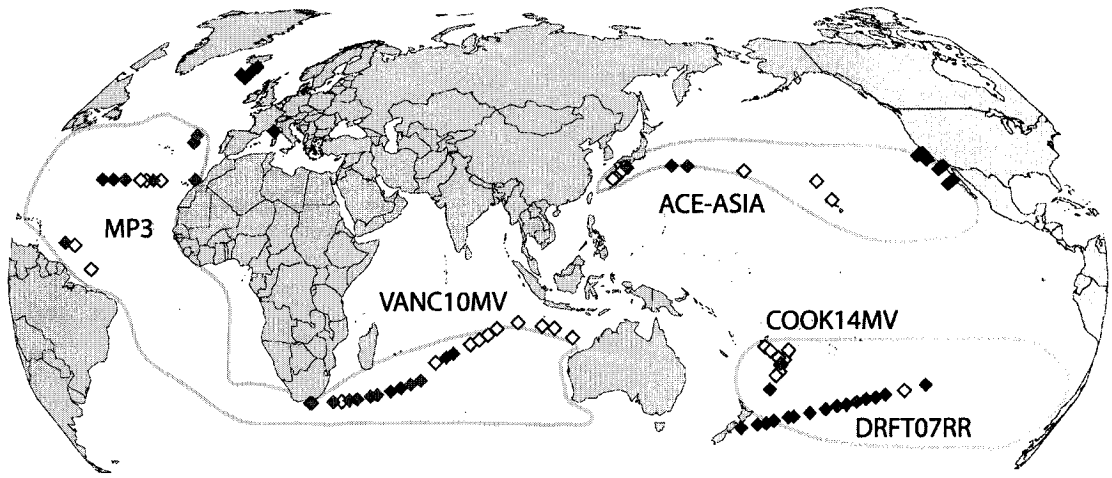

Figure 1. Global distribution of *Eucalanus hyalinus* s.l. and sample locations for this study. Solid black diamonds mark plankton tow locations containing only *E. hyalinus* s.s, grey diamonds mark tow locations containing both *E. hyalinus* s.s. and *E. spinfer*, and open diamonds mark tow locations in which only *E. spinifer* was collected. Regions outlined in grey illustrate the original distribution of *E. hyalinus* s.l., as described by Fleminger and Hulsemann (1973). Samples collected on the five major cruises indicated were obtained by towing obliquely between the surface and 400 to 1100 m depth.

Figure 2

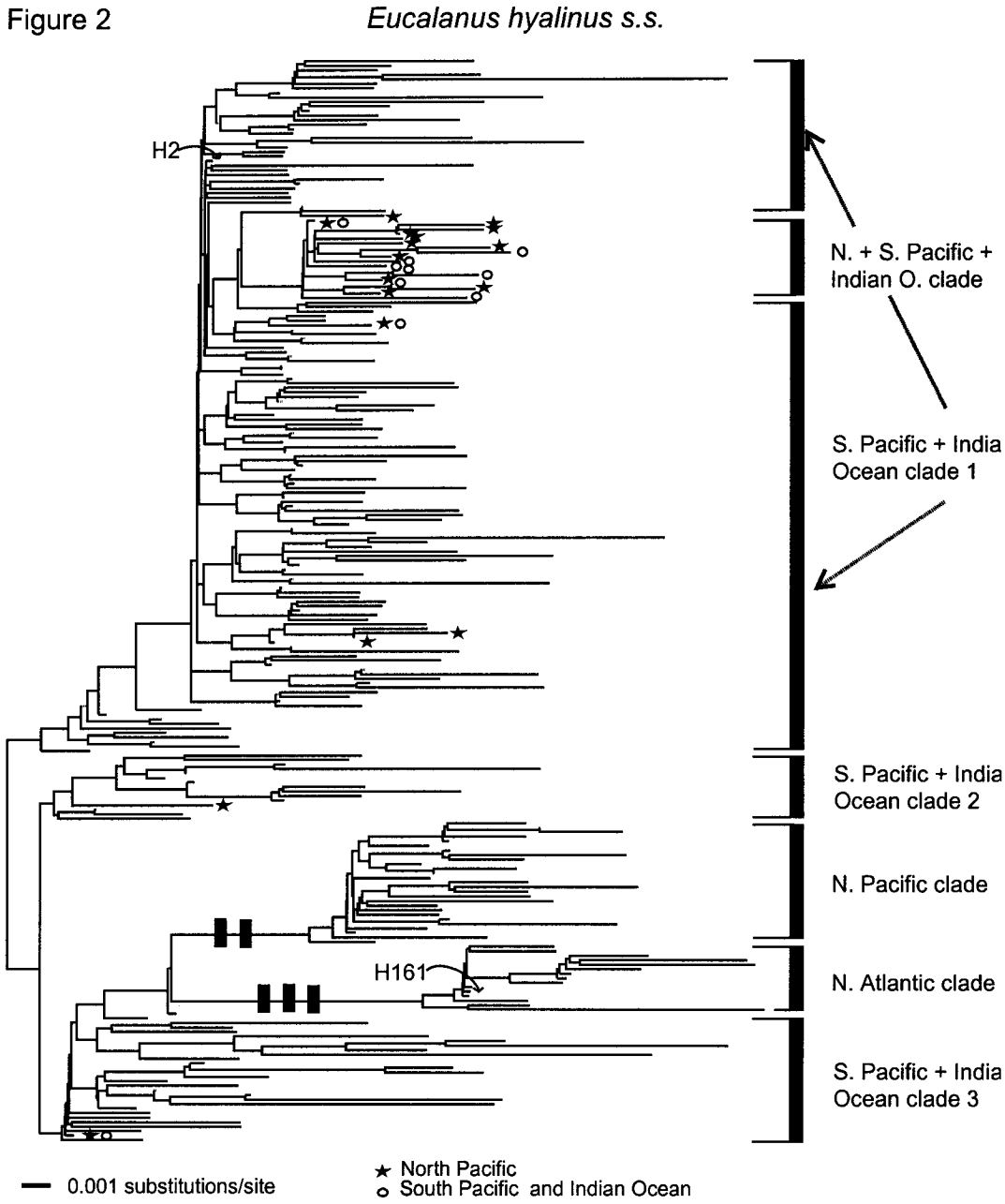

Figure 2. Neighbor-joining tree of 239 COI haplotypes sampled in *Eucalanus hyalinus* s.s. All unmarked sequences were collected in the region indicated by the clade label at right. Stars and open circles indicate haplotypes sampled in the North Pacific and South Pacific + Indian Ocean, respectively, although their position in the phylogeny may indicate similarity with a clade typical of another region. The only labeled haplotypes, H2 and H161, are the most common haplotypes in the South Pacific and North Atlantic, and are included in Figures 3 and 4. Solid bars indicate fixed substitutions along the lineage.

Figure 3

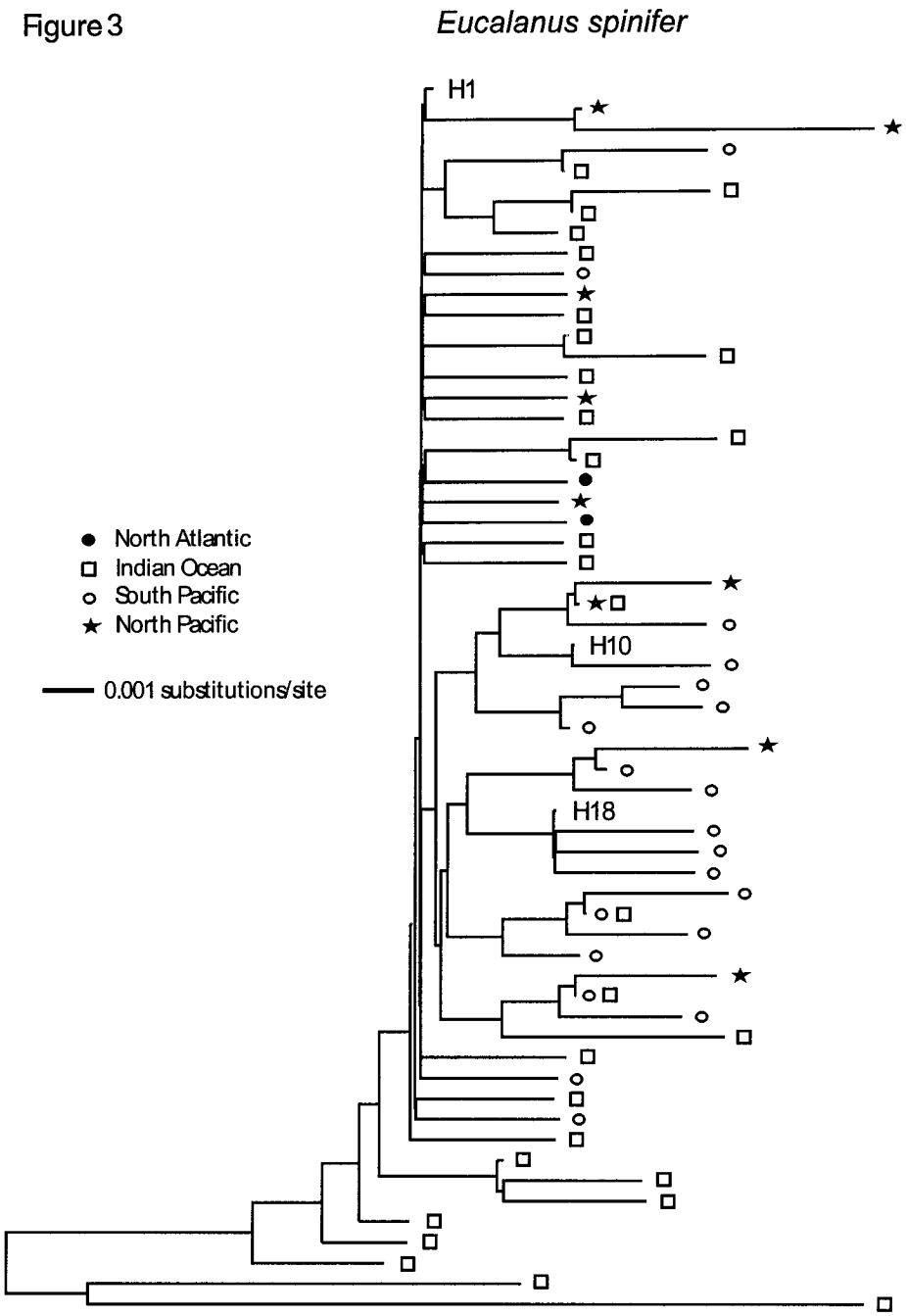

Figure 3. Neighbor-joining tree of 60 COI haplotypes sampled in *Eucalanus spinifer*. Solid circles, open squares, open circles, and stars indicate haplotypes sampled in the North Atlantic, Indian Ocean, South Pacific, and North Pacific, respectively. H1 is the most common haplotype globally, and H10 and H18 are endemic haplotypes in the Kuroshio Current and South Pacific regions.

Figure 4. Frequencies of haplotypes and pairwise Φ_{ST} estimates for regional population comparisons in *Eucalanus hyalinus* s.s. Haplotype clades described in Figure 3, with colors as indicated in the legend. Thickness of arrows is scaled to the magnitude of gene flow between regions. Sample sizes (N) within each region for the AMOVA included; * denotes significant values of Φ_{ST} ($\alpha = 0.05$). The pairwise Φ_{ST} for the North Atlantic and South Pacific comparison was 0.630* (not included). Populations in the Indian Ocean are 11, 12, 13, 14, 15, 16 (Table 1) from West to East, and in the South Pacific 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 from West to East.

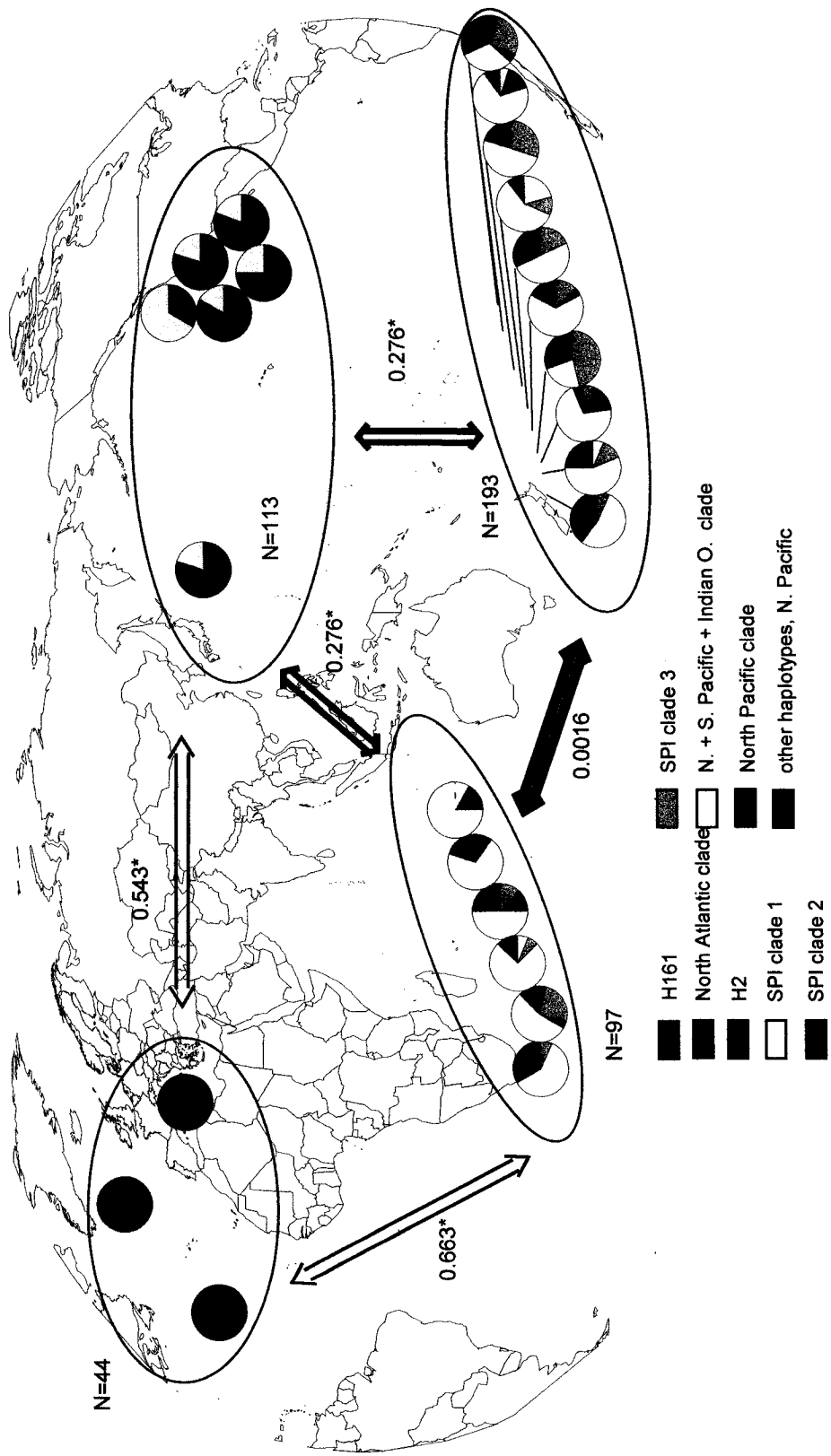

Figure 4.

Figure 5. Frequencies of haplotypes and pairwise Φ_{ST} estimates for regional population comparisons in *Eucalanus spinifer*. Haplotypes H1, H10, and H18 described on NJ-tree in Figure 4, with colors as indicated in legend. Thickness of arrows is scaled to the magnitude of gene flow between regions. Sample sizes (N) within each region for the AMOVA included; * denotes significant values of Φ_{ST} ($\alpha = 0.05$). The pairwise Φ_{ST} for the North Atlantic and South Pacific comparison was 0.198* (not included). Populations in the Indian Ocean are 13, 14, 20, 15, 16, 17, 18, 19 (Table 2) from West to East.

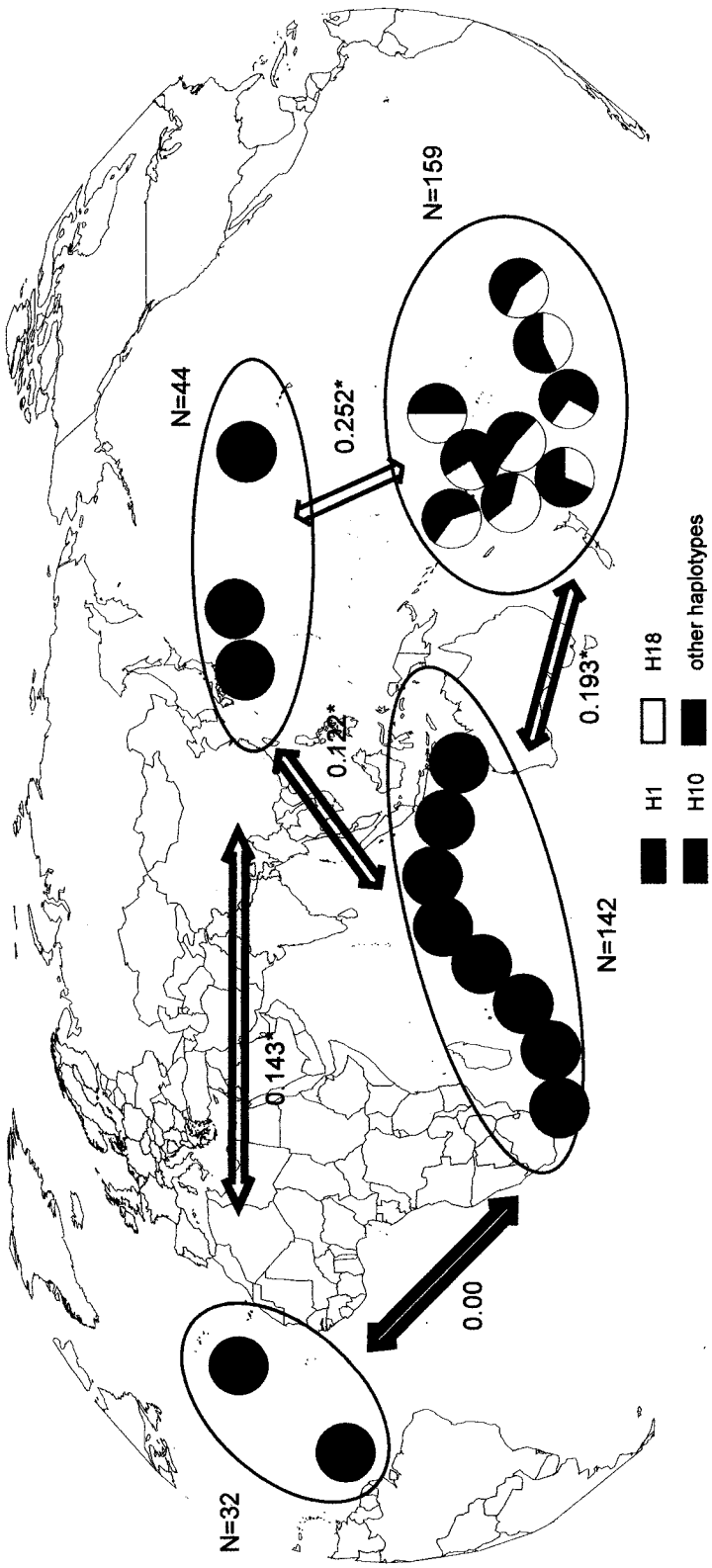

Figure 5.

Figure 6

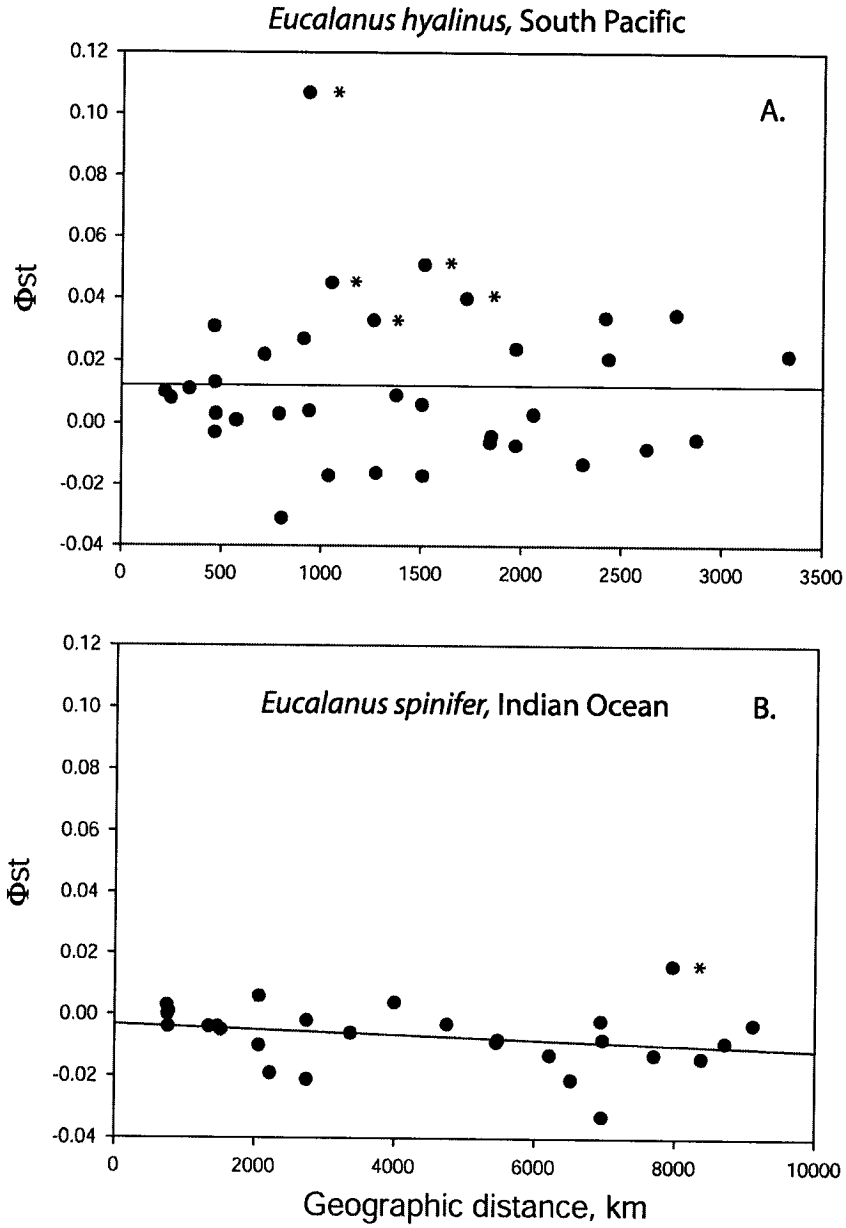

Figure 6. Plot of pairwise Φ_{ST} estimates vs. geographical distance (km) within oceanographic regions for (A) *Eucalanus hyalinus* populations within the South Pacific, and (B) *Eucalanus spinifer* populations in the Indian Ocean. In both cases, the slope and intercept were not significantly different from zero (South Pacific, *E. hyalinus*: RMA, $y = (2.59*10^{-5})\ x - 0.02$; OLS, $y = (8.65*10^{-9})\ x + 0.012$; Indian Ocean, *E. spinifer*: RMA, $y = (1.15*10^{-6})\ x - 0.00403$; OLS, $y = (-8.31*10^{-7})\ x - 0.00325$, 95% CI includes 0 in all cases). Φ_{ST} population comparisons significant at $\alpha = 0.05$ are marked by an asterisk (none are significant following Bonferroni correction).

Figure 7. The distribution of *E. hyalinus* s.s. and *E. spinifer* along an oceanographic transect in the Indian Ocean (cruise VANC10MV). Temperature (°C), salinity (psu), chlorophyll a (μg/L), and dissolved oxygen (ml/L) from 15 CTD casts, with station locations marked by black circles. Final panel indicates proportion of *E. hyalinus* and *E. spinifer* in plankton samples along the transect. Distance along transect plotted from the first cruise station, at 35° 03.04'S, 23° 44.28'E. Grey area denotes an absence of data below 225 m. In vivo fluorescence converted to chlorophyll a concentrations by the following equation: $y = (0.6629)*x + 0.02813$, after correction for baseline offset.

Figure 7.

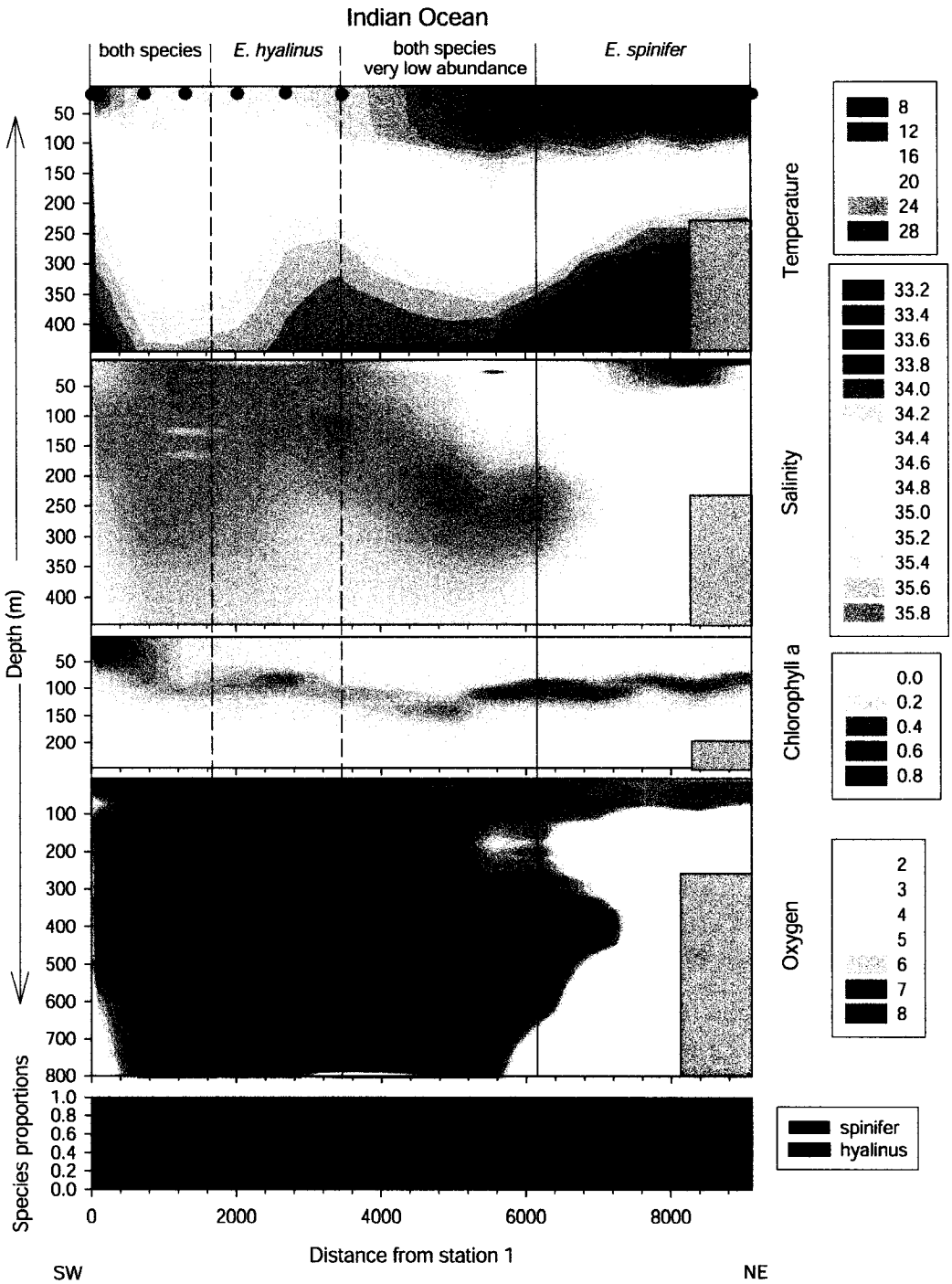

Figure 8.

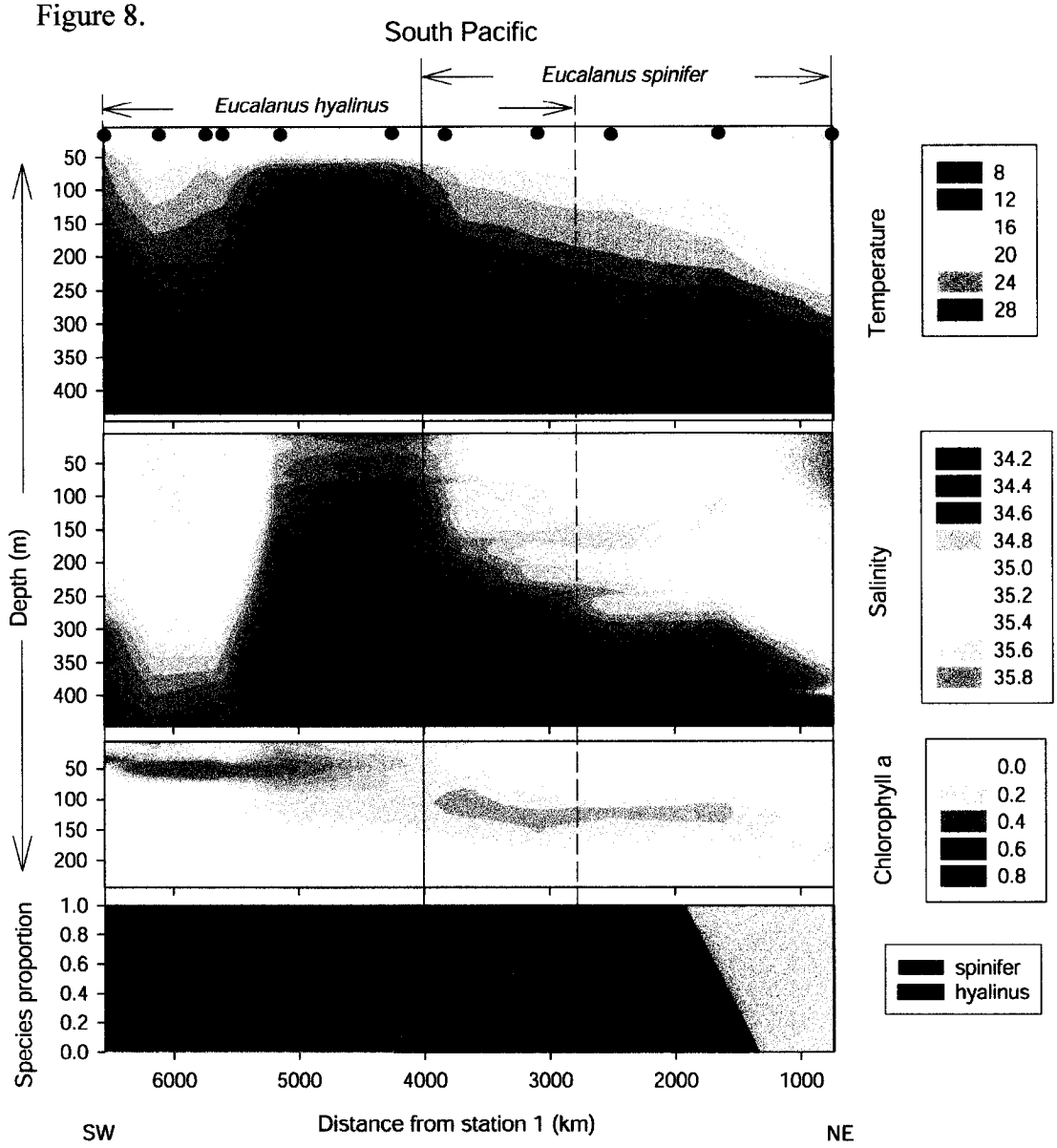

Figure 8. The distribution of *E. hyalinus* s.s. and *E. spinifer* along an oceanographic transect in the South Pacific (cruise DRFT07RR). Temperature (°C), salinity (psu), and chlorophyll a (μg/L) from 11 CTD casts, with station locations marked by black circles. Final panel indicates the proportion of *E. hyalinus* and *E. spinifer* in plankton samples along the transect. Grey region indicates an absence of data. Plankton tows were completed at CTD stations, as well as at intermediate locations along the cruise leg (21 tows, 11 casts). Distance along transect plotted from the first cruise station, at 27° 40.62'S, 111° 33.15' W, in the central South Pacific. Dissolved oxygen was not measured on this cruise. In vivo fluorescence converted to chlorophyll a concentrations by the following equation: y = (1.14604)*x –

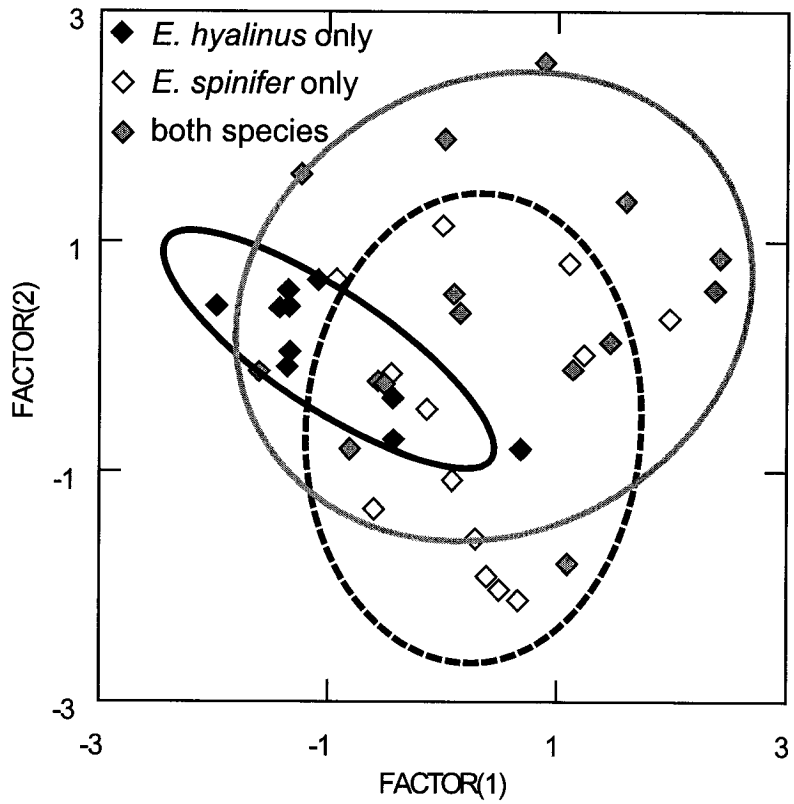

Figure 9. Plot of canonical scores from discriminant analysis of *E. hyalinus* and *E. spinifer* oceanographic distributions, with stations categorized by presence or absence of each species. CTD casts from stations at which only *E. hyalinus* or *E. spinifer* were collected marked by blue and red triangles, respectively. Open triangles indicate stations at which the two species were found to co-occur. Ellipses mark 95% confidence limits for each group.

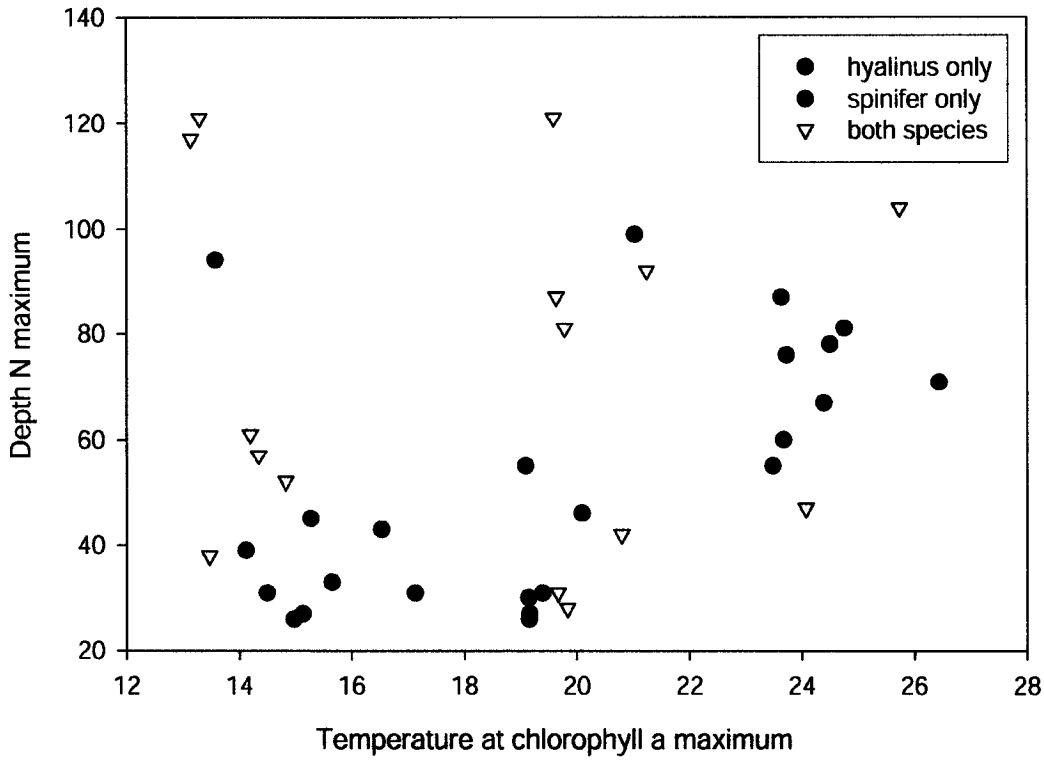

Figure 10. Plot of water temperature at the depth of the chlorophyll a maximum (°C) versus the depth of the Brunt-Väisala frequency maximum (m) for stations at which only *E. hyalinus*, *E. spinifer*, or both species occurred.

This chapter, in full, has been submitted for publication in Evolution. The dissertation author was the primary investigator and sole author of this paper.

Chapter V

Unique Intron Structure and Multiple Copy Number of Nuclear Elongation Factor 1-α in Marine Copepods

Abstract

Two unique spliceosomal introns (both ~50 bp) were discovered in nuclear elongation factor 1-α in the calanoid copepod genera *Eucalanus* and *Rhincalanus* (Copepoda: Calanoida). These intron positions have not been found in other well-characterized arthropod groups. It is inferred that the introns were recently and independently acquired within the family Eucalanidae. Elongation factor 1- α was also present in at least two functional and multiple non-functional gene copies within eucalanid copepods. Pseudogene copies were non-differentiable from functional genes by the presence and position of introns. Although EF1- α is demonstrated here to have an appropriate level of resolution for species-level phylogenetic inference in marine copepods, difficulty in identifying orthologous gene copies will severely limit the utility of this gene for systematic studies.

Introduction

Phylogenetic studies require the use of multiple, independently inherited molecular markers in order to accurately infer relationships among taxa. Single-copy nuclear gene loci are particularly useful for phylogenetic inference because they are

inherited independently of mitochondrial markers and identification of orthologous gene sequences is not complicated by the presence of paralogous gene copies. Many taxonomic groups, however, have been little studied in the phylogenetic context, and few nuclear genes have been identified that accurately resolve relationships within these groups. Calanoid copepods are one such group, and phylogenetic studies have focused on a few commonly used genes as molecular markers. The nuclear loci used to date are all ribosomal genes, including 28S rRNA (Braga et al. 1999), 18S rRNA (Bucklin 2003; Thum 2004), and ITS2 (Goetze 2003). Sequence data for protein-coding Elongation Factor 1- α and RNA polymerase II are also available for one copepod species, *Euytemora affinis*, due to its inclusion in higher-level phylogenetic studies of arthropods (Regier and Shutlz 1997; Regier and Shultz 1998). However, no protein coding nuclear genes have been used for species-level phylogenetic inference in the calanoid copepods. The goal of the present work was to test the utility of the nuclear gene Elongation Factor 1- α for phylogenetic inference of species-level relationships in the calanoid family Eucalanidae.

Elongation Factor 1- α has demonstrated utility to resolve phylogenetic relationships in alpha-level systematics, due to differentiation at silent nucleotide sites (Cho et al. 1995; Hedin and Maddison 2001), as well as in deeper divergences where amino acid substitutions provide phylogenetic resolution (Regier and Shutlz 1997; Regier and Shultz 1998; Regier et al. 2004). The gene has been used widely in phylogenetic studies of terrestrial (e.g., Caterino et al. 2000; Danforth et al. 2004; Vogler and Hughes 2004; Zakharov et al. 2004) and marine arthropods (Williams et

al. 2001), and has proved useful in most cases. EF 1- α is a highly conserved gene, which facilitates GTP dependent binding of tRNAs to the acceptor site of ribosomes. Highly conserved exon sequence enables the development of PCR primers that successfully amplify the gene across diverse taxa in phylogenetic studies. Although initially reported to be single-copy in many organisms, elongation factor 1- α is increasingly being recognized to occur in multiple copies in the nuclear genome. *Drosophila melanogaster* is known to have two functional copies of the EF1- α gene, with unequal expression levels in adults (Hovemann et al. 1988). Two functional copies have also been identified in *Alpheus* shrimp (Williams et al. 2001) and in *Apis* bees (Danforth and Ji 1998), approximately four functional copies have been reported in *Artemia* (Lenstra et al. 1986), in addition to a number of studies that report multiple copies which may include both functional genes and pseudogenes (France et al. 1999) (Hedin and Maddison 2001; Jordal 2002). In all cases, however, the level of nucleotide divergence observed between the 2 functional copies is quite high (10.6 to 22% in beetles, 24.9% *Apis* bees, 18.6% in *Drosophila*, 14 to 25% in *Alpheus* shrimp (Danforth and Ji 1998; Williams et al. 2001; Jordal 2002), and paralogous sequences have been readily identifiable.

Here I examine the phylogenetic utility of EF1- α in the calanoid copepod family Eucalanidae, a common family of calanoid copepods in marine temperate, subtropical, and tropical waters. A molecular phylogeny of the family has previously been inferred based on mitochondrial (16S rRNA) and nuclear (ITS2) gene loci (Goetze 2003), and is compared here to results for Elongation Factor 1- α.

Material and Methods

Frozen (liquid N_2) and alcohol preserved specimens were used in total RNA and genomic DNA extractions of 17 species in the Eucalanidae (Table 1). Alcohol preserved specimens were preserved in 95% non-denatured ethyl alcohol as unsorted bulk plankton material, prior to sample sorting and DNA extraction. All samples were stored in 95% ethyl alcohol at -20°C prior to DNA extraction. Genomic DNA was extracted from single adult females using the QIAGEN DNeasy tissue extraction kit. The only modifications to the manufacturer's protocol were to limit the 55°C lysis incubation step to 1-1.25 hours, to reduce the amount of elution buffer to 100µl, and to increase the duration of the elution incubation step to > 10 minutes. Primers used for PCR amplification of Elongation Factor 1-α were from Cho et. al. (1995), including M44-1 [5' –GCT GAG CG(CT) GA(AG) CGT GGT ATC AC – 3'], M46-1 [5'-GAG GAA AT(CT) AA(AG) AAG GAA-3'], and rcM53-2 [5'-GCA ATG TG(AG) GCT GTG TGG CA-3'], without inclusion of the M13 forward or reverse primers on the 5' end. The primer combination M44-1/rcM53-2 successfully amplified EF1-α across species in the Eucalanidae, and resulted in variable length fragments, ranging between ~850-1100 bp for different species in the family. Amplifications used touchdown PCR, following the protocol: 95°C for 2 min., stage 1 [95°C for 30 sec., 58°C for 30 sec., 72°C for 1min] for 10 cycles, stage 2 [95°C for 30 sec, 58°C to 48°C for 30 sec, 72°C for 1 min] over 20 cycles, decreasing the annealing temperature by 0.5° at each step, stage 3 [95°C for 30 sec., 48° C for 30 sec, 72°C for 1 min] for 5 cycles. PCR

products were present in either single or double bands indicating two differing length products in some species. Because the functional EF1- α copy was expected to be 914bp in length, only the longer of two products was removed from the agarose gel for cloning.

All PCR products were cloned prior to sequencing. PCR products were gel purified using the QIAquick gel extraction kit protocol before use in ligation reactions. Amplicons were inserted into the pCR® 2.1-TOPO® plasmid, and transformed into competent cells, following the protocol of the TOPO TA cloning kit (Invitrogen). Transformations included two controls, one with a supercoiled plasmid to test the efficacy of the transformation, and a second with dH_2O to test for possible contamination. Competent cells were spread on LB/ampicillin plates, and incubated overnight at 37°C. From 4 to 8 colonies from each cloning reaction were picked for overnight incubation (LB/ampicillin, growth phase). Plasmid DNA was extracted following a lysis by boiling preparation, as described in Maniatis et. al. (1989). An EcoRI restriction digest was used to check the sequence length of plasmid inserts. In some cases, plasmid DNA extracts were sequenced directly; in others, sequence inserts in plasmid DNA were PCR amplified before product purification and sequencing.

In order to determine which of the EF1-α copies corresponded to functional genes, expressed gene copies were compared to genomic DNA copies from single individuals. RNA/DNA extractions were completed on 7 individuals of five Eucalanid species. Following total RNA and DNA extractions from adult females,

cDNA was synthesized from EF1-α transcripts and compared to genomic DNA copies from the same individual. Tissue of a single frozen adult female was homogenized by bead beating, using 0.1mm zirconia/silica beads (www.Biospec.com), 40µl TRI Reagent® (Sigma), and beating for 20 seconds at 4.5 m/s. Homogenate was then stored on ice for 5 minutes to permit complete dissolution of nucleoprotein complexes. During the phase separation step, 8µl chloroform was added to the homogenate, samples were shaken for 15 seconds, and stored at room temperature for 5 minutes before centrifugation at maximum speed for 15 minutes (at 4°C).

For RNA precipitation, 20µl aeqeous phase was transferred to a 0.5 mL tube containing 20µl isopropanol. Samples were stored at room temperature for 10 minutes before centrifugation at 13,200 rpm for 10 minutes (room temperature). The RNA pellet was washed with 100µl of 75% ethanol (dilute with DEPC H_2O), centrifuged at 13,200 rpm for 5 minutes, before aspiration and drying at room temperature for 10 minutes. The pellet was resuspended in 20µl DEPC H_2O, and incubated at 60°C for 10 minutes. RNA was then treated with Dnase, using DNA-free™ (Ambion) and following manufacturer's protocols, to remove any contaminating DNA prior to cDNA synthesis. First strand synthesis of cDNA was completed using Superscript™ II RT (Invitrogen), following the manufacturer's protocols. The primer M44-1 was used as a gene specific primer in first strand cDNA synthesis.

Genomic DNA was isolated from the interphase and phenol phase separated from the initial sample homogenate. Organic and interphase were transferred to a new tube and 12µl 95% ethanol was added for DNA precipitation. Samples were stored at

room temperature for 3 minutes prior to centrifugation at 2,000 X g for 5 minutes (room temperature). The supernatant was removed and the DNA pellet was washed twice in 40µl 0.1 M sodium citrate and 10 % ethanol, with 30 minutes of storage time at each wash. DNA was washed in 75% ethanol (60 µl) for 15 minutes before centrifugation (10,200 rpm, 5 minutes), removal of the supernatant, and drying of the pellet. The DNA was resuspended in 20µl 8mM NaOH and 1.3µl 0.1 M HEPES. DNA, RNA, and cDNA samples were PCR amplified, using primers noted above. DNA and cDNA amplifications were sequenced directly; RNA amplifications were used to verify the efficacy of the Dnase treatment step, and the absence of any genomic DNA contamination prior to cDNA synthesis.

All DNA sequencing reactions were carried out on a MegaBACE 500 in 10µl volume, following recommended cycling protocols [30 cycles, 95°C 20 sec., 50°C 15 sec, 60°C 1 min]. M13 forward and reverse primers as well as the T7 plasmid primer were used in sequencing reactions, and clones were sequenced on both strands.

Both sequence strands were aligned, checked for errors, and combined into a consensus sequence using Sequencher 4.2 software (Gene Codes Corp.), before multiple sequence alignment of all clones in ClustalW (Thompson et al. 1994) and manual editing as necessary in MacClade (Maddison and Maddison 2000). The identity of all sequences was confirmed by Blast searching GenBank. DNA sequences were translated to protein in the software program DnaSP (Rozas et al. 2003), and verified against complete EF1-alpha sequences in Genbank. All phylogenetic inference was completed in PAUP* 4.0b 10. Model selection by AIC was performed

in Modeltest (Posada and Crandall 1998), and the symmetrical model with a proportion of invariant sites of 0.1758 and gamma corrected rate heterogeneity across sites (shape parameter = 0.5659) was selected. All phylogenetic inference and distance calculations used this model of molecular evolution.

Results

A total of 840 bp (280 amino acids) of sequence was obtained for the elongation factor 1- α gene from expressed cDNA copies of four eucalanid species (Table 1). This included sequence data for two species in the genus *Eucalanus*, one species in *Rhincalanus*, and one species in *Pareucalanus* (Table 1). Sequences obtained here included the central region of the gene, and lack 225 and 264 bases, respectively, from the 5' and 3' ends of the coding region of the mRNA transcript. Comparisons of cDNA and functional genomic DNA copies of EF1- α indicate that the number and location of introns varies across genera in the family. One intron was shared across *Eucalanus*, *Rhincalanus*, and *Pareucalanus* genera, with one additional unshared intron present in both *Rhincalanus* and *Eucalanus* (Fig. 1). The shared intron occurs in the same location as the conserved intron found in other arthropods at position 753bp from the start codon (including only coding regions, Fig. 1, Brady and Danforth 2004), and varies in length between 156 and 143 bp. The *Eucalanus*-specific intron begins at position 636 (Fig. 1, coding regions only), and is unique among previously studied arthropods. This intron was observed to be 56 and 50 bp in length for *E. hyalinus* and *E. bungii*, respectively. The *Rhincalanus*-specific intron, also

unique among previously studied arthropods, starts at position 904 (Fig. 1) and is 47 bp in length in *R. nasutus*.

Conserved sequence motifs mark the intron-exon boundary of all three introns, as well as across introns of all three genera. The 5' splicing junction of the *Eucalanus*-specific intron has the sequence A|AGGTAKWW, the shared intron C|AGGTRWWD, and the *Rhincalanus*-specific intron has G|AGGTATGA, all of which are similar in sequence to the consensus splice signal sequences for other arthropods (Mount et al. 1992; Weir and Rice 2004), and to other copepods in particular (Rawson et al. 2000). The position of the splicing site, however, is unusual, and appears to be shifted towards the 5' end of the molecule by two nucleotide sites relative to other organisms. The eucalanid 3' splicing site is also highly similar to consensus sequences for other organisms, and across all introns and genera has the sequence H|AGG. The position of the splicing site at the 3' end of the intron is also unusually shifted two nucleotide sites towards the 5' end of the molecule relative to other organisms.

Combined results from genomic DNA amplifications of 16 species, and cDNA amplifications from four species indicated that there are at least two functional copies of EF1- α in the Eucalanidae, in addition to a number of apparently non-functional, pseudogene copies (Fig. 2). Although only a single expressed gene copy was observed in *E. californicus* (5 clones), and two allelic copies were observed in *R. nasutus* (0.1% divergent, SYM+I+G corrected, 10 clones), both *E. hyalinus* and *P.* sp. were found to express more than two gene variants within an individual. These gene

copies differed by between 3.9 and 26.0 % sequence divergence (SYM+I+G corrected, Fig. 2.) It is currently unclear whether these gene copies represent alleles of two functional loci, or if more than two functional gene loci are present in the family. Sequences obtained from *P.* sp., in particular, seem to suggest that three gene loci may be involved, with cDNA copies C/D, A, and B (Fig. 2) all characterized by quite high levels of genetic divergence from one another (C/D – A: 17.1-21.4%, C/D – B: 25.2-26.0%, and B - A: 14%, SYM+I+G corrected). Although the presence of multiple, functional gene copies may not be unexpected, given similar prior observations in other marine crustaceans (France et al. 1999; Williams et al. 2001), the phylogenetic relationship between eucalanid functional gene loci demonstrates a genetic similarity not found in other organisms. As observed in figure 2, both functional and non-functional gene copies were inferred to cluster within each genus in the phylogenetic analysis. For example, all *Pareucalanus* functional and non-functional gene sequences were inferred to belong to a monophyletic clade with bootstrap support values of 98 and 96% for maximum parsimony (MP) and maximum likelihood (ML) analysis, respectively. Similar results were observed for functional and non-functional loci in the genus *Eucalanus* (88% support, ML and MP). Results for *Rhincalanus* and *Subeucalanus* were less well resolved.

Phylogenetic analysis including only known functional and putatively functional gene copies revealed that EF1- α successfully resolves relationships among species and genera with high resolution for some nodes (Fig. 3). In this analysis, gene copies were considered putatively functional if the number and length of introns

appeared to match functional gene copies for closely related species, and if no indels/frameshift mutations were identified in the exon sequence. In the genus *Eucalanus*, species *E. californicus* and *E. bungii* were found to be a sister species pair with bootstrap support of 99/98% (MP/ML), in congruence with results for other gene loci (Goetze, 2003). This sister species pair is 8.7% divergent at EF1- α, with 11 amino acid substitutions between species. Phylogenetic results for other species in the genus *Eucalanus* were also congruent with prior studies, although bootstrap support for monophyly of the genus was 77/72% (Fig. 3), somewhat lower than found in other gene loci (100%, Goetze, 2003). The species *Pareucalanus attenuatus* and *P. sewelli* were also found to be a sister species pair, although bootstrap support was only 65/67% (Fig. 3; 70/68% support for this node at 16S rRNA+ITS2). This sister species pair differed by 4.1% sequence divergence, and 8 amino acid substitutions. The position of *Pareucalanus* sp. was basal to this node, as found for other loci (Goetze, 2003). Monophyly of the genera *Subeucalanus* and *Pareucalanus* was well supported for EF1- α, with bootstrap support of 99/99% and 91/88%, respectively (Fig. 3). Relationships within *Rhincalanus* were poorly resolved, though it is uncertain whether gene sequences included for *R. rostrifrons* and *R. gigas* represent true functional copies. The outgroup taxon *Eurytemora affinis* had 90.4 to 93.6% amino acid identities with functional and putatively functional eucalanid gene copies.

Non-functional gene copies varied in the extent to which they were readily recognizable as pseudogenes. In some cases, non-functional gene loci lacked one or both introns that are expected to be present in functional genomic copies (Fig. 2).

Pseudogenes were also identifable in some cases by the presence of frameshift insertions or deletions within exons that would make the gene non-functional following translation (Fig. 2). However, many DNA sequences identified as putatively non-functional here were only distinguishable by the length of the central, shared intron (Fig. 2). In all observed functional sequences, the central intron was found to be >55bp in length. No genomic DNA sequences with introns shorter than 55bp were found in cDNA amplifications. However, exon sequence for these non-functional gene copies was often genetically very similar to functional genes, and would not otherwise be identifiable as non-functional. These sequences also had the expected number of introns, which were located in the same position as functional gene copies. Therefore, although the absence of introns appears to be conclusive evidence that a gene copy is non-functional, presence of the correct number and position of introns is insufficient evidence on which to conclude that the sequence is functional. Finally, it was observed that pseudogenes from within one genus were never found to have the intron structure of a different genus. For example, all *Pareucalanus* non-functional gene copies lacked introns in the *Rhincalanus*- and *Eucalanus*-specific positions.

Discussion

Two Recent Intron Gains?

Two unique spliceosomal introns in nuclear EF1- α were identified in the eucalanid genera *Rhincalanus* and *Eucalanus*. Although data for expressed gene

copies were obtained for multiple species only within the genus *Eucalanus*, it is hypothesized that these intron positions are conserved across species within the *Rhincalanus* and *Eucalanus* genera. Putatively functional gene sequences and non-functional gene copies obtained for other species in these genera from genomic DNA amplifications also contain these unique intron positions. These introns were also observed to be absent from all functional and non-functional gene sequences from species in other genera. A review of the literature of EF1- α intron positions for other arthropods finds that these introns are unique among well-characterized arthropods (Danforth and Ji 1998; Jordal 2002; Brady and Danforth 2004). The unique position of these introns cannot be explained by intron sliding, as both introns occur much farther than 5-10 bp away from any known intron positions. In addition to the unique introns, one central intron was found to occur across all genera in the Eucalanidae. Although only *Pareucalanus*, *Eucalanus*, and *Rhincalanus* are included in figure 1, putatively functional gene copies obtained from *Subeucalanus longiceps* and *S. crassus* suggest that the *Subeucalanus* genus also shares this intron position, and has the same intron structure as is found in the genus *Pareucalanus*. This shared, central intron also occurs in functional gene copies of beetles and bees (Danforth and Ji 1998; Jordal 2002; Brady and Danforth 2004), which implies that the presence of the intron may be ancestral for the Mandibulata, with intron loss in some groups.

The presence of two unique introns within the family can be explained by either intron gain or loss. Figure 4 illustrates that two independent intron gains is a more parsimonious explanation for the pattern observed than four intron losses within

the family. Additionally, the phylogenetic pattern observed here is identical to that used as 'strict' criteria for clear examples of recent intron gain in studies examining the mechanisms of intron insertion in model organisms (Logsdon 2004; Coghlan and Wofe 2004). Therefore, it appears probable that both introns were independently and recently acquired within the family Eucalanidae. Additional data from outgroups in the Calanoida, which lack these introns, would strengthen support for this conclusion. Examples of recent intron gain remain relatively few in the literature (Federova and Federova 2003), and the gains proposed here likely occurred more recently than many of the previous examples(<100 mya, Logsdon 2004). Given the recent origin of the *Rhincalanus* and *Eucalanus* introns, it may still be possible to identify their genomic progenitors based on sequence similarity to other regions of the genome.

The molecular evolution of intron gain in EF1- α in the Eucalanidae matches some expectations from studies of model organisms. An examination of the insertion sites for the newly acquired introns reveals that one of the two, the *Rhincalanus*-specific intron, appears to have been inserted at a 'proto-splice site', where exon sequence closely matches the preferred MAG|R insertion sequence (eucalanids: CHG|A, bar marks location of intron insertion, Logsdon 2004; Qiu et al. 2004). The *Eucalanus*-specific intron was inserted at exon sequence of ACA|A, which is less clearly identifiable as a preferred insertion site. Also, all three eucalanid EF1- α introns are phase 1 introns, in congruence with the common observation of phase autocorrelation within intron-containing genes (Qiu et al. 2004).

Finally, it has been noted that insects appear to have a particularly high diversity of spliceosomal introns, with vertebrate genomes predominantly characterized by evolutionary stasis in intron structure (Logsdon et al. 1998). Results presented here suggest that the evolutionary plasticity in intron structure of insect genomes may also occur more broadly in non-model arthropod classes.

Phylogenetic utility of Elongation Factor 1-α

The original goal of this study was to test and develop nuclear EF1- α as a phylogenetic marker for alpha-level systematics in calanoid copepods. Results presented here demonstrate that although EF1- α has an appropriate level of resolution for inferring relationships among closely related species (Fig. 3), phylogenetic inference will be severely impeded by the difficulty in identifying orthologous gene sequences (Fig. 2). The presence of many pseudogene loci, which may be little differentiated in exon sequence from functional gene copies, will result in PCR amplification of both functional and non-functional genes. The common approach of identifying orthologous loci by selecting gene sequences with homologous intron structure was found here to be an insufficient criterion for identifying functional gene copies. Many putatively non-functional gene copies contained the correct number and position of introns, but had an unusually short central intron. This feature was not observed in gene copies found expressed in total RNA of any eucalanid species. I conclude that distinguishing functional and non-functional gene copies from genomic amplifications is extremely difficult, if not impossible, to do accurately, and any

convincing phylogenetic study using this gene must amplify directly from expressed gene copies (RNA/cDNA). The presence of multiple pseudogene loci, which are poorly differentiated from functional gene copies, in the very distantly related taxa of *Alpheus* shrimp (Williams et. al. 2001) and eucalanid copepods suggests that EF1- α pseudogenes may well be present throughout much of the Crustacea.

In addition to the problem of pseudogenes, there is the added complication that although the multiple, functional gene loci found here demonstrate approximately the same level of genetic differentiation from one another as is found in multiple copies of other organisms (10-25% divergence), this level of differentiation is usually less than that observed between genera within the family (19 to 47%). This pattern resulted in the robust inference of multiple gene copies within monophyletic generic clades, but not necessarily as sister gene sequences (see Fig. 3). In order to accurately resolve species relationships within genera, it will therefore be necessary to obtain gene sequences of all functional loci for all species. This presents an added burden in cost and time to the systematic practitioner. However, this observation also suggests that phylogenetic inference including any of the gene copies would recover the true phylogenetic tree in studies at the generic level or higher.

In summary, although EF1- α appears to have undergone interesting evolution in intron structure within the family Eucalanidae, it is not an appealing candidate as a protein-coding nuclear gene for molecular systematic studies at the alpha-level in marine calanoid copepods. Future work will be needed to develop additional nuclear gene loci for accurate phylogenetic inference.

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Table 1: Geographic sources for specimens analyzed, nucleic acids extracted, and number of cDNA and DNA clones sequenced. Abbreviation for locations: ETP=Eastern Tropical Pacific, SW = southwest.

Species	Extraction	cDNA	DNA	Source
Eucalanus hyalinus	DNA, RNA	5	8	California Current [32.51° N, 117.16° W]
Eucalanus spinifer	DNA	-	5	South Pacific [34.02° S, 140.03° W]
Eucalanus inermis	DNA	-	3	Eastern Tropical Pacific [12.49° N, 141.59° W]
Eucalanus elongatus	DNA	-	9	Sulu Sea [7.00° N, 120.00° E]
Eucalanus californicus	RNA	5	0	California Current [32.51° N, 117.16° W]
Eucalanus bungii	DNA	-	11	Subarctic Pacific [56.42° N, 145.85° W]
Pareucalanus sewelli	DNA, RNA		4	Aegean Sea [38.47° N, 25.05° E]
Pareucalanus sp.	DNA, RNA	9	14	SW Pacific Ocean [23.89° S, 177.10° W; 23.52° S, 175.70° W; 25.79° S, 174.86° W]
Pareucalanus parki	DNA	-	5	California Current [34.23° N, 122.15° W]
Pareucalanus attenuatus	DNA	-	11	SW Pacific, ETP [20.52° S, 179.29° W; 4.44° S, 124.33° W]
Rhincalanus gigas	DNA	-	7	Southern Ocean [66.24° S, 171.00° W]
Rhincalanus rostrifrons	DNA	-	4	SW Pacific Ocean [13.29° S, 171.72° W]
Rhincalanus nasutus	DNA, RNA	10	5	California Current [32.51° N, 117.16° W]
Subeucalanus pileatus	DNA	-	7	North Atlantic [10.53° N, 56.54° W]
Subeucalanus longiceps	DNA	-	4	South Pacific [41.24° S, 175.40° W]
Subeucalanus crassus	DNA	-	5	SW Pacific Ocean [31.08° S, 177.09° W]
Subeucalanus subcrassus	DNA	-	1	SW Pacific Ocean [20.52° S, 179.29° W]

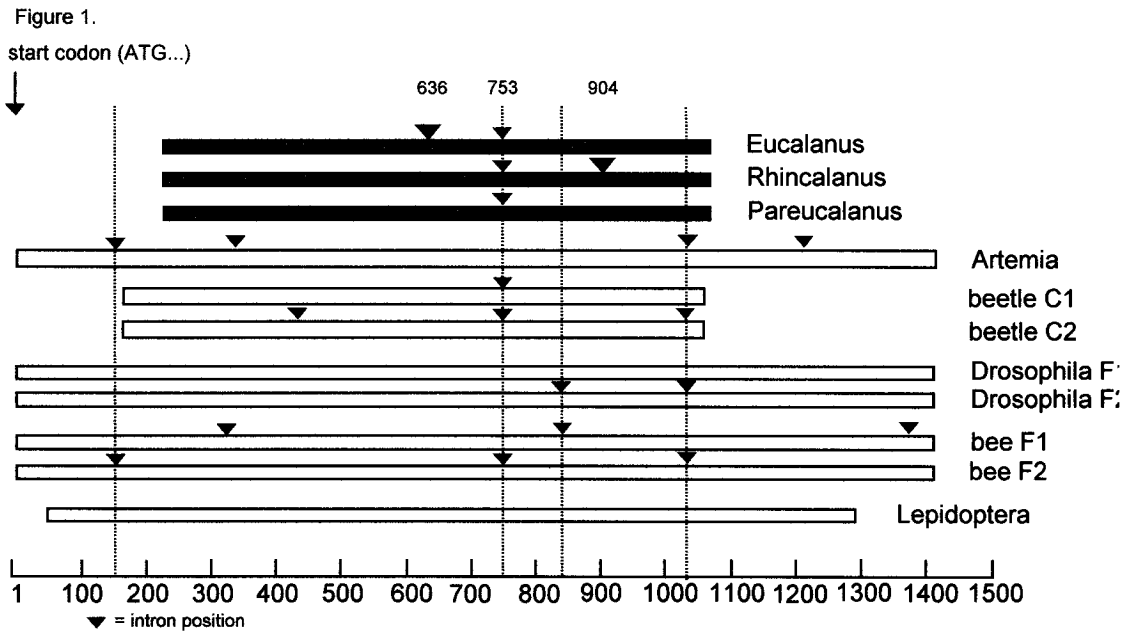

Figure 1. Intron positions in arthropod elongation factor 1-α gene loci, modified from Brady and Danforth (2004). Only coding regions are shown. Eucalanid copepods share one intron with other arthropod taxa at position 753 in addition to two unshared introns, which are specific to the genera *Eucalanus* and *Rhincalanus* (located at positions 636 and 904, respectively). Genus-specific introns marked by red triangles, with the shared intron indicated by a solid black triangle. Paralogous functional gene copies in *Drosophila*, *Apis* bees, and curculionid beetles are labeled as F1/F2 or C1/C2 respectively. Results from cDNA and genomic DNA clones from *Eucalanus hyalinus* A, *Rhincalanus nasutus* A, and *Pareucalanus* sp. A are included.

Figure 2. Maximum parsimony phylogram of unique elongation factor 1-α gene sequences obtained from genomic DNA and cDNA amplifications. cDNA gene copies are highlighted in red, all copies from genomic amplifications are in black. All sequences are labeled by species name. Genomic DNA copies that contain an indel(s), all of which result in a frameshift, are identified by green circles. Sequences lacking 1 or 2 introns are marked with 1 or 2 black stars, respectively. Genomic DNA copies are identified as either putatively functional (F), putatively non-functional (NF) or unknown (?), based on features of the gene sequence including the presence/absence of indels and introns, as well as the length of the central, shared intron. Numbers above branches are Maximum parsimony (MP)/Maximum likelihood (ML) bootstrap support values (%) for each node. Tree topology from the MP and ML bootstrap consensus tree (identical results).

Figure 2.

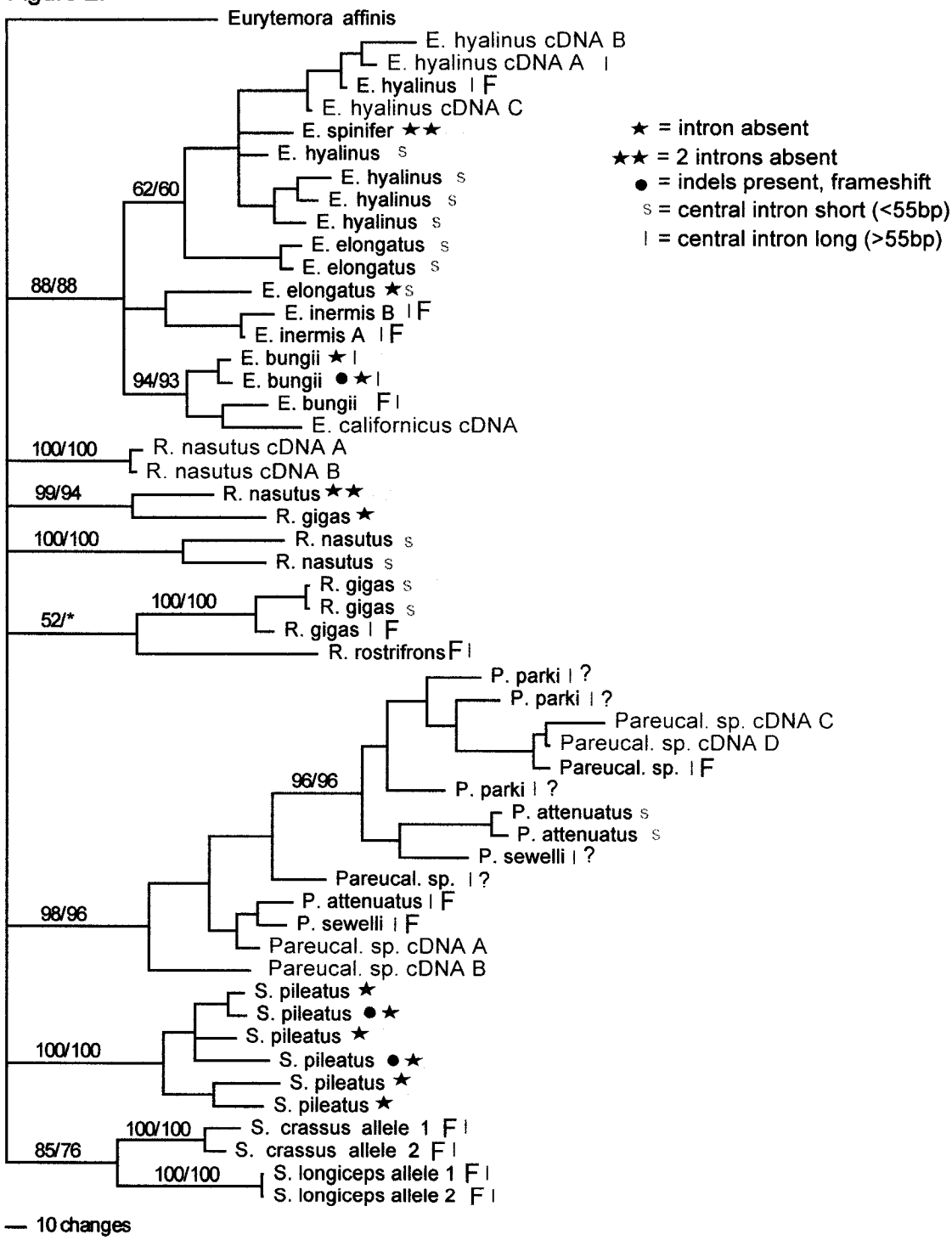

Figure 3.

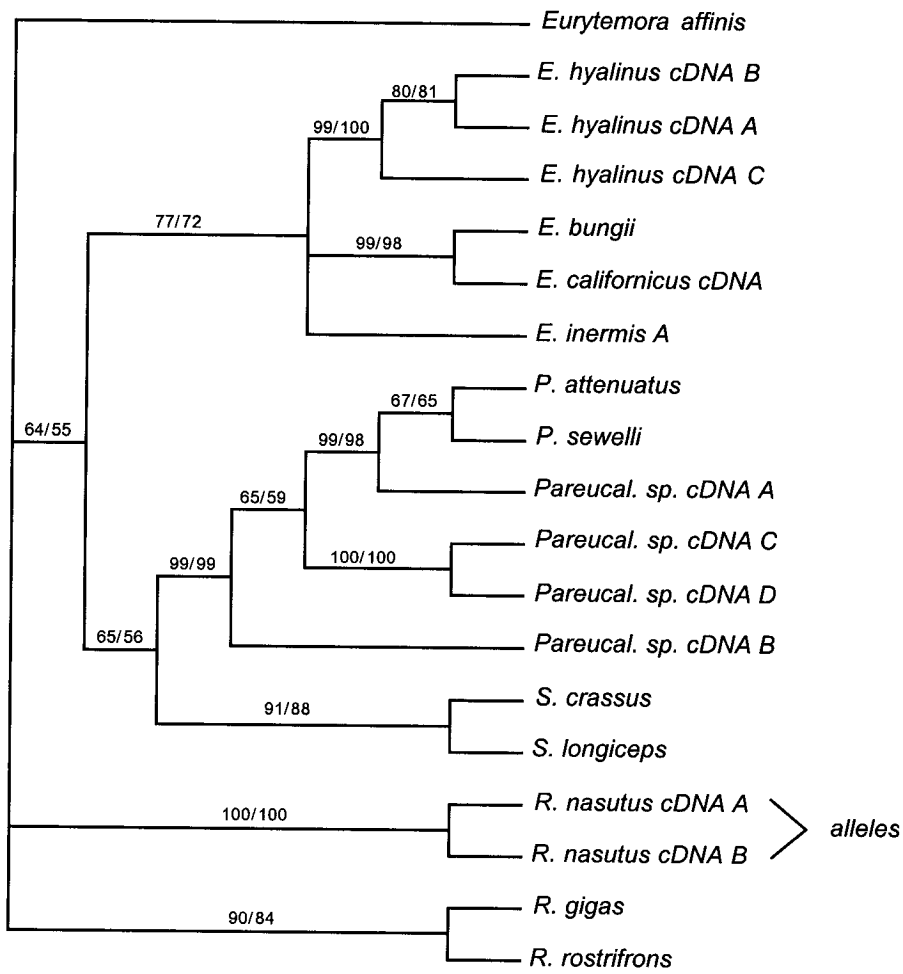

Figure 3. Maximum likelihood phylogeny of functional and putatively functional gene copies for species in the Eucalanidae. Numbers above branches are MP/ML bootstrap support values (%) for each node.

Figure 4.

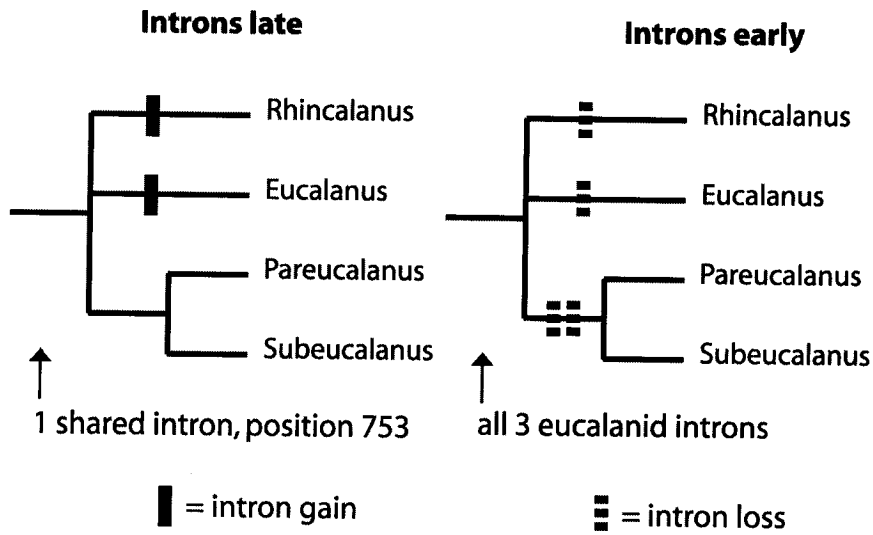

Figure 4. Character state transitions for intron positions in the Eucalanidae under 'Introns early' and 'Introns late' hypotheses. Small arrows (with text) indicate the ancestral character state in each case, with intron gains denoted by solid bars, and intron losses indicated by dashed bars. The shared intron at position 753 is assumed to predate the ancestor of the Eucalanidae, given the pattern of orthologous introns at this position across diverse arthropod taxa. Phylogeny based on 18S rRNA results (Goetze 2003).

Chapter VI

Conclusions and Comments

The long-term goal of the work presented here was to elucidate the biogeographic, oceanographic, and morphological factors that promote population divergence and speciation in open ocean zooplankton. Understanding the process of speciation in the sea has long been a goal of biological oceanographers and plankton ecologists (e.g., McGowan 1963; Fleminger 1967; Pierrot-Bults and Van der Spoel 1979). The research presented in the preceding chapters revisits this long-standing question with a suite of tools including molecular phylogenetics and population genetics, as well as large-scale oceanography and biogeography. The dissertation makes new contributions in at least two areas of plankton evolution.

The first major contribution is in the development and integration of molecular and morphological approaches to the phylogenetics and systematics of zooplanktonic organisms (**Chapters II, III**). One of the main objectives of the early phase of the research was to develop a model system in which the molecular phylogeny of the family had been well resolved, and all cryptic species had been identified. During the execution of this research molecular markers revealed the presence of twelve previously unrecognized eucalanid genetic lineages that were evolutionarily distinct from other populations with which they were previously conspecific. These results highlight the importance of validating species boundaries with molecular markers in studies of morphologically conservative planktonic organisms. Research on other

oceanic zooplankton has also identified cryptic taxa (Bucklin et al. 1996; de Vargas et al. 1999; de Vargas et al. 2001; Dawson and Jacobs, 2001; de Vargas et al. 2002; Saez et. al. 2003; de Vargas et al. 2004; Peijnenburg et al. 2004), suggesting that the phenomenon may be widespread, with current species diversity estimates 2-4 times lower than actual numbers for well-studied portions of the holozooplankton fauna.

Complete and accurate knowledge of the historical pattern of speciation of the family is a necessary foundation to understanding the factors that promote speciation in open ocean environments. **Chapter II** laid these foundations for the larger goals of the research. All of the cryptic eucalanid lineages identified represent previously unknown lineage splitting events, some of which clearly are speciation events. However, research reported in **Chapter II** was not globally complete, and additional sampling has been conducted since publication of that chapter (Fig. 1). In ongoing research I seek to establish whether the diversity described in **Chapter II** includes all cryptic lineages present globally in the family, and to document the biogeographic distributions of undescribed genetic lineages. One additional cryptic genetic lineage has been identified in the *Rhincalanus nasutus* cryptic species complex (Fig. 2). This genetic lineage is present in both the Gulf of Aqaba in the Red Sea, as well as in the Agulhas Current on the eastern coast of Southern Africa, suggesting that the genetic type may be distributed semi-continuously through the Red Sea and along the eastern coast of Africa. Additional sampling has also improved knowledge of the biogeographic distributions of other cryptic genetic lineages (Appendix B).

Considerable research remains to be done before one can say with certainty that the specific diversity of the family Eucalanidae has been fully described.

Morphologically cryptic, sibling species that are originally discovered through molecular markers may prove difficult or impossible to distinguish by morphological characters. Research presented in **Chapter III** examines the morphological divergence of a pair of cryptic sister species originally identified in research conducted for **Chapter II**. Two genetic forms were found to be morphologically distinguishable in the adult female by characters involving the shape of the anterior portion of the head, asymmetry of the antennules, length ratios along the antennules, and aspects of body size, including total length, prosome length, and lengths of the asymmetrical caudal rami. An old species name, *Eucalanus spinifer* T. Scott 1894, was removed from synonymy with *E. hyalinus* and applied to the smaller of the two genetic forms.

This chapter presents one of the first examples of a combined genetic and morphological species description in planktonic organisms, and hopefully demonstrates one way in which to more fully incorporate genetic data beyond inclusion of a few type sequences as part of a DNA barcoding exercise. Genetic data may revolutionize the field of taxonomy (Hebert et al. 2002; Tautz et al. 2003), as has been suggested, but it will not do so simply by serving as DNA 'type sequences' for reference specimens (Wheeler 2004). This approach fails to utilize the full power of genetic data to examine species boundaries and the extent of reproductive isolation between forms. The conceptually rich approach will use genetic data to systematically test hypotheses about species status, not merely as species identifiers or for inference

in higher level phylogenetics. Yet few examples are available in the literature as to how such an approach can be best developed and employed. Research presented in **Chapter III** provides one example, and I look forward to watching the field expand with a greater diversity of ideas for how best to merge genetic and morphological data in the practice of taxonomy.

Preliminary research on morphological divergence in the *Rhincalanus nasutus* species complex also suggests some morphological characters that may discriminate the different genetic forms. Although this work is far from being complete, I include it here because the results support the conclusion that cryptic genetic lineages will be identifiable by morphological characters in many cases once their presence is known. Initial work on integumental pore signatures of the California Current, Southwest Pacific, Kuroshio Current/Philippine Sea, and Sulu Sea populations finds that the presence, number, and shape of pores on the dorsal surface of the prosome may be diagnostic for different populations (Table 1, Fig. 3). The presence and level of development of spines on the dorsal and lateral posterior surface of the prosome segments also differs between populations (variability noted by Tanaka 1956a, Lang 1965, Bradford-Grieve 1994), and may be useful as lineage-specific characters. In sum, it appears likely that in many cases cryptic genetic lineages will be identifiable and describable by morphological criteria.

The second major contribution of the dissertation is in elucidating the large-scale patterns of genetic structure in conspecific oceanic zooplankton populations. Many oceanic zooplankton species have circumglobal biogeographic distributions, yet

little is currently known about genetic connectivity between disjunct populations throughout their global biogeographic range. Research presented in **Chapter IV** is one of the first global attempts to describe the genetic structure of oceanic marine holozooplanton, and examine the evidence for barriers to gene flow in open ocean environments. Results demonstrate that zooplankton species can be highly genetically structured on macrogeographic spatial scales, despite substantial gene flow within subtropical gyre systems. Habitat discontinuities at central water mass boundaries and continental landmasses were observed to act as effective barriers to gene flow in both *Eucalanus hyalinus* and *E. spinifer*. Species-specific differences in ecology between the sister species *E. hyalinus* and *E. spinifer* appeared to control patterns of gene flow between populations of each species worldwide. Results presented in **Chapter IV** provide some of the first data that demonstrate the presence and importance of barriers to gene flow for the development and maintenance of genetic differentiation of oceanic plankton populations. The next logical expansion from work presented in **Chapter IV** would be to include data from multiple, independently inherited molecular markers, in order to be able to examine the historical demography and timing of divergence of these holozooplankton populations.

Research in **Chapter VI** is primarily methodological, and was aimed at the development of new protein-coding nuclear genes for phylogenetic inference in calanoid copepods. The work does not bear on the problem of speciation in the sea, though it does provide useful information for other researchers interested in addressing this problem. Although the protein-coding nuclear gene elongation factor 1-α

successfully resolved many relationships among species within the Eucalanidae, difficulty in identifying orthologous gene copies make the gene unsuitable for phylogenetic studies. The gene was present in multiple functional and non-functional pseduogene copies. In addition, two unique spliceosomal introns were discovered within the genera *Rhincalanus* and *Eucalanus*, which have not been found in other arthropod taxa.

Research included in this dissertation is but a small fraction of the work that is required to fully understand the process of speciation in marine holozooplankton. In fact, much of the included research merely lays the necessary foundations for an intellectually focused effort on testing hypotheses about speciation in open ocean environments. This fact highlights the poverty of our current state of knowledge of the basic biology, taxonomy, biogeography, morphology, autecology, phylogeny, and population genetics of oceanic holozooplankton. The Eucalanidae are now close to being a good model system in which to examine the process of speciation. Research directions outlined below illustrate the questions that can now be fruitfully addressed in this system.

Future Research Directions

Firstly, there were two major objectives of the dissertation, which have not yet been fully accomplished. Following development of a species-level, taxonomically complete phylogeny of the family, I intended to test two hypotheses regarding the

degree of biogeographic range overlap and the pattern of morphological divergence between sister species pairs. The original null hypotheses were:

$H_0$1 = *There is no correlation between the extent of biogeographic range overlap and the level of genetic divergence between pairs of sister species.*

$H_0$2 = *There is no correlation between the level of genetic divergence between sister species pairs and the extent of divergence in morphological characters likely to be important in either ecological niche specialization or reproductive islolation.*

Testing these hypotheses was to address the following questions: (1) Does biogeographic range overlap between sister species evolve as a function of time since the speciation event? (2) Under what biogeographic circumstances does speciation take place? (3) Are ecological or reproductive characters more important in the speciation process? and (4) Do morphological characters continue to evolve after speciation?

However, the observation of potentially large numbers of cryptic lineages presents severe problems for interpretation of previously described biogeographic distributions for species in the family Eucalanidae (Lang 1965; Lang 1967; Fleminger 1973; Fleminger and Hulsemann 1973). Some previously described distributions remain accurate, others include records from multiple sibling lineages. Ongoing work (see above) describing the biogeographic distributions of newly discovered cryptic genetic lineages will enable the testing of hypothesis **$H_0$1** above. This work will also identify any remaining cryptic genetic lineages, providing greater confidence that the specific diversity of the family has been adequately described. Once this work has been completed, and it is certain that the specific diversity of the family has been well characterized, it will be possible to test **$H_0$2** above. Many of the cryptic lineages

identified are recently divergent lineages, and will be important taxa to include in testing $\mathbf{H_o2}$ above. It would be possible to test $\mathbf{H_o2}$ even in the absence of knowledge about morphological characters by which to discriminate closely related lineages, as the morphological characters that would need to be examined would be standardized across all taxa in the study.

Regarding the larger question of what further research needs to be done to understand the process of speciation in oceanic holozooplankton, the possible research paths are many. Research in this dissertation focused primarily on genetic divergence in allopatry, although testing $\mathbf{H_o1}$ above would have been more inclusive of other modes. However, many researchers believe sympatric and parapatric speciation to be important, if not dominant, modes of speciation in pelagic systems (e.g., Pierrot-Bults and Van Der Spoel 1979; Briggs 1999; Norris 2000; Norris and de Vargas 2000). How can we examine these alternative modes of speciation?

I expect, for a variety of reasons, that studying these alternative speciation modes will be more challenging (if not largely intractable) in holozooplankton populations than in terrestrial, or coastal marine benthic invertebrate systems. Many of the approaches used in studying sympatric and parapatric speciation will not be available to researchers studying planktonic organisms. First, speciation modes with selection playing a major role might be expected to proceed to completion rapidly, due to large population size and high levels of standing genetic diversity. As a result, we can expect to find few zooplanktonic examples of partially complete speciation events, which form some of the most compelling study systems for sympatric speciation in

freshwater aquatic ecosystems (e.g., sticklebacks, cichlids). Second, it is challenging to study the same biological population over a period of time longer than a few days, due to difficulties in tracking water parcels in the pelagic ocean. This will prevent direct analysis of the constancy of selection pressures through time, and patterns of population response to shifts in the selective environment or availability of ecological niches. Third, most truly oceanic zooplankton are difficult to culture in the laboratory. This means that it will be difficult to conduct mate preference or crossing experiments, and developing a mechanistic understanding of links between resource use and mating ecology may be nearly impossible. In sum, there are a number of factors that contribute to making the study of speciation more difficult for planktonic organisms, particularly for sympatric or ecological speciation modes, than in other organisms.

However, under both sympatric and parapatric speciation, selection will be a dominant evolutionary force. One future line of research that seems particularly fruitful is in attempting to understand the roles of natural and sexual selection in population divergence and speciation. Whether detected at the molecular level (selection on particular loci or alleles), the organismal level (differential mortality, mate selection, reproductive success), or the population or species level (selection in macoevolutionary time, shifts in alleles frequencies through time/space), a sophisticated understanding of how selection acts on plankton populations will be a necessary precursor to interpreting its role in the speciation process.

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Table 1. Summary of preliminary results for integumental pore analysis of the *Rhincalanus nasutus* cryptic species complex. R = right, L = left side of the body, for paired pore sets. Labels A1 = antennule, A2 = antenna, Mx2 = Maxilla, Mxpd = Maxilliped indicate the location of the pore set along the cephalosome. Number of pores in pore set, a = absent, s = single, d = double, t = triple, q = quadruple. ? = uncertain, and NS = not scored, usually due to difficulty in observing this part of the specimen. II, III, and IV indicate second, third, and fourth prosome segments, with #/10 indicating the number of individuals (out of 10) that have spines in this location. PL = prosome length, reported as mean (range). There may also be 'population'-specific lateral pores on the cephalosome near maxillae and maxillipeds.

		← Pores →			Spines →		Dorsal	
Population	N	A1/A2	Mx2	Mxpd	Dorsal	Lateral	Pore shape	PL (mm)
California	10	**L:** 10/10 s	**L:** 6/10 q, 3/10 t, 1/10 NS	**L:** 9/10 a, 1/10 s ?	**II:** 0/10 **III:** 8/10 **IV:** 10/10	**II:** 3/10 **III:** 10/10 **IV:** 10/10	round	3.53 (3.16-3.72)
		R: 10/10 s	**R:** 8/10 q, 1/10 t, 1/10 NS	**R:** 10/10 a				
Sulu Sea	10	**L:** 10/10 s	**L:** 9/10 q, 1/10 d	**L:** 9/10 s, 1/10 a	**II:** 5/10 **III:** 10/10 **IV:** 10/10	**II:** 8/10 **III:** 10/10 **IV:** 10/10	round/ intermediate?	3.59 (3.44-3.72)
		R: 10/10 s	**R:** 7/10 q, 3/10 d	**R:** 9/10 s 1/10 a				
SW Pacific	10	**L:** 9/10 s, 1/10 d	**L:** 10/10 d	**L:** 10/10 a	**II:** 8/10 **III:** 10/10 **IV:** 10/10	**II:** 10/10 **III:** 10/10 **IV:** 10/10	triangular	4.25 (4.00-4.72)
		R: 10/10 s	**R:** 9/10 d, 1/10 s	**R:** 10/10 a				
Kuroshio	10	**L:** 10/10 d	**L:** 9/10 d, 1/10 s	**L:** 9/10 a, 1/10 NS	**II:** 10/10 **III:** 10/10 **IV:** 10/10	**II:** 10/10 **III:** 10/10 **IV:** 10/10	intermediate	3.87 (3.40-4.28)
		R: 9/10 d, 1/10 NS	**R:** 9/10 d, 1/10 s	**R:** 10/10 a				

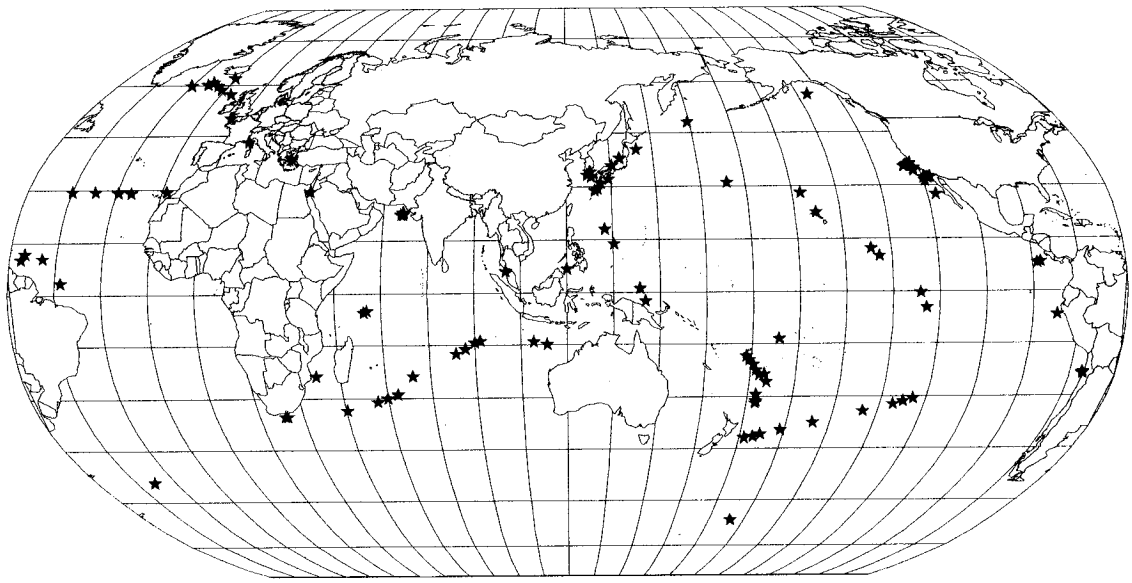


Figure 1. Extended sampling coverage since publication of Chapter II. Red stars mark locations from which additional specimens have had DNA extracted (not including *E. hyalinus* and *E. spinifer*) or been sequenced, black stars mark locations from the published study in Chapter II.

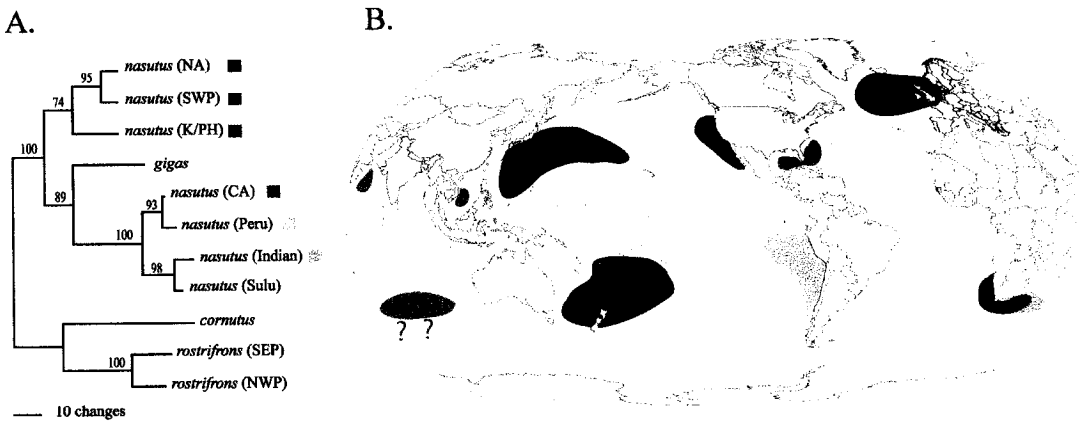


Figure 2. Ongoing results for genetic lineages in the *Rhincalanus nasutus* species complex. (A) Maximum parsimony phylogram, with bootstrap values above each node. (B) Geographic distribution of genetic lineages, with color-coding as in A. Dark grey indicates regions where *R. nasutus* is known to occur, but for which no specimens are currently available. Colored question marks indicate regions suspected to contain the lineage indicated by the color of the question mark, but from which no specimens are currently available. *R. nasutus* Indian Ocean and Red Sea lineage is 2.8% (uncorrected p-distance) divergent from the Sulu Sea population.

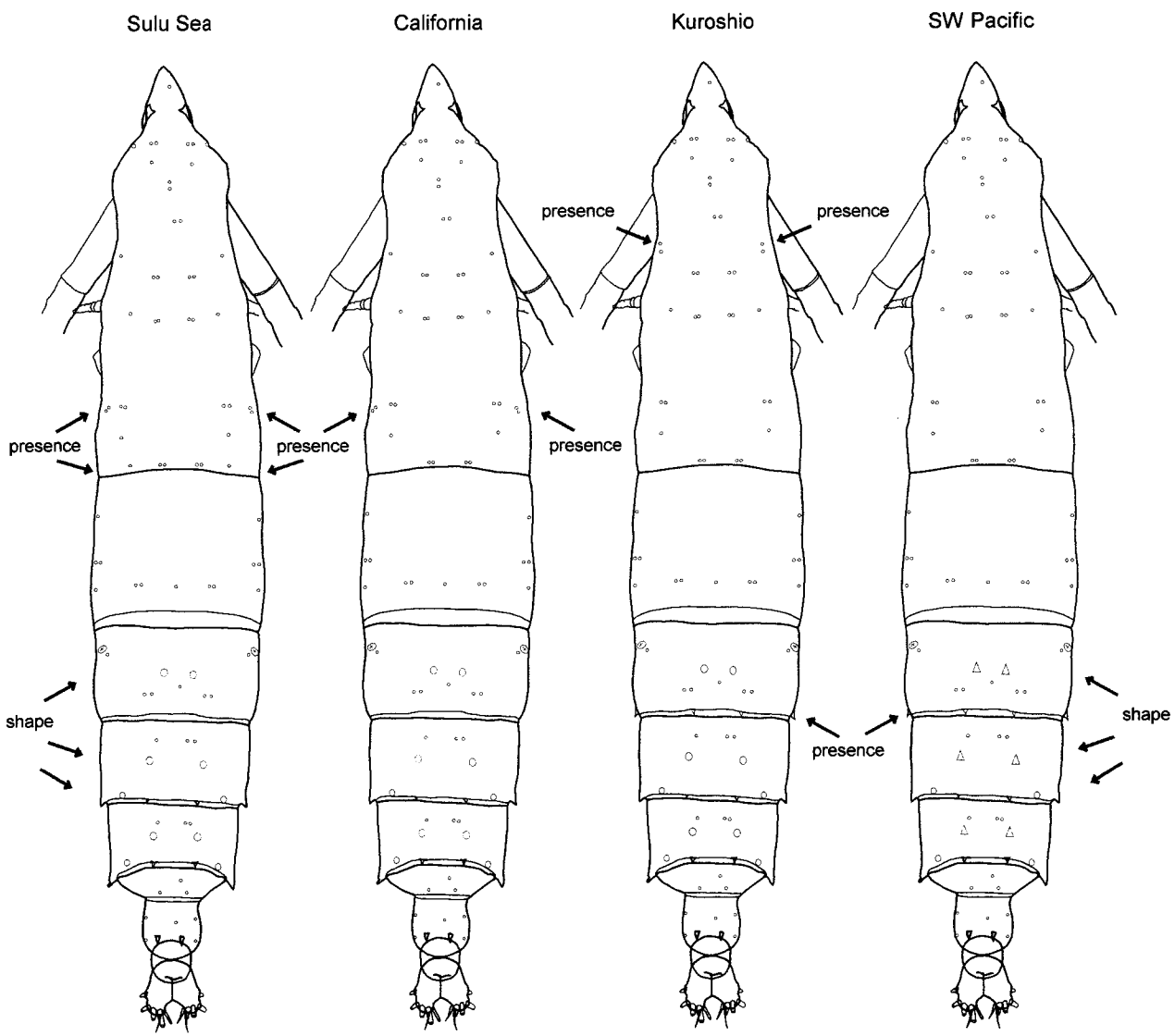


Figure 3. Preliminary results from integumental pore analysis of *Rhincalanus nasutus* genetic lineages. The genetic lineages appear to be distinguishable by the presence of unique pores or paired sets of pores, the presence and extent of development of spines on the lateral and dorsal posterior margin of prosome segments 2, 3 and 4, and by the shape of the paired large pores on the dorsal surface of prosome segements 2, 3, and 4. 'Population'-specific pores, pore shapes, and presence of spines indicated in red. Work completed by Crystal Grover.

Appendix A. Listing of ethanol preserved and frozen plankton samples acquired and used in the dissertation. Listed are: cruise name, station number, date collected, latitude, longitude, maximum depth of tow (m), mesh size of plankton net used (μm), preservative, collector, and region for samples in which no information on latitude, longitude is available. ETOH = 95% non-denatured ethyl alcohol.

Cruise	Station Number	Date	Latitude	Longitude	Depth	Mesh	Preservation	Collector	Region
VANC10MV	VANC10MV-01	5/16/2003	-35.05	23.73	566	333	ETOH	GOETZE	
VANC10MV	VANC10MV-02	5/16/2003	-35.07	24.50	920	333	ETOH	GOETZE	
VANC10MV	VANC10MV-03	5/18/2003	-34.83	32.04	947	333	ETOH	GOETZE	
VANC10MV	VANC10MV-04	5/19/2003	-34.67	34.76	947	333	ETOH	GOETZE	
VANC10MV	VANC10MV-05	5/20/2003	-34.36	37.73	950	333	ETOH	GOETZE	
VANC10MV	VANC10MV-06	5/20/2003	-34.05	40.51	910	333	ETOH	GOETZE	
VANC10MV	VANC10MV-07	5/21/2003	-33.30	45.36	950	333	ETOH	GOETZE	
VANC10MV	VANC10MV-08	5/22/2003	-32.85	47.74	1090	333	ETOH	GOETZE	
VANC10MV	VANC10MV-09	5/23/2003	-31.83	52.61	950	333	ETOH	GOETZE	
VANC10MV	VANC10MV-10	5/24/2003	-30.85	56.30	980	333	ETOH	GOETZE	
VANC10MV	VANC10MV-11	5/25/2003	-29.85	59.84	950	333	ETOH	GOETZE	
VANC10MV	VANC10MV-12	5/26/2003	-28.74	63.39	950	333	ETOH	GOETZE	
VANC10MV	VANC10MV-13	5/28/2003	-23.59	69.01	1030	333	ETOH	GOETZE	
VANC10MV	VANC10MV-14	5/29/2003	-22.09	72.74	950	333	ETOH	GOETZE	
VANC10MV	VANC10MV-15	5/30/2003	-21.04	75.14	1040	333	ETOH	GOETZE	
VANC10MV	VANC10MV-16	5/30/2003	-19.75	78.01	950	333	ETOH	GOETZE	
VANC10MV	VANC10MV-17	5/31/2003	-18.43	80.92	1040	333	ETOH	GOETZE	
VANC10MV	VANC10MV-18	6/1/2003	-17.18	83.68	950	333	ETOH	GOETZE	

VANC10MV	VANC10MV-19	6/2/2003	-15.76	86.78	1010	333	ETOH	GOETZE	
VANC10MV	VANC10MV-20	6/5/2003	-13.96	89.94	950	333	ETOH	GOETZE	
VANC10MV	VANC10MV-21	6/5/2003	-13.49	91.77	1150	333	ETOH	GOETZE	
VANC10MV	VANC10MV-22	6/6/2003	-12.86	94.29	1100	333	ETOH	GOETZE	
VANC10MV	VANC10MV-23	6/7/2003	-12.22	96.79	850	333	ETOH	GOETZE	
VANC10MV	VANC10MV-24	6/9/2003	-13.21	104.66	1050	333	ETOH	GOETZE	
VANC10MV	VANC10MV-25	6/10/2003	-13.85	109.04	1070	333	ETOH	GOETZE	
VANC10MV	VANC10MV-26	6/11/2003	-14.49	113.46	1050	333	ETOH	GOETZE	
VANC10MV	VANC10MV-27	6/12/2003	-16.58	115.38	1030	333	ETOH	GOETZE	
COOK14MV	COOK14MV-01	10/6/2001	-18.27	178.46	650	333	ETOH	GOETZE	
COOK14MV	COOK14MV-02	10/7/2001	-19.53	179.45	650	333	ETOH	GOETZE	
COOK14MV	COOK14MV-03	10/7/2001	-20.87	-179.49	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-04	10/7/2001	-22.23	-178.43	850	333	ETOH	GOETZE	
COOK14MV	COOK14MV-05-01	10/7/2001	-23.89	-177.10	300	333	CRYO	GOETZE	
COOK14MV	COOK14MV-05-02	10/7/2001	-23.86	-177.08	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-06	10/8/2001	-23.52	-175.70	900	333	ETOH	GOETZE	
COOK14MV	COOK14MV-07	10/9/2001	-23.22	174.73	900	333	ETOH	GOETZE	
COOK14MV	COOK14MV-07(2)	10/9/2001	-23.22	-174.72	495	333	CRYO	GOETZE	
COOK14MV	COOK14MV-08	10/9/2001	-22.76	-174.54	900	333	ETOH	GOETZE	
COOK14MV	COOK14MV-09	10/10/2001	-23.83	-174.41	1000	333	ETOH	GOETZE	
COOK14MV	COOK14MV-10	10/11/2001	-23.84	-174.22	1000	333	ETOH	GOETZE	
COOK14MV	COOK14MV-11	10/12/2001	-24.19	-175.00	600	333	CRYO	GOETZE	
COOK14MV	COOK14MV-12	10/12/2001	-24.19	-175.03	1500	333	ETOH	GOETZE	
COOK14MV	COOK14MV-13	10/13/2001	-23.86	-175.04	900	333	ETOH	GOETZE	
COOK14MV	COOK14MV-14	10/13/2001	-24.96	-175.25	950	333	ETOH	GOETZE	

COOK14MV	COOK14MV-15	10/14/2001	-25.79	-174.86	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-16	10/15/2001	-25.74	-175.30	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-17	10/15/2001	-26.87	-175.65	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-18	10/15/2001	-27.93	-175.97	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-19	10/16/2001	-29.09	-176.15	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-20	10/16/2001	-30.22	-176.59	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-21	10/16/2001	-31.08	-177.09	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-22	10/17/2001	-31.97	-177.43	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-23	10/17/2001	-32.00	-177.35	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-24	10/19/2001	-30.40	-178.20	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-25	10/19/2001	-29.23	-177.61	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-26	10/20/2001	-27.76	-176.81	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-27	10/20/2001	-26.12	-175.73	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-28	10/21/2001	-24.39	-175.22	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-29	10/22/2001	-23.23	-174.73	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-30	10/22/2001	-22.89	-175.97	500	333	ETOH	GOETZE	
COOK14MV	COOK14MV-31mv	10/24/2001	-22.00	-173.99	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-32	10/24/2001	-20.62	-173.20	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-33	10/24/2001	-19.32	-172.89	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-34	10/25/2001	-17.99	-172.59	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-35	10/25/2001	-17.10	-172.11	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-36	10/26/2001	-16.68	-172.25	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-37	10/27/2001	-16.02	-172.24	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-38	10/27/2001	-15.42	-172.23	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-39	10/27/2001	-14.80	-172.20	950	333	ETOH	GOETZE	

COOK14MV	COOK14MV-40	10/27/2001	-14.18	-172.22	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-41	10/27/2001	-13.29	-171.72	950	333	ETOH	GOETZE	
COOK14MV	COOK14MV-42	10/28/2001	-13.59	-171.75	950	333	ETOH	GOETZE	
DRFT07RR	DRFT07RR-01	12/17/2001	-27.68	-111.55	800	333	ETOH	GOETZE	
DRFT07RR	DRFT07RR-02	12/17/2001	-27.99	-113.08	800	333	ETOH	GOETZE	
DRFT07RR	DRFT07RR-03	12/18/2001	-29.34	-118.95	800	333	ETOH	GOETZE	
DRFT07RR	DRFT07RR-04	12/19/2001	-30.66	-124.77	925	333	ETOH	GOETZE	
DRFT07RR	DRFT07RR-05	12/20/2001	-31.35	-127.84	910	333	ETOH	GOETZE	
DRFT07RR	DRFT07RR-06	12/21/2001	-32.05	-130.99	880	333	ETOH	GOETZE	
DRFT07RR	DRFT07RR-07	12/22/2001	-33.39	-137.13	1030	333	ETOH	GOETZE	
DRFT07RR	DRFT07RR-08	12/23/2001	-34.04	-140.06	950	333	ETOH	GOETZE	
DRFT07RR	DRFT07RR-09	12/23/2001	-34.73	-143.27	1030	333	ETOH	GOETZE	
DRFT07RR	DRFT07RR-10	12/24/2001	-35.39	-146.30	1050	333	ETOH	GOETZE	
DRFT07RR	DRFT07RR-11	12/24/2001	-36.08	-149.48	1080	333	ETOH	GOETZE	
DRFT07RR	DFRT07RR-12	12/25/2001	-36.73	-152.59	1080	333	ETOH	GOETZE	
DRFT07RR	DRFT07RR-13	12/26/2001	-37.45	-156.01	1000	333	ETOH	GOETZE	
DRFT07RR	DRFT07RR-14	12/27/2001	-38.53	-161.24	1000	333	ETOH	GOETZE	
DRFT07RR	DRFT07RR-15	12/28/2001	-39.62	-166.69	1160	333	ETOH	GOETZE	
DRFT07RR	DRFT07RR-16	12/28/2001	-39.97	-168.41	1160	333	ETOH	GOETZE	
DRFT07RR	DRFT07RR-18	12/28/2001	-40.88	-173.00	1040	333	ETOH	GOETZE	
DRFT07RR	DRFT07RR-19	12/30/2001	-41.40	-175.66	1000	333	ETOH	GOETZE	
DRFT07RR	DRFT07RR-20	12/31/2001	-41.97	-178.57	950	333	ETOH	GOETZE	
DRFT0-7RR	DRFT07RR-21	1/1/2002	-42.95	176.26	480	333	ETOH	GOETZE	
0106TRAN	AS1	6/14/2001	38.78	25.08		333	ETOH	GOETZE	
0106TRAN	CAN1	6/22/2001	29.61	-13.80		333	ETOH	GOETZE	

MP3	MP3-02-06-00	6/27/2001	29.16	-23.60		333	ETOH	GOETZE	
MP3	MP3-03-01-00	6/27/2001	29.26	-25.47		333	ETOH	GOETZE	
MP3	MP3-04-06-00	6/28/2001	29.34	-27.99		333	ETOH	GOETZE	
MP3	MP3-05-01-00	6/28/2001	29.42	-29.95		333	ETOH	GOETZE	
MP3	MP3-06-06-00	6/29/2001	29.55	-31.93		333	ETOH	GOETZE	
MP3	MP3-07-01-00	6/29/2001	29.62	-33.68		333	ETOH	GOETZE	
MP3	MP3-08-03-00	6/30/2001	29.71	-35.94		333	ETOH	GOETZE	
MP3	MP3-09-01-00	6/30/2001	29.79	-37.47		333	ETOH	GOETZE	
MP3	MP3-11-01-00	7/1/2001	29.87	-41.54		333	ETOH	GOETZE	
MP3	MP3-12-06-00	7/2/2001	29.95	-45.04		333	ETOH	GOETZE	
MP3	MP3-14-01-00	7/8/2001	12.06	-55.44		333	ETOH	GOETZE	
MP3	MP3-16-01-00	7/9/2001	11.45	-52.40		333	ETOH	GOETZE	
MP3	MP3-17-07-00	7/10/2001	11.26	-50.95		333	ETOH	GOETZE	
MP3	MP3-18-01-00	7/10/2001	10.95	-49.65		333	ETOH	GOETZE	
MP3	MP3-20-01-00	7/11/2001	10.20	-46.59		333	ETOH	GOETZE	
MP3	MP3-21-04-00	7/12/2001	9.96	-45.51		333	ETOH	GOETZE	
MP3	MP3-21-20-00	7/14/2001	10.22	-45.52		333	ETOH	GOETZE	
MP3	MP3-23-02-00	7/19/2001	10.53	-56.54		333	ETOH	GOETZE	
MP3	MP3-23-16-00	7/20/2001	10.48	-56.56		333	ETOH	GOETZE	
MP3	MP3-23-18-00	7/21/2001	10.44	-56.53		333	ETOH	GOETZE	
MP3	MP3-23-24-00	7/21/2001	10.37	-56.61		333	ETOH	GOETZE	
MP3	MP3-23-28-00	7/22/2001	10.33	-56.51		333	ETOH	GOETZE	
MP3	MP3-23-33-00	7/22/2001	10.32	-56.61		333	ETOH	GOETZE	
MP3	MP3-34-03-00	8/2/2001	3.99	-43.12		333	ETOH	GOETZE	

MP3	MP3-40-01-00	8/5/2001	6.23	-48.24		333	ETOH	GOETZE	
MP3	MP3-44-10-00	8/7/2001	7.23	-52.44		333	ETOH	GOETZE	
MP3	MP3-45-01-00	8/8/2001	8.60	-53.22		333	ETOH	GOETZE	
STAR00	M00-49	9/4/2000	12.82	-141.99	200	505	ETOH	PL	
STAR00	M00-51	9/5/2000	10.50	-139.18	200	505	ETOH	VL	
STAR00	M00-53	9/6/2000	8.02	-136.35	200	505	ETOH		
STAR00	M00-55	9/7/2000	5.90	-133.63	200	505	ETOH		
STAR00	M00-57	9/8/2000	3.75	-131.05	200	505	ETOH		
STAR00	M00-59	9/9/2000	2.03	-128.97	200	505	ETOH		
STAR00	M00-61	9/10/2000	0.00	-126.54	200	505	ETOH		
STAR00	M00-63	9/11/2000	-3.21	-126.22	200	505	ETOH		
STAR00	M00-65	9/12/2000	-4.74	-124.56	200	505	ETOH		
STAR00	M00-67	9/13/2000	-4.53	-121.24	200	505	ETOH		
STAR00	M00-69	9/14/2000	-4.36	-118.44	200	505	ETOH		
STAR00	M00-71	9/15/2000	-4.25	-115.87	200	505	ETOH		
STAR00	M00-73	9/16/2000	-4.05	-112.63	200	505	ETOH		
STAR00	M00-75	9/17/2000	-1.64	-111.50	200	505	ETOH		
STAR00	M00-77	9/18/2000	1.21	-110.82	200	505	ETOH		
STAR00	M00-79	9/19/2000	4.11	-110.05	200	505	ETOH		
STAR00	M00-81	9/20/2000	3.20	-107.26	200	505	ETOH		
STAR00	M00-83	9/21/2000	3.85	-104.80	200	505	ETOH		
STAR00	M00-85	9/22/2000	3.75	-101.51	200	505	ETOH		
STAR00	M00-87	9/23/2000	4.73	-98.07	200	505	ETOH		
STAR00	M00-89	9/24/2000	5.68	-95.15	200	505	ETOH		
STAR00	M00-91	9/25/2000	7.09	-92.49	200	505	ETOH		

STAR00	M00-92	9/26/2000	6.96	-91.32	200	505	ETOH		
STAR00	M00-94	9/27/2000	8.04	-88.78	200	505	ETOH		
STAR00	M00-96	9/28/2000	8.75	-87.63	200	505	ETOH		
CD151	56101#12	9/20/2003	23.28	66.70	40		ETOH	CWHITCRAFT	
CD151	56107#9	9/30/2003	23.20	66.57	40		ETOH	CWHITCRAFT	
CD151	56112#2	10/1/2003	23.21	66.57	40		ETOH	CWHITCRAFT	
CD151	56116#12	10/5/2003	22.89	66.61	40		ETOH	CWHITCRAFT	
CD151	56136#9	10/12/2003	22.87	66.00	40		ETOH	CWHITCRAFT	
CD151	56141#9	10/15/2003	23.00	66.41	40		ETOH	CWHITCRAFT	
	GulfAQ-01	9/14/2003	29.46	34.93	630		ETOH	VFARSTEY	
IMECOCAL	120.35	7/7/2001	28.05	-114.89			ETOH	CICESE	
IMECOCAL	120.45	7/7/2001	27.72	-115.54			ETOH	CICESE	
IMECOCAL	117.35	10/13/2001	28.62	-115.25			ETOH	CICESE	
IMECOCAL	117.75	10/15/2001	27.29	-117.85			ETOH	CICESE	
IMECOCAL	117.60	10/14/2001	27.79	-116.88			ETOH	CICESE	
IMECOCAL	117.45	10/13/2001	28.29	-115.91			ETOH	CICESE	
IMECOCAL	117.80	10/15/2001	27.12	-118.18			ETOH	CICESE	
IMECOCAL	120.45	7/6/2001	27.72	-115.54			ETOH	CICESE	
IMECOCAL	117.30	10/13/2001	28.79	-114.93			ETOH	CICESE	
IMECOCAL	117.50	10/14/2001	28.12	-116.23			ETOH	CICESE	
IMECOCAL	117.70	10/14/2001	27.46	-117.53			ETOH	CICESE	
IMECOCAL	117.55	10/14/2001	27.96	-116.56			ETOH	CICESE	
IMECOCAL	117.40	10/13/2001	28.46	-115.58			ETOH	CICESE	
IMECOCAL	117.65	10/14/2001	27.62	-117.21			ETOH	CICESE	
MBARI, S401	67.75	8/7/2001	35.95	-123.84			ETOH	DFIELD	

MBARI, S401	67.85	8/8/2001	35.62	-124.54			ETOH	DFIELD	
MBARI, S401	67.90	8/8/2001	35.45	-124.90			ETOH	DFIELD	
MBARI, S401	67.80	8/9/2001	35.78	-124.19			ETOH	DFIELD	
MBARI, S401	67.70		36.12	-123.48			ETOH	DFIELD	
CALCOFI 0204	93.3 40.0	3/29/2002	32.51	-118.21	200	505	ETOH		
CALCOFI 0204	93.3 60.0	3/29/2002	31.85	-119.57	200	505	ETOH		
CALCOFI 0204	90.0 60.0	4/2/2002	32.40	-119.96	200	505	ETOH		
S401 MBARI	67.60	8/7/2001	36.45	-122.77				DFIELD	
CALCOFI 0204	93.30	3/28/2002	32.85	-117.53	200	505	ETOH		
	93.3 50	3/29/2003	32.17	-118.89					
CALCOFI 0204	93.3 45.0	3/29/2002	32.35	-118.56	200	505	ETOH		
CALCOFI 0204	93.3 55.0	3/29/2002	32.01	-119.23	200	505	ETOH		
IMECOCAL	117.40	7/22/2002	28.46	-115.58			ETOH		
IMECOCAL	117.50	7/21/2002	28.12	-116.23			ETOH		
IMECOCAL	117.55	7/21/2002	27.96	-116.56			ETOH		
IMECOCAL	117.65	7/20/2002	27.62	-117.21			ETOH		
CalCOFI 9901	90.60	1/14/1999	32.42	-119.96	850	333	ETOH	CLJ	
CalCOFI 9901	80.60	1/27/1999	34.15	-121.15	990	333	ETOH	CLJ	
?	Vial_2	5/1/2001	-23.00	71.00	100		ETOH	ESCRIBANO	
?	Vial_3-5	5/1/2001	-23.50	71.00	500		ETOH	ESCRIBANO	
	PLYMOUTH-1	11/27/2001	50.42	4.08			ETOH	RHARRIS	
?	205/04	8/1/2001	34.00	127.33		333	ETOH	Y-S KANG	
?	314/03	8/1/2001	33.00	127.33		333	ETOH	Y-S KANG	
?	314/01	8/1/2001	33.00	128.67		333	ETOH	Y-S KANG	
	314/04	8/1/2001	33.83	127.00		333	ETOH	Y-S KANG	

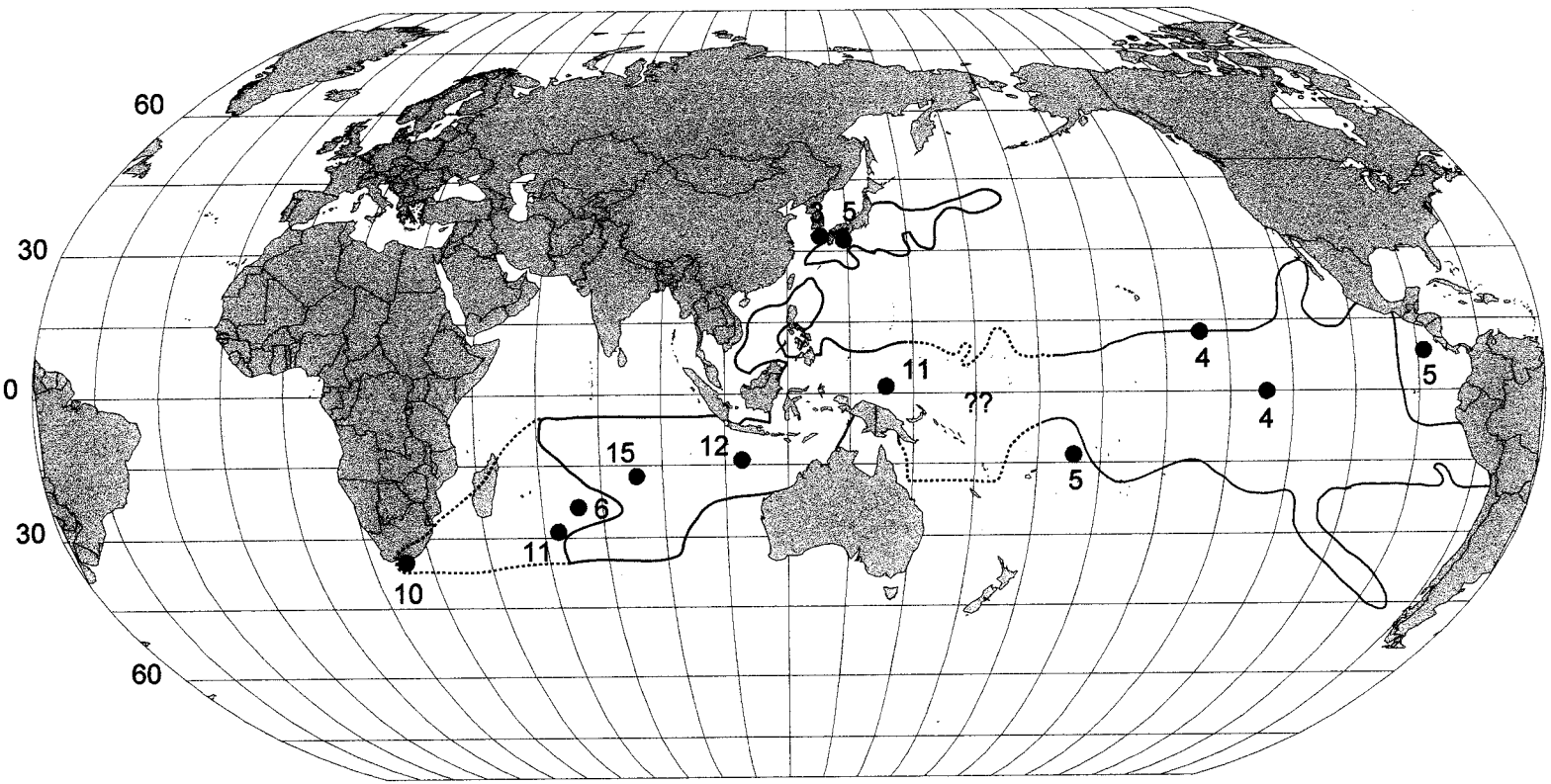
	205/05	8/1/2001	34.50	128.33		333	ETOH	Y-S KANG	
	206/03	8/1/2001	34.67	129.33		333	ETOH	Y-S KANG	
KOREA	314/01	10/19/2001	33.00	128.67	100	333	ETOH	Y-S KANG	
KOREA	205/03	10/13/2001	34.42	128.50	30	333	ETOH	Y-S KANG	
KOREA	205/04	10/13/2001	34.00	127.33	100	333	ETOH	Y-S KANG	
KOREA	205/05	10/13/2001	34.33	128.17	100	333	ETOH	Y-S KANG	
KOREA	314/04	10/19/2001	33.83	127.00	100	333	ETOH	Y-S KANG	
KOREA	206/03	10/12/2001	34.50	128.67	100	333	ETOH	Y-S KANG	
KOREA	205/03	10/13/2001	34.42	128.50	50	333	ETOH	Y-S KANG	
KOREA	314/04	10/19/2001	33.83	127.00	100	333	ETOH	Y-S KANG	
??	IOS2026	8/23/2000	51.31	127.24	300		ETOH	GALBRAITH	
??	IOS2005	8/5/2000	48.39	125.35	115		ETOH	GALBRAITH	
??	IOS2005	9/5/2000	48.38	126.07	250		ETOH	GALBRAITH	
??	U1165	10/20/1997	46.67	178.48	100		ETOH	GRIEVE	
??	U1225	10/30/1997	42.33	178.50	100		ETOH	GRIEVE	
??	U1235	11/1/1997	42.32	178.40	100		ETOH	GRIEVE	
??	U1602	11/25/2000	43.43	178.50	100		ETOH	GRIEVE	
??	-	8/19/1996	45.93	171.07	800		ETOH	GRIEVE	
ANDERSEN	3009A F0	9/30/2000	43.53	7.47	860	333	ETOH	VANDERSEN	
ANDERSEN	3009B F9-F8-F7	9/30/2000	43.53	7.47	75	333	ETOH	VANDERSEN	
ANDERSEN	3009B F0	9/30/2000	43.53	7.47	1000		ETOH	VANDERSEN	
HOT 117	2?? ALOHA	7/24/2000	22.75	-158.00	195		ETOH	LANDRY	
BIOCOM02	23	2/2/2001			0-100	333	ETOH	CARPENTER	
BIOCOM02	37	2/9/2001	9.48	41.81	0-20	333	ETOH	CARPENTER	
BIOCOM02	37	2/9/2001	9.48	41.81	60-80	333	ETOH	CARPENTER	

BIOCOM02	37	2/9/2001	9.48	41.81	150-200	333	ETOH	CARPENTER	
BIOCOM02	39	2/10/2001	11.13	42.29	20	333	ETOH	CARPENTER	
BIOCOM02	41	2/11/2001	10.35	44.79	0-100	333	ETOH	CARPENTER	
BIOCOM02	??	2/14/2001	9.80	49.57	0-200	202	ETOH	CARPENTER	
BIOCOM02	49	2/17/2001	11.05	56.19	0-200	202	ETOH	CARPENTER	
BIOCOM02	51	2/19/2001	11.41	55.00	0-200	202	ETOH	CARPENTER	
?	ST-3	5/24/2001	31.27	131.87	750	333	ETOH	OHTSUKA	
?	ST-6	5/26/2001	29.86	130.79	740	333	ETOH	OHTSUKA	
?	ST-11	5/28/2001	29.17	129.80	740	333	ETOH	OHTSUKA	
	IOS0117	7/3/2001	51.95	144.95			ETOH	GALBRAITH	
	OSO0134	7/10/2000	51.00	144.89			ETOH	GALBRAITH	
	OSO1067	7/6/2001	50.00	144.93			ETOH	GALBRAITH	
	OSO1053	6/29/2001	56.07	145.14			ETOH	GALBRAITH	
	OSO1057	7/1/2001	53.97	145.11			ETOH	GALBRAITH	
	OSO1055	7/1/2001	54.99	145.14			ETOH	GALBRAITH	
DYFAMED	DYFAMED-1	6/29/2001	43.42	7.86	400	200	ETOH	GASPARINI	
DYFAMED	DYFAMED-2	7/29/2001	43.42	7.86	500	200	ETOH	GASPARINI	
DYFAMED	DYFAMED-3	8/29/2001	43.42	7.86	400	200	ETOH	GASPARINI	
	VANDERSEN-1	9/8/2001	41.43	22.05	400		ETOH	VANDERSEN	
	VANDERSEN-2	9/20/2001	40.10	19.37			ETOH	VANDERSEN	
	VANDERSEN-3	9/20/2001	40.05	19.35			ETOH	VANDERSEN	
	VANDERSEN-4	9/23/2001	42.22	19.87			ETOH	VANDERSEN	
	FROST-1	1/1/2001	54.00	38.00			ETOH	FROST/WARD	
	FROST-2	5/27/2001	38.17	138.68	200	330	ETOH	FROST/IGUCHI	
	FROST-3	5/27/2001	37.00	137.23	200	330	ETOH	FROST/IGUCHI	

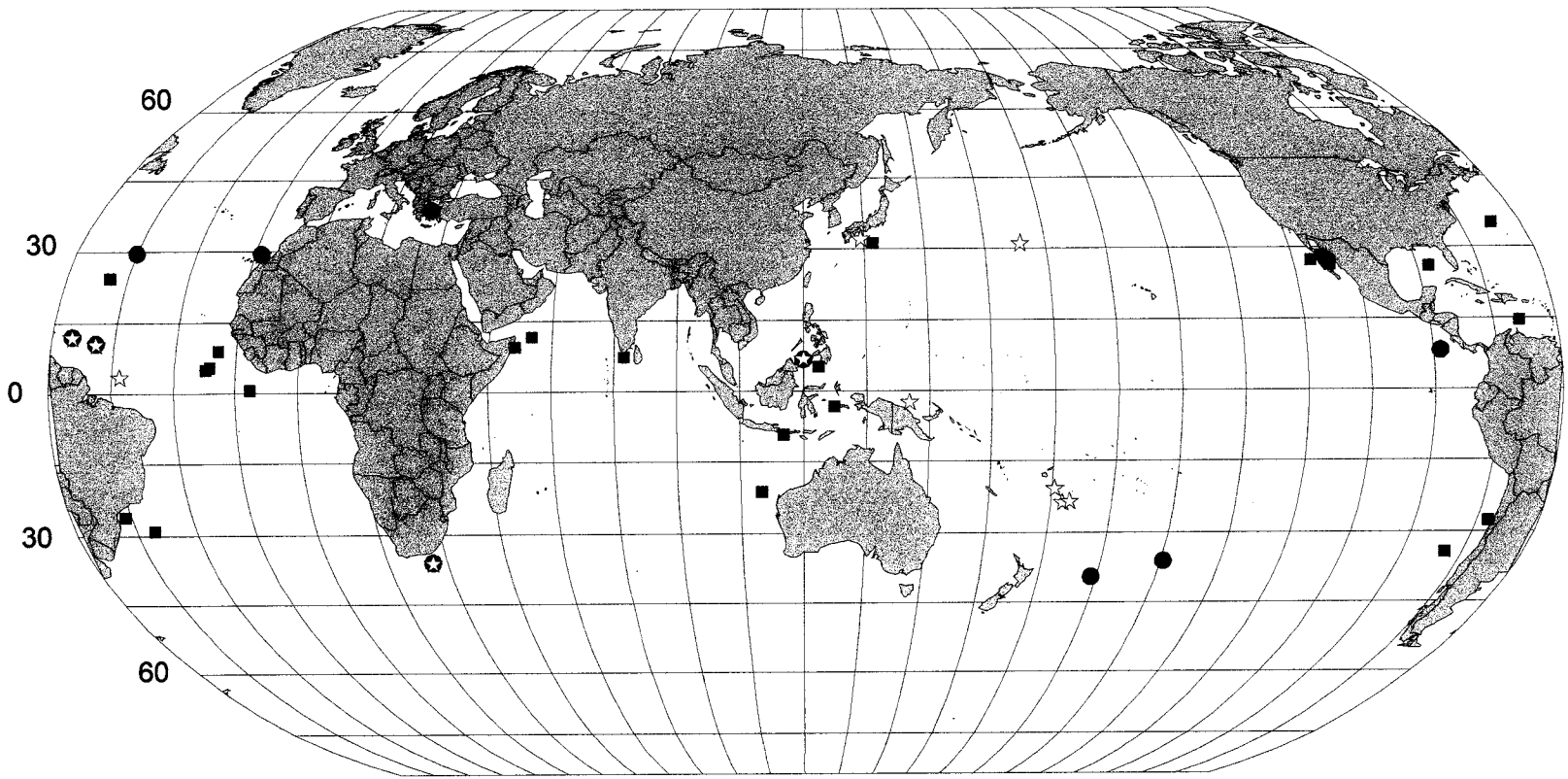
	FROST-4	5/22/2001	36.17	135.85	200	330	ETOH	FROST/IGUCHI	
OSO1034	FROST-5	5/17/2001	47.75	164.98		330	ETOH	FROST/BOWER	
?	BC7	5/8/2001	58.96	16.98			ETOH	IRIGOIEN	
	EC3	5/12/2001	61.19	22.95			ETOH	IRIGOIEN	
	CG4	5/14/2001	61.24	16.61			ETOH	IRIGOIEN	
	ADH	5/21/2001	57.49	11.53			ETOH	IRIGOIEN	
	D (-IB21)		63.21	19.99			ETOH	IRIGOIEN	
	F	5/16/2001	61.65	15.22			ETOH	IRIGOIEN	
	FL2	5/15/2001	62.47	15.95			ETOH	IRIGOIEN	
	C	5/13/2001	60.08	19.84			ETOH	IRIGOIEN	
	BJ2	5/22/2001	58.58	13.86			ETOH	IRIGOIEN	
ACE-ASIA	AA-31	4/8/2001	38.50	134.10		333	ETOH	SSTORMS	
ACE-ASIA	AA-19	4/3/2001	32.26	134.23		333	ETOH	SSTORMS	
ACE-ASIA	AA-14	3/29/2001	32.86	149.52		333	ETOH	SSTORMS	
ACE-ASIA	AA-8	3/22/2001	31.24	173.92		333	ETOH	SSTORMS	
ACE-ASIA	AA-13	3/28/2001	32.75	154.93		333	ETOH	SSTORMS	
ACE-ASIA	AA-17	4/2/2001	32.80	136.58		333	ETOH	SSTORMS	
ACE-ASIA	AA-2	3/16/2001	28.21	162.14		333	ETOH	SSTORMS	
KY01-03	D9-0		48.57	164.72	300	330	ETOH	MACHIDA	
WK0106	ST. 29		41.02	144.84	150	330	ETOH	MACHIDA	
TR0108	ST. 25		40.00	145.43	150	330	ETOH	MACHIDA	
TR0108	ST. 29	9/1/2001	41.00	144.70	150	330	ETOH	MACHIDA	
SOFEX	SOF-1	1/24/2002	52.40	171.60	110	200	ETOH	LANDRY	
SOFEX	SOF-2	2/13/2002	66.40	171.00	300	200	ETOH	LANDRY	
SOFEX	SOF-3	2/20/2002	52.56	166.95	250	200	ETOH	LANDRY	

	Pulau Payar	3/23/2002	6.06	100.05	surface	300	ETOH	FYUSOFF	
	D11	5/3/2002	60.30	30.75			ETOH	XIRIGOIEN	
NATL	I3(1)	5/22/2002	60.30	30.90			ETOH	XIRIGOIEN	
NATL	I3(2)	5/22/2002	148.50	82.18			ETOH	XIRIGOIEN	
ARIES	ARIES 1	5/23/2002	59.00	19.00			ETOH	XIRIGOIEN	
MBARI S302	77.70	6/28/2002	34.38	-122.24				DFIELD	
COOK07MV	01	4/8/2001	14.88	145.15	300	333	ETOH	TBAIZ	
COOK11MV	02	8/11/2001	18.32	132.74	575	333	ETOH	WILSON	
COOK11MV	03	8/12/2001	14.05	135.46	575	333	ETOH	WILSON	
COOK11MV	04	8/15/2001	1.71	143.72	575	333	ETOH	WILSON	
COOK11MV	05	8/16/2001	2.40	145.37	575	333	ETOH	WILSON	
KN162-10	KN162-10	2/6/2001	23.79	37.27	350	333	ETOH	JC	
	FERRARI	4/4/2001	27.53	79.97	80	110	ETOH	FERRARI	
IOS0117	OSO1059	7/2/2001	52.95	144.96	1200		ETOH	GALBRAITH	
KH00-1	ST. 26		7.00	120.00			ETOH	R. MACHIDA	
GC 98 11/12	Est:14 Sta:335	12/1/1998				333	ETOH	RLARA	Gulf of CA
GLOBEC	Fm9	4/6/2002						DFIELD	Oregon coast
DELPH.ALGOA	C05329-071	3/1/2000							Agulhas, Cape region
MESO 3	CR-10	6/15/2002							Central CA
MESO 3	FM-11	6/1/2002							Central CA
MESO 3	HH7	5/31/2002							Central CA
	C07266	7/8/2001							Agulhas, Cape region
FU9508	3	8/24/1995	27.98	112.15	300	202	ETOH	PCVERDIN	
FU9508	1	8/22/1995	30.53	113.57	200	202	ETOH	PCVERDIN	
CalCOFI 9901	San Pedro Basin	1/15/1999	33.50	118.29	810	333	ETOH	CLJ	

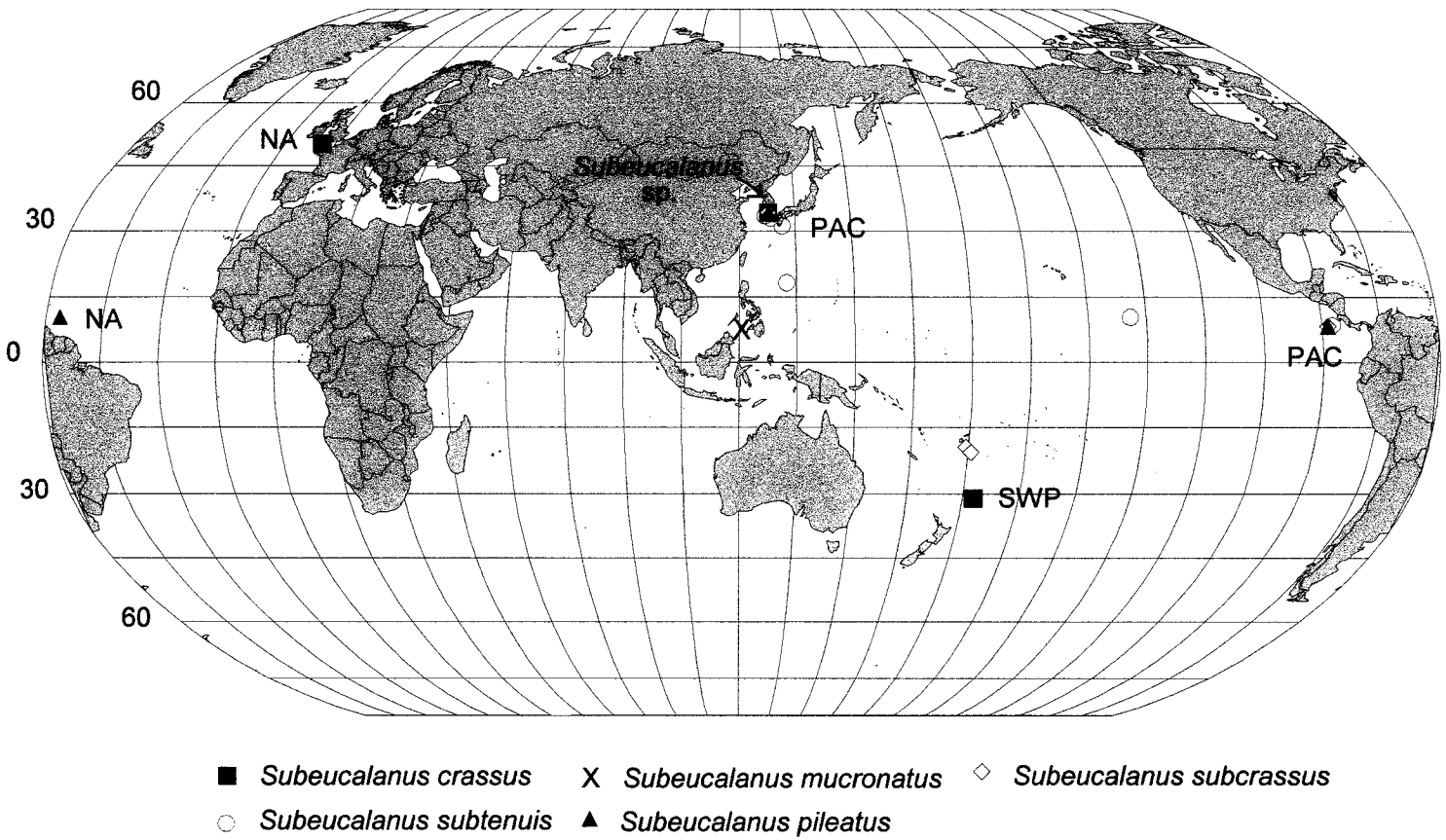
?	ED18		51.25	130.50			ETOH	GALBRAITH	
BIOMASA 0108-09	PAYON-1	8/31/2001	6.78	82.55	100	300	ETOH	AYON	



Appendix B-1. Geographic distribution of two genetic lineages in *Rhincalanus rostrifrons*. Circles mark locations from which DNA sequence data have been obtained for *R. rostrifons* s. l. Red is lineage *R. rostrifrons* (EP), and blue is *R. rostrifrons* (WP) from Chapter II. Distribution of *R. rostrifrons* s.l. after Lang, 1965. Numbers are the numbers of individuals sequenced from each location.



Appendix B-2. Geographic distributions of three genetic lineages morphologically cryptic within *Pareucalanus sewelli* (see Chapter II). Two genetic lineages of *P. sewelli*, (NA) and (PAC) indicated in blue and red circles respectively, with yellow stars marking sampling locations for highly divergent *Pareucalanus* sp. Black squares mark original locations of *P. sewelli* s.l. from Fleminger, 1973. DNA sequence data have not yet been obtained from additional specimens collected across the Indian Ocean. *Pareucalanus* sp. co-occurs with *P. attentuatus* in the western tropical Pacific.



Appendix B-3. Geographic distribution of genetic lineages in the genus *Subeucalanus*, and locations of genetic samples used in Chapter II. Three genetic lineages in *Subeucalanus crassus* are marked SWP, NA, and *Subeucalanus* sp. Two genetic lineages in *S. pileatus* are marked NA and PAC. No cryptic lineages have yet been found in *S. subtenuis*, *S. subcrassus*, and *S. mucronatus*. Locations at which they have been sampled are indicated.