Molecular evolutionary relationships of the octopodid genus *Thaumeledone* (Cephalopoda: Octopodidae) from the Southern Ocean

J.M. STRUGNELL[1,2]*, M.A. COLLINS[1] and A.L. ALLCOCK[2]

[1]*British Antarctic Survey, NERC, High Cross, Madingley Rd, Cambridge CB3 0ET, UK*
[2]*Ecology and Evolutionary Biology, School of Biological Sciences, Queen's University Belfast, Belfast BT9 7BL, UK*
jan.strugnell@gmail.com

Abstract: Recent trawling in the Southern Ocean has yielded individuals of a number of species of the deep sea octopod genus *Thaumeledone*. This paper provides the first molecular study of the genus, employing molecular sequences from five mitochondrial (12S rDNA, 16S rDNA, COI, COIII, *cytochrome oxidase b*) and a single nuclear gene (*rhodopsin*) and includes representatives of each of the known Southern Ocean species. *Thaumeledone rotunda*, believed to be circumpolar in distribution and found in relatively deep water is the sister taxa to *T. gunteri*, known only from South Georgia. A notable level of sequence variability was evident between a *T. peninsulae* individual recently captured from the Powell Basin, and two *T. peninsulae* individuals captured from the continental slope, north of the South Shetland Islands. This is likely to represent population level intraspecific variation within this species.

Introduction

The genus *Thaumeledone* comprises benthic octopods known from relatively deep waters in the Southern Hemisphere. *Thaumeledone* are small, squat bodied octopuses and possess a single row of suckers on their arms. They possess a distinctive deep purple pigmentation on the oral surface of the web (Allcock *et al.* 2004). The terminal organ of the hectocotylised arm has a large calamus, giving the terminal organ a club-like appearance.

Six species of *Thaumeledone* are currently recognized (Allcock *et al.* 2004). One species, *T. brevis* (Hoyle 1885), is known only from the type material which was captured in deep water off Montevideo, Uruguay, in 1876. Many other specimens have been previously identified as *T. brevis*, particularly from very deep water in the Southern Ocean, but Allcock *et al.* (2004) recognized these to be distinct from the type material of *T. brevis* and re-identified these specimens as *T. rotunda* (Hoyle 1885). Two species, *T. marshalli* O'Shea, 1999 and *T. zeiss* O'Shea, 1999 are known only from New Zealand waters. The remaining two species, *T. gunteri* Robson, 1930 and *T. peninsulae* Allcock *et al.* 2004 occur south of the Polar Frontal Zone in Antarctic waters (Fig. 1). *Thaumeledone gunteri* is known only from around South Georgia, whilst *T. peninsulae* was previously only known from the continental slope of the Antarctic Peninsula and adjacent islands. Both species are restricted to slope waters, neither occurring at the extreme depths at which *T. rotunda* is found.

Nothing is known of the evolutionary history within the genus *Thaumeledone*. A single specimen (of unknown species) was used within a phylogenetic study of the Octopoda (Carlini *et al.* 2001) and found *Thaumeledone* sp. to be the sister taxa to *Megaledone setebos* (Robson 1932), a large octopus species restricted to Antarctic waters.

Recent trawling programmes aboard RRS *James Clark Ross*, RV *Polarstern* and fisheries surveys around South Georgia have yielded a collection of individuals of the Southern Ocean species of *Thaumeledone* (Allcock *et al.* 2004). Tissue samples were collected from a number of these individuals upon capture and provide a unique opportunity to undertake a molecular phylogenetic study of the group.

The aim of this study was to resolve the phylogenetic relationships within Southern Ocean *Thaumeledone* species.

Materials and methods

A large proportion of specimens used within this study were collected from a number of research cruises to the Antarctic Peninsula, the Scotia Sea and South Georgia over the past ten years aboard RV *Polarstern* particularly during the EASIZ and ANDEEP programmes, and aboard RV *Dorada* and MV *Cordella* as part of the annual fish surveys around South Georgia (Fig. 1). Details of these cruises can be found in Allcock *et al.* (2004). In addition to this, as part of the BIOPEARL expedition, RRS *James Clark Ross* fished in the Scotia Sea between February and April 2006. A specimen of *T. peninsulae* was captured from ~1500 m from the Powell Basin using an Agassiz trawl. This specimen has been deposited in the National Museums of
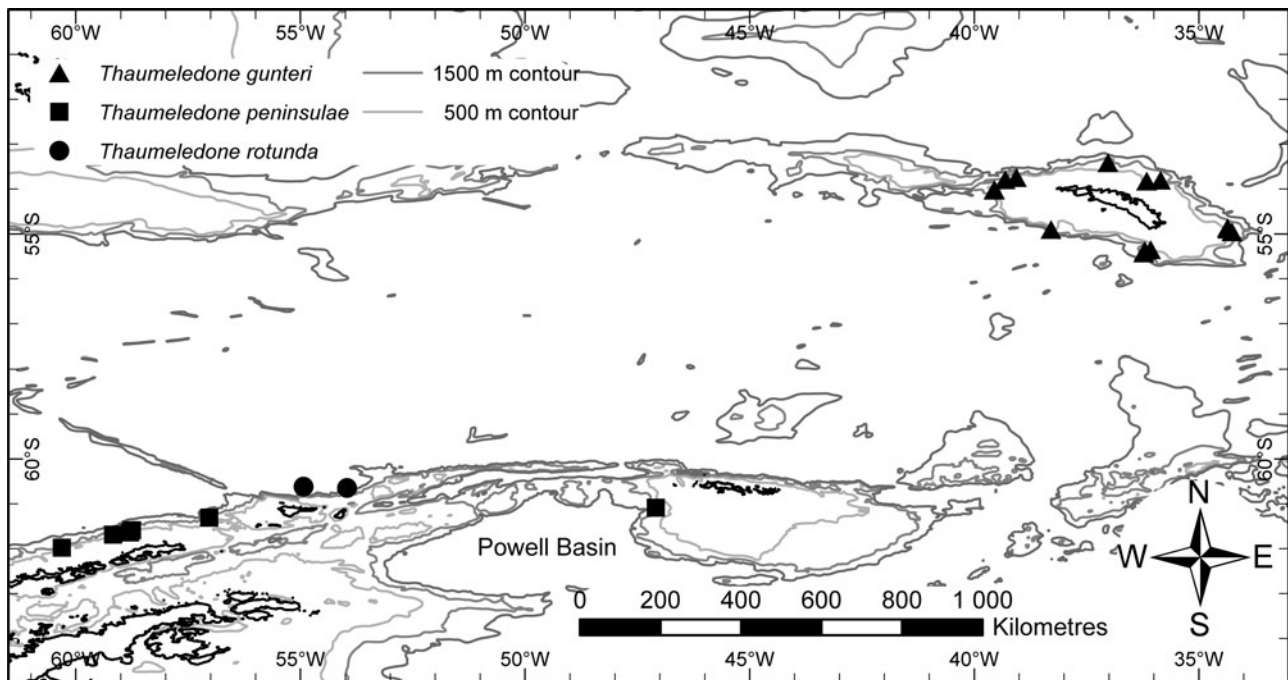
Fig. 1. Map of the Scotia Arc showing capture locations of *Thaumeledone* during recent cruises (ANTARKTIS XIV/2 in 1996, EASIZ III in 2000, ANDEEP I in 2002, BIOPEARL in 2006 and South Georgia Fish Surveys, 1994, 1997, 2003, 2004).

Scotland under catalogue number NMSZ 2007009. A specimen of *Velodona togata* Chun, 1915 was also obtained for the study and was captured by a commercial prawn trawler off the coast of Durban, South Africa from 400–500 m in February 2005. This specimen was included in the study because a larger phylogenetic study of the Octopodiformes found it to be a suitable outgroup (data not shown).

Specimens were examined either alive, when possible, or freshly dead. Tissue samples were taken from a small number of *Thaumeledone* specimens and from specimens of other closely related species in the vicinity and preserved in 70% ethanol for subsequent application of molecular techniques. Where possible, these specimens were then fixed in 4% formalin and deposited in either the Natural History Museum, London (BMNH), the Smithsonian Institute (USNM) or the National Museums of Scotland (NMSZ).

Morphological analysis

Standard counts and measurements are recommended for morphological description of octopus species and these were recorded for the newly captured specimen of *T. peninsulae* from the Powell Basin. These counts and measurements have already been published for several specimens of *T. rotunda*, *T. peninsulae* and *T. gunteri* (Allcock *et al.* 2004) and for *T. zeiss* and *T. marshalli* (O'Shea, 1999). From the recorded and published

information, a range of morphometric indices (see Roper & Voss 1983) were calculated: mantle width index (MWI), head width index (HWI), funnel length index (FuLI), free funnel length index (FFuLI), arm width index (AWI), arm sucker index (ASI), web depth index (WDI), arm length index of arms 1, 2, and 3 (ALI1, ALI2, ALI3; ALI4 was not used because of a number of damaged 4th arms), mantle arm index (MAI), opposite arm index (OAI), ligula length index (LLI), calamus length index (CaLI), and spermatophore length index (SpLI). Two matrices of counts and indices were then compiled including information from Southern Ocean species only. In the first, the number of indices and counts used was maximized by including only mature male specimens. This matrix comprised 16 variables (all the indices mentioned above plus the hectocotylised arm sucker count) for nine individuals: four *T. peninsulae* (including the Powell Basin specimen), three *T. gunteri* and two *T. rotunda*. In the second matrix the number of individuals included was maximized at the expense of variables. Missing values are not permitted in the analysis so variables must be dropped if individuals are included for which these variables are not available. The second matrix comprised 11 variables (OAI, LLI, CaLI, SpLI and hectocotylised arm sucker count were excluded) and 21 individuals: ten *T. peninsulae* (including the Powell Basin specimen), seven *T. gunteri* and four *T. rotunda*. A third matrix was then compiled which also included data from the New Zealand species. It comprised 11 variables (MWI, HWI, FuLI, FFuLI, ASI, WDI, ALI1-4 and

Table I. Cephalopod tissue samples used in this study.

Species	Longitude	Latitude	Station number	Depth (m)	Museum catalogue number	Date
Adelieldone polymorpha	54.56 W	61.16 S	61/048-1^	277–343	NMSZ 2002037.029	30/01/02
Thaumeledone gunteri	35.51 W	53.47 S	SG03/63§	679–719	NMSZ 2004083	31/01/03
Thaumeledone gunteri	39.55 W	54.01 S	93*	364–394	-	04/02/94
Thaumeledone peninsulae	55.72 W	61.23 S	PB-AGT-1<	1521–1530	NMSZ 2007009	17/03/06
Thaumeledone peninsulae	58.75 W	61.59 S	42/048^	766–824	USNM 1021039	28/11/96
Thaumeledone peninsulae	59.17 W	61.69 S	42/045^	792–862	USNM 1020683	27/11/96
Thaumeledone rotunda	53.96 W	60.65 S	61/046-8^	2895–2896	NMSZ 2002038 001	02/02/02
Thaumeledone rotunda	54.93 W	60.62 S	42/039^	3213–3222	USNM 1020991	25/11/96
Velodona togata	31.50 E	29.67 S		400–500 m	-	8–26/02/05

* = MV *Cordella*, ^ = RV *Polarstern*, § = RV *Dorada*, < = RV *James Clark Ross*.

MAI) and 29 individuals: ten *T. peninsulae* (including the Powell Basin specimen), six *T. gunteri*, four *T. rotunda*, four *T. zeiss* and five *T. marshalli*. Matrices were imported into the statistical package Primer, normalized, and the Euclidean distance between samples was calculated. The multivariate distance between individuals was visualized in a non-metric multidimensional scaling (MDS) plot. ANOSIM was used to test for significant differences between species. The table of pairwise ANOSIM test statistics summarizes the distances between species based on the combined influence of a number of morphological variables. We used these pairwise test statistics to generate a morphological distance matrix. Mantel tests were used to test for significance congruence between the morphological and genetic distance matrices.

Molecular analysis

DNA was extracted from a selection of the tissues samples taken (Table I) and used in molecular sequence analysis. The DNA extraction protocol followed that of Taggart *et al.* (1992). Briefly, 375 µl of 0.2M EDTA, 0.5% sodium lauroylsarcosine (pH 8.0) and 10 µl proteinase K (20 mg ml⁻¹) were added to the tissue sample (~0.1 g) and incubated overnight at 55°C. The following day 10 µl RNAse (20 mg ml⁻¹) was added to each tube and incubated for 1 hour at 37°C. The solution was extracted once with phenol and once with chloroform:isoamyl alcohol (99:1). DNA was precipitated using 3 volumes of 92% ethanol and then washed overnight in 70% ethanol before being resuspended in 30 µl of sterile TE (pH 8.0) and stored at 4°C.

Primers for five mitochondrial genes (12S rDNA, 16S rDNA, COI, COIII, *cytochrome oxidase b*) were taken from the literature (Simon *et al.* 1990, 1991, 1994, Folmer *et al.* 1994, Strugnell *et al.* 2004, Guzik *et al.* 2005) with the 12S rDNA and COI primers modified slightly to match cephalopod sequences on GenBank. Primers used for the nuclear gene, *rhodopsin*, were designed in the conserved regions of cephalopod and invertebrate sequences of this gene present on GenBank and are available from the authors on request.

PCR reactions were carried out in 25 µl volumes. Thermal cycling conditions consisted of a denaturation step at 94°C for 2 min, followed by 35 cycles at 94°C for 40 sec, 50°C for 40 sec, and 72°C for 90 sec. A final extension step of 72°C for 10 min was added in each case. Annealing temperatures varied according to the primers used and are available from the authors on request. Amplified products were purified using the QiaGen PCR purification kit (QiaGen Ltd., UK) following manufacturers instructions. Purified PCR products were commercially sequenced by Macrogen Inc (Korea) in both directions using the same primers used for PCR amplification.

DNA sequences were compiled and aligned by eye in Se-Al v2.0a11 Carbon (Rambaut 2002). It was necessary to introduce gaps to align sequences of 12S rDNA, 16S rDNA and *rhodopsin*. The sequence data for each gene were concatenated into a single dataset. Of the 2612 characters used in the analysis, 350 (13.4%) were found to be variable.

PAUP v4.0b10 (Swofford 1998) was used to perform full heuristic searches. Starting trees were generated by neighbour joining (NJ) (Saitou & Nei 1987). A GTR (Γ+I) likelihood model incorporating rate heterogeneity (six rate categories) was used. Branch swapping was performed using TBR (tree-bisection-reconnection). Parameters were then re-estimated and finally branch swapping was performed using NNI (nearest-neighbour interchange). Substitution model parameter values were A = 0.33, C = 0.14, G = 0.14, T = 0.39, A↔C = 2.21, A↔G = 13.54, A↔T = 4.69, C↔G = 1.56, C↔T = 23.36 G↔T = 1.00, I = 0.71, Γ = 3.72. ML bootstrap values of clade support were generated using the above parameters using 1000 replicates.

MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003) was used to calculate marginal posterior probabilities using the GTR+I+Γ model of nucleotide substitution for each partition. Model parameter values were treated as unknown and were estimated in each analysis. Random starting trees were used and analyses were run 1 million generations, sampling the Markov chain every 100 generations. The analysis was performed twice, in each case starting from a different random tree to ensure the analyses were not trapped in a local optima.

The program Tracer v1.3 (Rambaut & Drummond 2003) was used to ensure that the Markov chains had reached stationarity and to also determine the correct 'burn-in' for
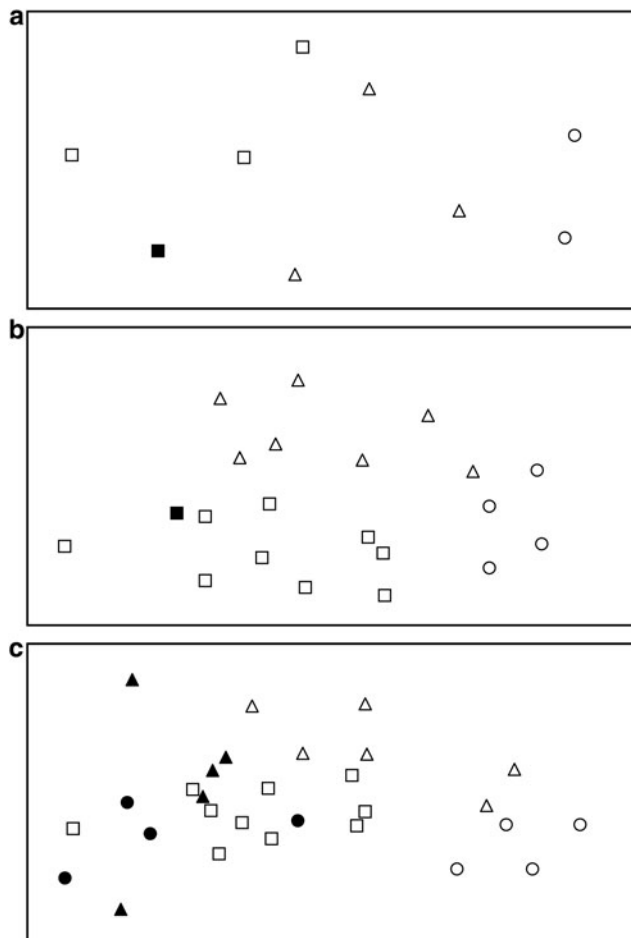
Fig. 2. a. MDS ordination based on 16 counts and morphometric indices from mature male specimens. **b.** MDS ordination based on 11 morphometric indices from male and female specimens of a range of maturities. **c.** MDS ordination based on 11 morphometric indices from male and female specimens of a range of maturities. □ = *Thaumeledone peninsulae*, ■ = *T. peninsulae* specimen from the Powell Basin, Δ = *T. gunteri*, ○ = *T. rotunda*, ● = *T. zeiss*, ▲ = *T. marshalli*.

the analysis (i.e. the number of initial generations that must be discarded before stationarity is reached).

Pairwise distances were calculated using the values for GTR+I+Γ calculated during the maximum likelihood analysis using PAUP v4.0b10 (Swofford 1998) and were separated into three categories: 1) difference among individuals in the same species, 2) differences among species in the same genus (not including intraspecific differences), 3) differences among species in different genera. For each of these categories, values were plotted against the proportion pairwise distance.

Results

Morphological analysis

The recently captured specimen of *T. peninsulae* caught from the RRS *James Clark Ross* (NMSZ 2007009) from the

Powell Basin region is a mature male (dorsal mantle length 47 mm, total length 110 mm) and has been well preserved. The arms are approximately equal in length (*c.* 60 mm each) and width (6 mm). The suckers are small (3 mm) with *c.* 33 suckers on each arm. The web depth per sector ranges from 22–31 mm. The funnel organ is VV shaped. Gills are 8–9 mm in length and have 5–6 lamellae per demibranch. The third right arm is hectocotylised and is slightly shorter than its opposite number (52 mm) with 22 suckers. The hectocotylus is club-like in appearance. The ligula is 7 mm in length and the calamus is 4 mm. The reproductive system contained three large spermatophores, one of which was measured (55 mm).

Multivariate combinations of morphological variables can apparently distinguish among Southern Ocean species of *Thaumeledone* (Fig. 2a & b). When data were maximized for variables (Fig. 2a), the newly captured *T. peninsulae* appears to be slightly separated from other *T. peninsulae* specimens. When data were maximized for number of individuals (Fig 2b), the newly captured *T. peninsulae* is on the edge of the *T. peninsulae* cluster, but clearly still within this grouping. There are insufficient data points to give significant pairwise differences between species when only males are included, however ANOSIM reveals significant pairwise differences between all species pairs in the dataset maximized for number of individuals. A distance matrix based on these pairwise differences showed no significant congruence with a matrix based on genetic distance (test statistic *rho* = -0.5, *P* = 0.845).

Multivariate combinations of morphological variables do not appear to distinguish between all *Thaumeledone* species once the New Zealand species are included (Fig. 2c). Although the global ANOSIM test statistic is significant (global test statistic = 0.499, *P* < 0.001), pairwise tests show that there are no significant differences between *T. peninsulae* and *T. zeiss* (test statistic = 0.175, *P* = 0.126), between *T. peninsulae* and *T. marshalli* (test statistic = 0.23, *P* = 0.081) and between *T. marshalli* and *T. zeiss* (test statistic = 0.125, *P* = 0.159). All other pairwise comparisons are significant.

Molecular analysis

Sequences generated in this study are available from GenBank under accession numbers EU086512-086515, EU071456-071459, EU071438-071440, EU071432-071434, EU071445-071447, EU071443, AF299266, AY557521, EF102113, EF102215, EF102194, EF102173, EF102153 and EU148453-EU148478. The phylogenetic tree is rooted using *Adelieledone polymorpha* (Robson 1930) as additional phylogenetic analyses also containing the species, *Vampyroteuthis infernalis* Chun, 1903, *Octopus vulgaris* Cuvier, 1797 and *Enteroctopus dofleini* Wülker, 1910, found *Adelieledone* to be basal to *Thaumeledone* (data not shown). Furthermore, previous
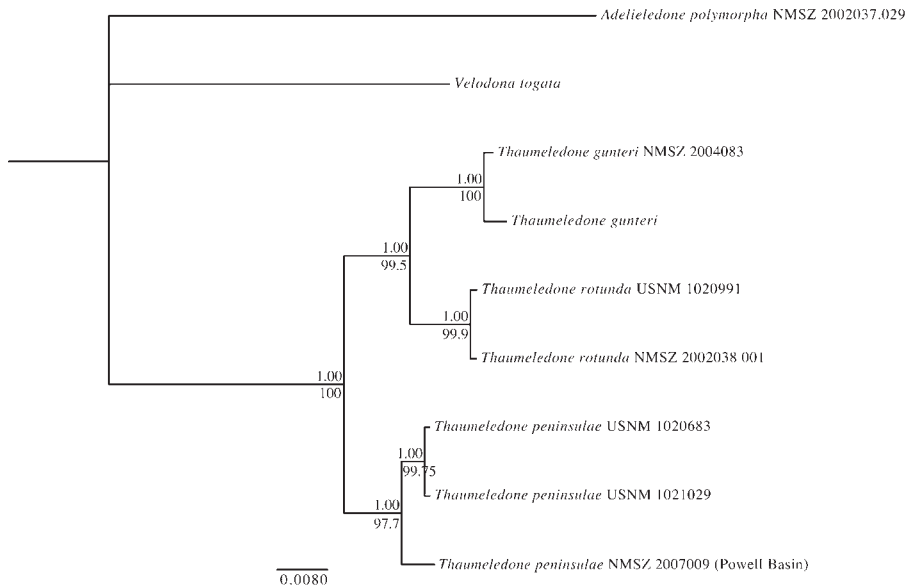
Fig. 3. Bayesian analysis tree depicting the phylogenetic relationship of five species (nine individuals) of Octopoda. The analysis employed five mitochondrial (12S rDNA, 16S rDNA, COI, COIII, *cytochrome oxidase b*) and one nuclear gene (*rhodopsin*). Bayesian support values are indicated above the nodes, maximum likelihood bootstrap values are indicated below the nodes.

phylogenetic studies (Carlini *et al.* 2001, Strugnell *et al.* 2004) have confirmed the basal position of *Adelieledone*. All relationships within the phylogenetic tree are highly supported by Bayesian posterior probabilities (PP) and maximum likelihood bootstrap (BS) values (Fig. 3). *Velodona togata* is basal to the *Thaumeledone* clade. The *Thaumeledone* species are divided into two clades (PP = 1.00, BS = 1.00). The first of these clades is highly supported (PP = 1.00, BS = 99.5) and comprises a sister taxa relationship between *T. gunteri* and *T. rotunda*.

The second clade contains a sister taxa relationship between two *T. peninsulae* individuals (USNM 1020683, USNM 1021039) from off the South Shetland Islands (PP = 1.00, BS = 99.75) and the *T. peninsulae* individual (NMSZ 2007009) from the Powell Basin (PP = 1.00, BS = 97.7).

Proportional pairwise comparisons (GTR+I+Γ) between genera were markedly higher (0.094–0.130) than comparisons of species within genera (0.020–0.033). Intraspecific pairwise comparisons ranged from 0.0006 within *T. rotunda* to 0.008 for comparisons between *T. peninsulae* from off the South Shetland Islands and the *T. peninsulae* individual from the Powell Basin (Fig. 4).

Molecular sequences for five of the six gene fragments sequenced were identical between the two *T. peninsulae* individuals from off the South Shetland Islands (Table II). These two individuals differed only in one base pair in the gene COI. Interestingly, these two *T. peninsulae* individuals possessed some sequence difference from the *T. peninsulae* individual from the Powell Basin for each of the five mitochondrial genes, ranging from 0.4% sequence difference for 16S rDNA to 1.7% difference for COIII. For COI the difference was 0.61% or four base pairs. The
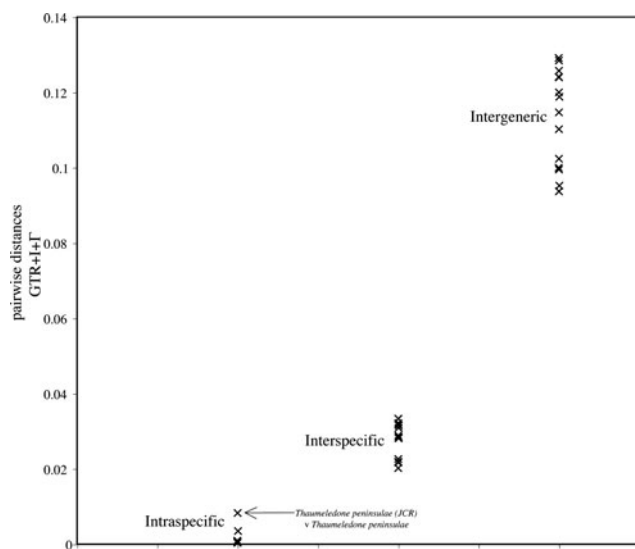

Fig. 4. Pairwise distances (GTR+I+Γ) among five species and nine individuals of Antarctic octopods.

Table II. Nucleotide differences between individuals of *T. peninsulae* (percentage difference is shown in brackets).

Gene	*Thaumeledone peninsulae* (USNM 1020683)	*Thaumeledone peninsulae* (USNM 1021039)	*Thaumeledone peninsulae* (NMSZ 2007009)
12S rDNA	0	0	2 (0.49%)
16S rDNA	0	0	1 & 1 insertion (0.4%)
COIII	0	0	9 (1.70%)
COI	1	1	4 (0.61%)
cytochrome oxidase b	0	0	2 (0.61%)
rhodopsin	0	0	0

molecular sequence of the nuclear gene *rhodopsin* was identical among all three *T. peninsulae* individuals.

Discussion

This study provides the first molecular phylogenetic analysis of the genus *Thaumeledone*. All relationships within the phylogeny are highly supported. The sister taxa relationship of *T. gunteri* and *T. rotunda* is intriguing. Allcock *et al.* (2004) suggested a close phylogenetic relationship between *T. peninsulae* and *T. gunteri*, citing the shared features of large irregular papillae covering the body surface, similar arm and web formulae, a similar funnel organ and loop in the rectum. Furthermore, *T. peninsulae* and *T. gunteri* have been previously captured from relatively close geographical locations and from similar depths. In contrast, *T. rotunda* is known from significantly deeper waters and is thought to be circumpolar in distribution. It also differs morphologically from the other two species in a number of features including the possession of a W shaped funnel organ, no loop in the rectum and notably smaller posterior salivary glands (Allcock *et al.* 2004). When discussing unpublished *T. brevis* records from the Southern Ocean (i.e. *T. rotunda* using the latest taxonomic revision), Voss (1988) stated '*T. gunteri* is probably a synonym'. However, the morphology of these species differs widely (see Allcock *et al.* 2004) and there is no evidence that Voss had examined the type material. At that time *T. gunteri* was known only from type material.

Despite some morphological similarities between *T. peninsulae* and *T. gunteri*, the molecular phylogenetics show a close sister taxa relationship between *T. gunteri* and *T. rotunda*. This suggests that at least some of the morphological features unique to *T. rotunda* may have evolved in conjunction with its distribution in deeper waters. For example the reduction in posterior salivary gland size (associated with paralysis of prey) may be due to the greater propensity of small and soft bodied prey items in the deep sea than in shallower depths (Voss 1988), and thus a reduced requirement for toxic agents to subdue these prey items. Reduction of such features in *T. rotunda* is consistent with the concept that reductions and losses of characters in many deepwater octopods has occurred convergently (Voight 1993).

The amount of sequence divergence between *Thaumeledone* species (ranging from 2–3.3%) is a relatively small level of congeneric divergence compared to other published studies. For example Moore (1995) found 6.5% average sequence divergence within genera for the gene COI for moths, whilst Hebert *et al.* (2004) reported 7.93% average divergence within genera for birds. However, the congeneric divergence of *Thaumeledone* is consistent with other Southern Ocean octopodid genera, with the genus *Pareledone* possessing a 1–2% sequence divergence (Allcock *et al.* 2007).

That differences were seen in the sequences of COI however, is worthy of comment. The gene COI has been chosen as the DNA barcoding marker. If sequence differences commonly occur in this gene in species with restricted larval dispersal, it may prove less than ideal for this purpose. Only further sequencing of individuals drawn from several populations of such species will reveal the suitability of COI as a barcoding marker for *Thaumeledone*.

A number of other taxa in the Southern Ocean have been reported to have undergone a recent radiation, for example groups of notothenioid fish, (e.g. *Trematomus*), isopods, amphipods, pycnogonids and ascidians (see Rogers 2007 for a review). It has been suggested that much of this diversification may have occurred as recently as the last 100 000 years and has resulted from climatic changes associated with expansion and contraction of ice sheets generating cycles of population fragmentation, allopatric speciation and secondary contact (Rogers 2007).

The phylogenetic tree and the plot of pairwise comparisons shows a clear difference is present between the *T. peninsulae* specimens from off the South Shetland Islands and the *T. peninsulae* individual from the Powell Basin. The overall percentage sequence difference between the Powell Basin specimen and those from off the South Shetland Islands is greater than that between other intraspecific comparisons but is less than the interspecific comparisons. It is also notable that the overall pairwise sequence divergence among these three specimens (0.8%) is greater than average intraspecific sequence differences observed for other species (e.g. moths 0.25%, Moore 1995). However, we believe these differences to be due to population level divergence and we do not believe that the differences observed are great enough to warrant erecting a new species. The morphological evidence mirrors the molecular genetic evidence. Although the MDS plots place the Powell Basin *T. peninsulae* (NMSZ 2007009) on the edge of the *T. peninsulae* cluster, (particularly when maximized for variables), this specimen is still within the *T. peninsulae* grouping. That it appears slightly more distinct in Fig. 2a could potentially be attributed to slightly greater differences between the male characters used in this analysis. However, additional analysis (not shown) indicates that including more specimens simply extends the spread of the cluster and the Powell Basin specimen therefore appears to be slightly less peripheral in Fig. 2b.

Differences between populations might be expected over these geographic distances in this species. *T. peninsulae* is known to produce large eggs (13 mm reported by Allcock *et al.* 2004) and it is likely that they produce demersal, crawl away young (Hochberg *et al.* 1992) which have limited dispersal capabilities. Given that the Powell Basin specimen was captured 300 nautical miles to the east of the other *T. peninsulae* specimens, it is likely that the differences observed reflect a relatively small amount of genetic mixing between these two locations.

Obtaining additional DNA specimens of the two New Zealand species of *Thaumledone*, *T. marshalli* and *T. zeiss* will greatly aid in our understanding of the evolutionary history of the genus. Morphometric analysis fails to separate the two New Zealand species from one another and from *T. peninsulae* and it is unlikely that such data would prove phylogenetically useful, even if they provided separation among species, since there was no congruence between morphological and molecular matrices for the Southern Ocean species. Codeable morphological characters are scarce and many (e.g. the size of the salivary glands) seem to reflect environmental influences (e.g. habitat depth) rather than evolutionary history. The addition of molecular sequences for these taxa is therefore essential to determine the origins and the genetic divergence within the genus. Bearing in mind the population level differences seen over relatively small distances in this study, it is also likely that future trawling efforts in the Southern Ocean, particularly on the slope waters of sub-Antarctic islands, will discover further species of *Thaumeledone*.

Acknowledgements

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