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ORIGINAL PAPER

Genetic diversity of *Nymphon* (Arthropoda: Pycnogonida: Nymphonidae) along the Antarctic Peninsula with a focus on *Nymphon australe* Hodgson 1902

A. R. Mahon · C. P. Arango · K. M. Halanych

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Abstract Sea spiders are conspicuous, and often abundant, members of the Antarctic benthic community. Nymphonidae (Pycnogonida) in Southern Ocean waters comprise over 240 species which are often difficult to assign due to their intraspecific 'highly variable' morphology. In particular, *Nymphon australe,* the numerically dominant species in Antarctic waters is known to have a high level of phenotypic variation in external morphology and is also reported to have a circumpolar distribution. Circumpolarity seems contradictory to the pycnogonid's brooding lifestyle and presumably limited dispersal. Here we examine the genetic diversity of several *Nymphon* species collected in the Antarctic Peninsular region. Concomitantly, we assess the genetic structure of *N. australe* to gain insight into *Nymphon* dispersal capacity. Cytochrome *c* oxidase subunit I (COI) and 16S ribosomal gene data suggest a recent common history and/or recent gene-flow of *N. australe* populations across nearly 800 km of the Antarctic Peninsula. Furthermore, these data support that the Antarctic Peninsula region may hold two previously unrecognized species of *Nymphon*.

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A. R. Mahon · K. M. Halanych
The Department of Biological Sciences, Auburn University,
101 Life Sciences Building, Auburn, AL 36849, USA
e-mail: ken@auburn.edu

C. P. Arango
Queensland Museum, P.O. Box 3300, South Brisbane,
QLD 4101, Australia
e-mail: claudia.arango@qm.qld.gov.au

A. R. Mahon (✉)
Department of Biological Sciences,
The University of Notre Dame, Galvin Hall,
Notre Dame, IN 46556, USA
e-mail: armahon@gmail.com

Introduction

Pycnogonids, or sea spiders, are a group of exclusively marine arthropods that are found worldwide from shallow waters to abyssal depths (King 1973) with over 1,300 recognized species placed in nine or ten families (Dunlop and Arango 2005; Arango and Wheeler 2007). Nearly 20% of all pycnogonid species occur in the Southern Ocean, making this region highest in relative richness of sea spiders (Child 1995). Like other taxa (e.g., Hempel 1985; Clarke and Johnson 2003; Clarke et al. 2005), this region contains high endemism with more than 50% of sea spiders being endemic to the region (Munilla 2001). Nymphonidae, specifically *Nymphon*, has the largest number of reported specimens and the majority of species recorded in general pycnogonid collections in the Southern Ocean region (Child 1995). Nearly 70 of the 270 described species are represented in the region (Bamber and El Nagar 2008). Members of *Nymphon* and in general of Nymphonidae tend to be morphologically uniform and have been traditionally recognized as a monophyletic group (King 1973; Arango 2002), but, improved and more extensive taxon sampling might be needed to resolve the relationship with their putative sister family, the Callipallenidae, and their position in Pycnogonida phylogeny (Arango 2003; Arango and Wheeler 2007; Nakamura et al. 2007).

Although dispersal capabilities of sea spiders are presumed to be limited because they are benthic brooders, many Antarctic pycnogonids are reported to have circumpolar distributions and limited local or regional endemism on the Antarctic continental shelf (King 1973). Males possess ovigerous legs used to carry fertilized eggs until hatching. Sea spiders have been reported from plankton samples (e.g., Hedgpeth 1962; Mauchline 1984; see Bogomolova and Malakhov 2003), but such captures are rare and likely accidental in nature (Arnaud and Bamber 1987). Furthermore, rafting as a mechanism for dispersal of brooding Antarctic benthic invertebrates has been repeatedly proposed (Dell 1972; Simpson 1977; Pearse 1979; Picken 1980; Dayton et al. 1970; Thiel and Gutow 2005), but empirical evidence is lacking. *Nymphon australe*, the most ubiquitous species, is reported to have a circumpolar distribution, extending to more temperate waters in the southern hemisphere and is the most frequently captured pycnogonid in benthic trawls in the Southern Ocean (Child 1995).

Such a wide distribution is notable for a brooding organism, and in general, Antarctic circumpolar distributions have been reported to be the result of dispersal by the Antarctic circumpolar current (ACC; e.g., Bargelloni et al. 2000). Alternatively, Thajte et al. (2005) hypothesized that glaciation may have resulted in circumpolar species distributions if species were good colonizers and could exploit unglaciated or recently deglaciated areas. However, they also speculated that cryptic species might result in species with poor dispersal capabilities (e.g., pycnogonids); a speculation for which empirical evidence is mounting (e.g., Held 2003).

Child (1995) noted extreme morphological variation within *N. australe* and difficultly identifying species due to the high level of morphological diversity observed and lack of diagnostic apomorphies, even within single sampling locations. Child (1995) further noted that other pycnogonid species could share large amounts of morphological variation, but that they have not been taken in sufficient numbers necessary for comparison. To our knowledge, no molecular studies of Southern Ocean pycnogonids have been undertaken to test morphological taxonomic hypotheses.

Because *Nymphon* reproductive biology, in the context of Antarctic climatic history, seems to favor the formation of cryptic species and is counter to the reported circumpolar distribution of member species, we explored the genetic diversity of *Nymphon* in the Antarctic Peninsula region with special attention to genetic structure within *Nymphon australe*. To this end, we utilized molecular data from the mitochondrial cytochrome *c* oxidase subunit I (COI) and the ribosomal 16S gene within a phylogenetic and phylogeographic framework attempting to identify cryptic species and elucidate genetic patterns within the numerically dominant species, *N. australe*, in the Antarctic Peninsular region.

Materials and methods

Sampling

Samples were collected in 2004 and 2006 aboard the *ASRV Laurence M. Gould* using a Blake trawl, wire dredge, or epibenthic sled. Pycnogonids were preserved either in >70% ethanol or were placed at −80°C prior to return to Auburn University. Ninety-one individuals of Nymphonidae were used from 18 sampling locations along the Antarctic Peninsula (Fig. 1). Of these, 57 individuals of *Nymphon australe* were obtained from 6 of the 18 sampling locations. Ideally, more individuals would have been employed in analyses but collecting in Antarctic waters presents logistical challenges which limit sample size. Supplementary Table 1 gives the operational taxonomic units (OTUs) used in this study, locality information and GenBank accession numbers. Specimens were morphologically examined and identified to species level following common procedures in pycnogonid taxonomy (e.g., Gordon 1944; Child 1995; Fry and Hedgpeth 1969). All specimens were identified without knowledge of genetic results. Specimen vouchers have been deposited at the Smithsonian Institution National Museum of Natural History (provided in associated GenBank entries).

Molecular techniques

DNA extractions were performed using a Qiagen DNeasy® Tissue Kit (Qiagen Inc., Valencia, CA) following the manufacturer's recommendations. An ~600 bp fragment of the cytochrome *c* oxidase subunit I (COI) gene was amplified using the Folmer et al. (1994) primers and a reaction cocktail consisting of 0.75U Taq Polymerase and 10× PCR buffer (Eppendorf), 2.5 mM $Mg(OAc)_2$, 10 nmol of each dNTP, DNA template, primers, and water to 25µl. The PCR cycling program included an initial incubation at 95°C for 2 min and 40 cycles of 94°C for 30 s, 45°C for 1 min, and 68°C for 1 min. This was followed by a final extension at 68°C for 7 min. An ~500 bp fragment of the 16S rDNA (16S) was amplified using the primers 16SarL (Palumbi et al. 1991) and LR-J-12887 (Simon et al. 1994) with reagents as mentioned above. The 16S PCR cycling program included a 2 min incubation at 95°C, followed by 40 cycles of 94°C for 30 s, 45°C for 1min, 68 C for 1 min, and a final extension of 68°C for 7 min. Amplified PCR products were gel purified using a Qiagen QIAquick® Gel Extraction Kit (Qiagen Inc., Valencia, CA) following the manufacturer's recommendations.

Purified PCR products were bi-directionally sequenced on a Beckman CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Resulting sequences were assembled and screened using Sequencher 4.6 (Gene Codes

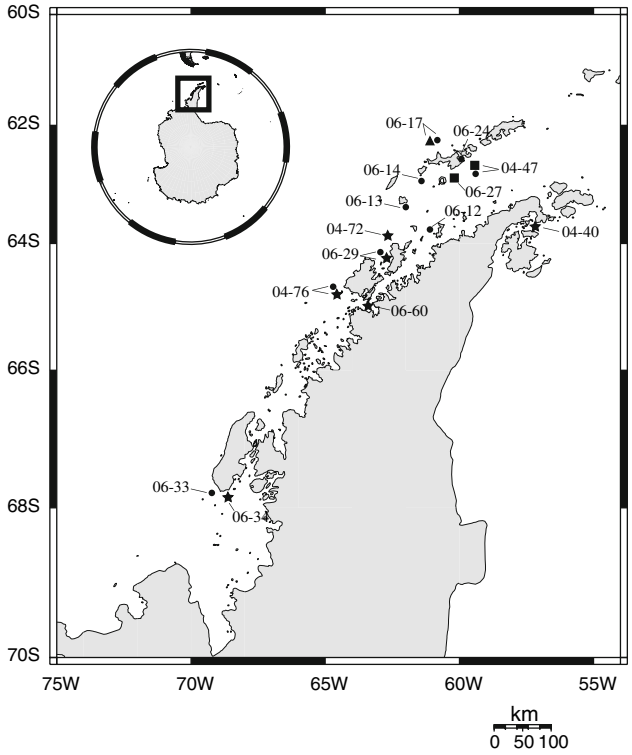

Fig. 1 Collection localities of Nymphonidae specimens. *Stars* indicate collection locations of *Nymphon australe* (morphological identification); *Squares* indicate collection locations of *Nymphon* sp. 1; The *triangle* indicates the collection location of *Nymphon* sp. 2; *Circles* indicate collection locations of all other species of Nymphonidae collected for this investigation

Corporation, Ann Arbor, MI). Sequences were aligned using BioEdit 7.0.1 (Hall 1999) and COI nucleotide sequences were translated to protein sequences to further check for sequencing errors. An incongruence length differences (ILD) test (Farris et al. 1995) on a limited dataset indicated that COI and 16S partitions were congruent ($P > 0.05$), thus they were combined for all analyses reported herein.

Data analyses

To reconstruct topologies, a haplotype dataset was created using Collapse v1.2 (Posada 2006; available at http://darwin.uvigo.es/software/collapse.html). Reconstructions were performed with Bayesian inference using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) implementing the GTR + I + G model of substitution, suggested by MrModeltest 2.2 (Nylander 2004). Although the same model of substitution was recovered for both dataset, the 16S and COI were run as separate unlinked partitions to allow for differences in parameter estimations during the run. Two sets of four chains (3 hot, 1 cold) were run for 2×10^6 generations and sampled every 100 generations. Stationarity was evaluated by examining convergence in log-likelihood values resulting in the first 50,000 generations being discarded as burn-in. A 50% majority-rule consensus tree was calculated from the remaining trees and nodal support values (i.e., posterior probabilities) were obtained to assess reliability of the recovered nodes. *Pentanymphon antarcticum* was used to root the resultant tree.

Uncorrected pairwise genetic distances were calculated in Mega 3.1 (Kumar et al. 2004) for the combined COI + 16S dataset. Using groups designated based on the MrBayes consensus topology, within and between group values were calculated as uncorrected (p) distances.

For the *Nymphon australe* clade, parsimony networks were constructed in TCS v.1.21 using 95% connection limits with gaps treated as missing data (Clement et al. 2000). Reticulations in resulting networks were broken using the rules of Crandall et al. (1994). Unfortunately, several of our localities included few individuals limiting the application of other statistical methods (e.g., AMOVAs, Φ_{st}, etc).

For *Nymphon australe* samples, Tajima's D (Tajima 1989) and Fu's F_S (Fu 1996) neutrality tests were conducted using DNaSP 4.0 (Rozas et al. 2003). Significantly negative values for Tajima's D and Fu's F_S reflect an excess of rare polymorphisms in a population, which indicates either positive selection or an increase in population size (Aris-Brosou and Excoffier 1996). Also, a Mantel test was performed in Arlequin v3.11 (Schneider et al. 2000) to investigate the correlation between genetic connectivity and geographic distance in *N. australe*. For this test, the shortest over water distances were employed (Supplementary Table 2).

Results

The final concatenated dataset consisted of 1,100 bp (COI, 597 bp; 16S, 503 bp) for 91 individuals. Aligned data is available at TREEBASE (http://www.treebase.org). Within Nymphonidae, 371 (34%) of characters were variable and 315 (28.5%) were parsimony informative. Within *Nymphon australe*, there were 27 variable sites (14 parsimony informative) with per site nucleotide diversity of $\pi = 0.00365$ and haplotype diversity of $H_d = 0.910$.

Figure 2 shows the Bayesian analysis topology for which "species" level nodes were well-supported with posterior probabilities above 0.95 under the GTR + I + G model of nucleotide substitution. Seven *Nymphon* lineages recovered in the tree correspond to individuals that could unambiguously be placed as recognized species based on morphological information. However, two novel lineages were recovered representing two previously unknown *Nymphon* taxa (called *Nymphon* sp. 1 and *Nymphon* sp. 2 here after) that were initially identified as *Nymphon* cf. *australe*

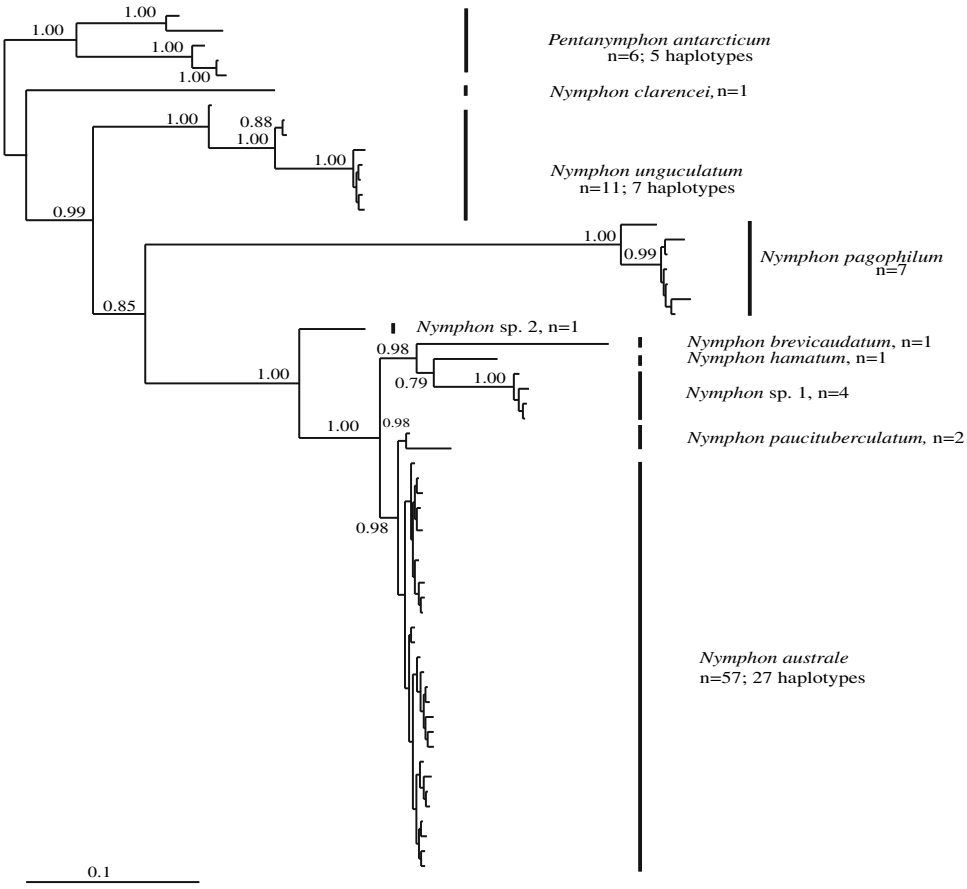

Fig. 2 Bayesian analysis of combined COI + 16S collapsed haplotype dataset using the GTR + I + G model of substitution calculated in MrModeltest (Nylander 2004). *Values* next to nodes indicate posterior probabilities. Total *number of samples* included in the investigation are indicated. Details of the analyses are given in the text

prior to molecular analyses based on morphology. Using the Child 1995 key, these specimens fell within the *N. australe-N. eltaninae* couplet, but the combination of characteristics observed do not allow to assign the specimens to either of the species. In the Bayesian analysis (Fig. 2), *Nymphon* sp. 1 was placed sister to *N. hamatum* and *Nymphon* sp. 2 was found to be basal to a clade that included five other *Nymphon* species.

Uncorrected pairwise distances (p) are shown in Table 1. Average within group genetic distance for "species" groups with multiple individuals was $p = 1.78\%$, with the largest average uncorrected p value (4.62%) found in *Pentanymphon antarcticum*. Between group values ranged from a minimum of 0.30% between *Nymphon paucituberculatum* and *N. australe* to a maximum of 16.39% between *N. clarencei* and *N. pagophilum*.

For the *Nymphon australe* clade ($n = 57$), 27 unique haplotypes from six sampling locations spanning a nearly 800 km range from Marguerite Bay to the northeastern tip of the Antarctic Peninsula were observed. Supplementary Table 3 contains haplotype frequency data in relation to sampling location. Additionally, Supplementary Fig. 1 combines the haplotype designation information from Supplementary Table 3 and shows where each haplotype is found within the resultant Bayesian tree topology. The parsimony network constructed using TCS v.1.21 (Clement et al. 2000) is shown in Fig. 3. In addition, to help delineate species boundaries, we ran a TCS analysis including all *Nymphon* OTUs. Recovered networks (not shown) corresponded with recognized species with the exceptions of samples morphologically identified as *Nymphon cf. australe* producing two networks (i.e., *Nymphon* sp. 1 and *Nymphon* sp. 2).

Neutrality tests on the *N. australe* concatenated dataset found negative values for both Tajima's D (not significant) and Fu's F_S tests (Tajima's $D = -1.100$, $P > 0.10$; Fu's $F_s = -15.926$). The results of the Mantel test were not significant ($P = 0.290$), showing no statistical support for a relationship between genetic connectivity and geographic distance.

Discussion

Based on mitochondrial data, the pycnogonid family Nymphonidae in the Antarctic Peninsula region harbors more diversity than previously recognized. Because the extent of gene flow is intimately tied to rates of evolutionary change and likelihood of speciation (e.g., Mayr 1970; Vermeij 1978; Slatkin 1985; Grant and Silva-Tatley da 1997; Pechenik 1999), we might expect that taxa with high species diversity

might show considerable intraspecific genetic structure (i.e., due to limited gene flow leading to future isolation). Alternatively, species that exhibit broad gene flow may be predicted to show limited speciation potential and lack intraspecific structure. In the current investigation, genetic patterns within the dominant species, *Nymphon australe*, showed high genetic diversity and shared nearly identical haplotypes over ranges of 500–800 km, a pattern suggestive of recent gene flow. However, 24 out of 27 haplotypes are restricted to single localities suggesting limited movement of individuals.

Interspecific relationships

The topology presented here in Fig. 2 represents a first step toward understanding the evolutionary relationships of Nymphonidae in the Antarctic Peninsular region, although further taxon sampling is clearly needed. Species determination and delineation within Nymphonidae can be difficult because of the high degree of variability of certain morphological characters and the limited number of characters that can be utilized in systematic, or taxonomic analyses (see discussion in Hedgpeth 1947, 1955; King 1973; Arango 2002). Within the Southern Ocean, especially the Antarctic Peninsula region, several species of *Nymphon* with similar morphology have been reported (Arnaud and Bamber 1987; Child 1995). Of interest, Child (1995) grouped some, but not all, *Nymphon* species into an "australe" group and a "hamatum" group based on morphology. However mitochondrial data do not support these designations, with *N. hamatum* falling inside species designated as belonging to the "australe" group. However, Child (1995, p.5) also commented that "*Nymphon australe* apparently serves as the 'keystone' species with a pattern from which most of the other (and subsequent?) species evolved, diversified, and from which each has diverged over time." In a current phylogenetic context, Child (1995) hypothesized that *N. australe*'s morphology is largely plesiomorphic, suggesting that diagnosis of *N. australe* may be problematic and that unrecognized lineages may exist.

Thus, discovery of high levels of diversity within the Nymphonidae in this study is not altogether surprising. Previous studies have shown that levels of species diversity for high latitude organisms could have increased through survival of organisms in glacial refugia during the last glacial maximum (Wares and Cunningham 2001). In regions of glaciation, bottleneck events and subsequent population expansions are thought to be common (e.g., Marko 2004). Such a situation would explain lack of genetic structure combined with the presence of multiple closely related species. Additionally, in North America and Europe, some studies have shown that postglacial colonization events have reduced the genetic diversity of organisms (e.g., Avise

Table 1 Average uncorrected pairwise distances (*p*) within and between clades

	Nymphon australe	*Nymphon pauciturberculatum*	*Nymphon* sp1	*Nymphon hamatum*	*Nymphon brevicaudatum*	*Nymphon* sp2	*Nymphon pagophilum*	*Nymphon unguiculatum*	*Nymphon clarencei*	*Pentanymphon antarcticum*
Nymphon australe	**0.37%**									
Nymphon pauciturberculatum	0.30%	**2.05%**								
Nymphon sp. 1	5.57%	5.34%	**0.26%**							
Nymphon hamatum	4.42%	4.35%	5.19%	–						
Nymphon brevicaudatum	8.77%	7.42%	9.49%	8.89%	–					
Nymphon sp. 2	5.59%	5.66%	8.61%	8.11%	11.43%	–				
Nymphon pagophilum	15.05%	14.23%	15.37%	15.32%	16.22%	16.12%	**1.47%**			
Nymphon unguiculatum	13.93%	13.02%	14.14%	13.77%	14.44%	9.32%	15.66%	**1.80%**		
Nymphon clarencei	14.95%	13.97%	13.98%	14.75%	14.65%	14.65%	16.39%	12.40%	–	
Pentanymphon antarcticum	11.63%	10.45%	12.10%	11.59%	12.18%	11.89%	13.59%	9.22%	9.88%	**4.62%**

Within group distances shown along diagonal in bold ("–" indicate single individual in taxon)

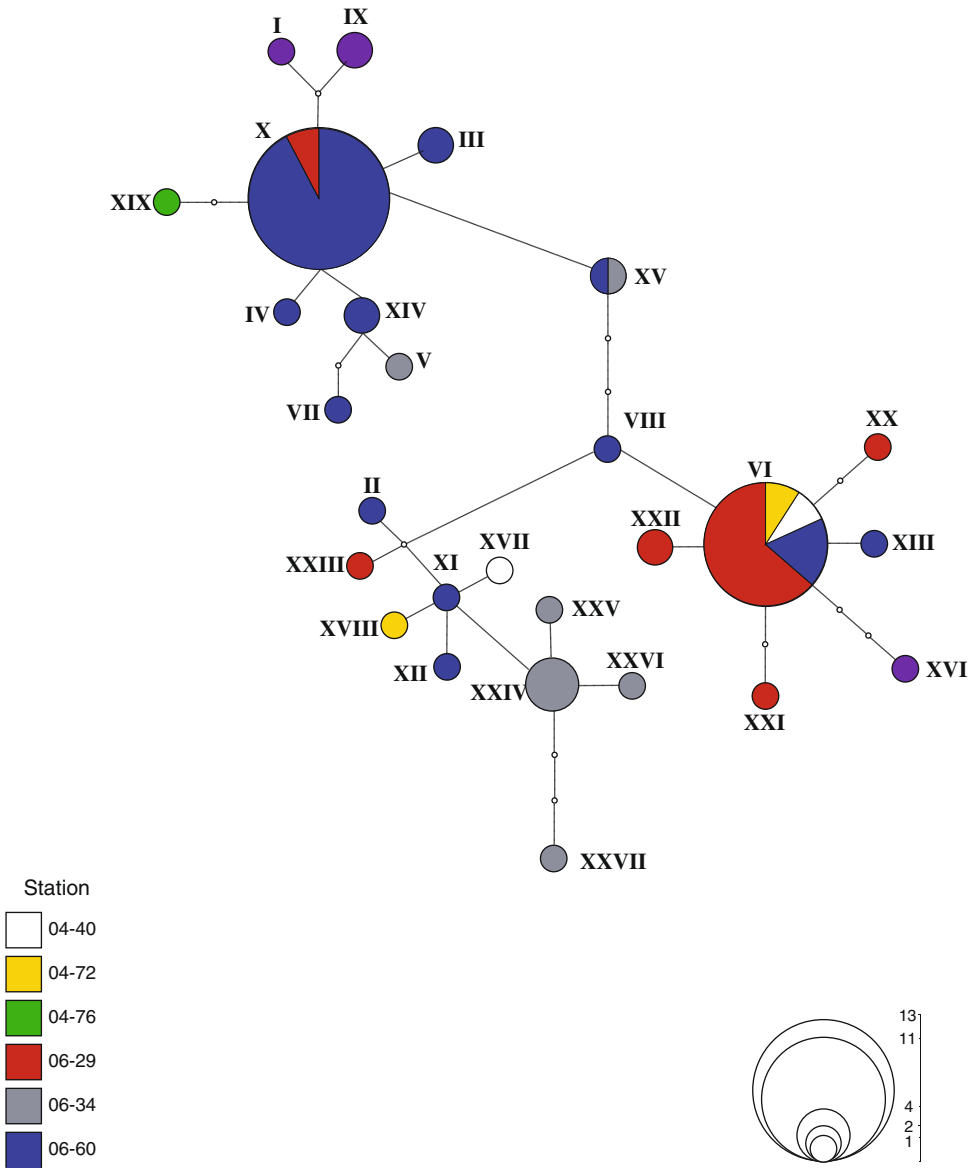

Fig. 3 Haplotype networks for *Nymphon australe* produced by TCS 1.21 (Clement et al. 2000). Connection limits of 95% were used between haplotypes and gaps were treated as missing data. Station numbers correspond to cruise station numbers from 2004 (04-xx) and 2006 (06-xx) aboard the *ASRV Laurence M. Gould*; these data are located in Supplementary Table 1. Roman numerals next to each haplotype are congruent to data presented in Supplementary Table 3 and Supplementary Fig. 1

et al. 1987; Templeton 1998; Avise 2000; Hewitt 2001; Marko 2004). For brooding organisms (or organisms with benthic developmental stages) like members of the Nymphonidae, historic refugia in glaciated regions could be an important factor for the distributions of marine species at high latitudes (Stewart and Lister 2001; Marko 2004). The *Nymphon* lineage appears to conform to the expectations of Pearse and Bosch (1994) and Thajte et al. (2005) who state that low dispersal potential, combined with historical glacial processes, have created several closely related species occupying the same geographic region.

The levels of genetic diversity in mitochondrial data presented here are, to some degree, consistent with the morphological plasticity of *Nymphon australe* noted by Child (1995). Interestingly, *Nymphon* sp. 1 and *Nymphon* sp. 2 were initially morphologically identified as *Nymphon* cf. *australe* without knowledge of molecular results. However, *Nymphon* sp. 1 and *Nymphon* sp. 2, discerned by molecular tools, are morphologically similar to *N. australe*, and possibly Child's (1995) conclusions were unknowingly based on pseudocryptic species. A number of factors suggest these OTUs are distinct evolutionary lineages that need further attention to determine species status. First, they are interspersed between currently recognized *Nymphon* species (Fig. 2). Second, the TCS parsimony analysis, using a 95% cutoff value, placed these two taxa as distinct networks. Lastly, when compared to *N. australe,* uncorrected *p* values for *Nymphon* sp.1 (5.57%) and *Nymphon* sp. 2 (5.59%) were similar to other interspecific divergence values (Table 1). Also of interest, the status of *N. paucituberculatum* deserves examination. This taxon was found sister to *N. australe* (Fig. 2) but showed an average uncorrected

p distance to *N. australe* of only 0.30%. This value was less than intraspecific diversities reported within other taxa, but it was not found within the *N. australe* haplotype network.

Detailed morphological studies of variation within these organisms may provide new insights into the diagnosis of *N. australe* and related species. As the majority of pycnogonids collected in the Southern Ocean are purported to be *N. australe*, this 'cryptic' speciation should be thoroughly investigated in future studies to elucidate the potential number of species misidentified as *N. australe*.

Intraspecific genetic variation

The dominant Nymphonidae in the Southern Ocean, *Nymphon australe,* shows historical genetic connectivity over an 800 km sampling area along the Antarctic Peninsula and share identical haplotypes over 500 km apart. This pattern is unexpected when one compares pycnogonid dispersal capabilities to speciation potential. Brooding in *N. australe* presumably reduces gene flow (Poulin and Feral 1996) and genetic structure is thought to accumulate easier in species with brooding life history stages rather than those with planktonic development (Berger 1973, 1977; Ament 1979; Ward 1990; McMillian et al. 1992; Duffy 1993; Hunt 1993; Hellberg 1996; Poulin and Feral 1996; Hoskin 1997; Arndt and Smith 1998; Pechenik 1999; Ayre and Hughes 2000; Kyle and Boulding 2000; Marko 2004; Sotka and Palumbi 2006).

Pearse and Bosch (1994) suggest that species with a brooding life history have low dispersal capabilities over long periods of time and thus are likely endemic to a given region and/or have since speciated in that location. This supposition is compatible with Thajte et al. (2005) assertion about the effect dispersal and glaciation can potentially have on genetic patterns. Their work suggested that organisms could have survived the last glacial period by migration to the deep sea or occupation of shallow water niches and reinvaded the shelf waters of Antarctica during the following interglacial period (Thajte et al. 2005). This hypothesis could potentially explain the high levels of cryptic (or pseudocryptic) species found in the region (e.g., Held 2003; Held and Wägele 2005; Wilson et al. 2007, Hunter and Halanych 2008).

Population genetic markers for *Nymphon australe* suggest a complex interplay of genetic evolutionary forces shaping the patterns of diversity and structure in this species. The results of this work suggest that a number of haplotypes are restricted to a given locality, suggesting that dispersal is limited (Fig. 3). However, where many recently bottlenecked species show a single dominant haplotype with several singletons separated by one or two nucleotide mutations, the haplotype network of *N. australe* (Fig. 3) reveals a fair amount of diversity. This is not a typical situation, as others have reported that at high latitudes, there is low genetic diversity with a few haplotypes dominating large areas, a characteristic of recent range extensions from refugia (e.g., Hewitt 1999; Marko 2004). Potential explanations for this distribution and genetic connectivity could include long-distance dispersal, which has been discussed for other species without planktonic larvae (e.g., Johannesson 1988; Johannesson and Warmoes 1990; Vermeij et al. 1990). Marko (2004) and others note that dispersal of such organisms is likely bolstered by rafting on macroalgal mats. Unfortunately, for the current *N. australe* dataset, more samples need to be taken before we can reliably quantify the patterns of gene flow and rates of population expansion in this species.

From the data presented in this investigation, the role of intraspecfic evolutionary forces relative to speciation processes is not clear in this group of Southern Ocean sea spiders. Additionally, a greater sampling of *Nymphon australe*, both in numbers of organisms and sampling localities, in the Southern Ocean is needed to determine if the species conforms to its proposed circumpolar distribution. Furthermore, future investigations should incorporate the roles of rafting and oceanic currents when attempting to explain the wide dispersal observed here.

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