CSIRO PUBLISHING

Invertebrate Systematics, 2012, **26**, 548–560
http://dx.doi.org/10.1071/IS12023

DNA barcoding in *Nautilus pompilius* (Mollusca : Cephalopoda): evolutionary divergence of an ancient species in modern times

Rachel C. Williams[A], *Stephen J. Newman*[B] *and William Sinclair*[A,C,D]

[A]Centre for Wildlife Conservation, Faculty of Science and Natural Resources, University of Cumbria, Penrith, CA11 0AH, United Kingdom.
[B]Western Australian Fisheries and Marine Research Laboratories, Department of Fisheries, Government of Western Australia, PO Box 20, North Beach, WA 6920, Australia.
[C]Centre for Environmental Management, CQUniversity, Rockhampton, Qld 4702, Australia.
[D]Corresponding author. Email: billy.sinclair@cumbria.ac.uk

Abstract. DNA barcoding studies to elucidate the evolutionary and dispersal history of the current populations of *Nautilus pompilius* allow us to develop a greater understanding of their biology, their movement and the systematic relationships between different groups. Phylogenetic analyses were conducted on Australian *N. pompilius*, and COI sequences were generated for 98 discrete accessions. Sequences from samples collected across the distribution were sourced from GenBank and included in the analyses. Maximum likelihood revealed three distinct clades for *N. pompilius*: (1) populations sourced from west Australia, Indonesia and the Philippines; (2) populations collected from east Australia and Papua New Guinea; (3) western Pacific accessions from Vanuatu, American Samoa and Fiji, supporting previous findings on the evolutionary divergence of *N. pompilius.* A minimum spanning tree revealed 49 discrete haplotypes for the 128 accessions, from a total of 16 discrete sampling locations. Population similarity reflects oceanic topographic features, with divergence between populations across the *N. pompilius* range mirroring geographical separation. This illustrates the success of DNA barcoding as a tool to identify geographic origin, and looks to the future role of such technology in population genetics and evolutionary biology.

Additional keywords: *coxI*, conservation, population genetics.

Received 11 April 2012, accepted 13 September 2012, published online 19 December 2012

Introduction

The collation of evidence from fossils and molecular data demonstrates that in the early Cambrian Period, the major classes of molluscs diverged (Maloof *et al*. 2010; Smith and Caron 2010; Kröger *et al*. 2011). Nautiloids mark the first appearance of cephalopods as a separate molluscan entity over 500 million years ago (Jereb and Roper 2005) and remain the only living representatives of the large extinct group of ammonites, belemnites and nautiloids (Holland 1987; Suzuki *et al*. 2000; Bonnaud *et al*. 2004). Wray *et al*. (1995) postulated that the evolution of the ancestral progenitor population of modern *Nautilus pompilius* (Linneaus, 1751) has divided into three geographically distinct clades: one consisting of western Australian/Indonesian populations, one from the western Pacific and one from eastern Australia/Papua New Guinea. Recent work using a partial sequence of the cytochrome c oxidase subunit I (COI) gene region corroborated these predictions (Sinclair *et al*. 2011), identifying separation between the western Australian/Indonesian clade and the eastern Australian/Papua New Guinean clade. Conclusions indicate that this separation occurred due to the influence of geography and dispersal capacity (Hewitt 2004; Santos *et al*. 2006; Silva *et al*. 2010). The work of Bonacum *et al*. (2011) on the western Pacific clade also suggested that the divergence demonstrated within this clade appeared to be driven by geographic isolation. Large expanses of open water inhibit their dispersal; their reported inability to travel below ~800 m without imploding due to increasing water pressure (Saunders and Wehman 1977; Kanie *et al*. 1980; Wani 2004), and their vulnerability to predators when travelling across large expanses of open water, restricts the movement of *Nautilus* to the reef slopes (O'dor *et al*. 1993). As an additional limiting factor of their range, temperatures exceeding 25°C can be lethal to *Nautilus* within several days (Saunders and Spinosa 1979); however, they are able to adapt to adjusting temperatures and can move from 6 to 24°C water in several hours (Carlson *et al*. 1984). Limited dispersal distances between reefs emphasises the geographical isolation between populations (O'dor *et al*. 1993). As such, gene flow is restricted, resulting in documented diversification

Journal compilation © CSIRO 2012

between populations, the pattern of which appears to be occurring along geographic boundaries (Sinclair *et al*. 2011).

Dispersal constraints on marine species can strongly influence the genetic structure of a population (Hewitt 2004; Santos *et al*. 2006; Wieters *et al*. 2008; Silva *et al*. 2010). Marine organisms found in small and isolated populations can experience negative fitness effects as a result of limited connectivity and dispersal (Palumbi 1994; Wieters *et al*. 2008; Nunes *et al*. 2009). Isolation can also occur as a result of geography; physical barriers restrict movement and gene flow, causing populations to differentiate by genetic drift (Hindar *et al*. 1991). Currents and topology can exert a strong influence on the biogeography of the ocean fauna (Neumann 1960; Mitchell 1975; Genin *et al*. 1986; Maravelias 1999) by acting as barriers to dispersal (Thornhill *et al*. 2008).

Although physical separation can create a degree of diversity within the species, it also results in small isolated populations, with no means of rapid dispersal; the lifecycle of *N. pompilius* is not consistent with broadcast spawning dispersal patterns demonstrated by many marine organisms. *Nautilus* lay their eggs as discreet units attached to benthic substrate, and the resulting zygote is one of the largest known invertebrate eggs, taking ~11 months to hatch (Saunders and Landman 2010). The absence of a planktonic stage and a restrictive temperature regime for hatching success do not support a wide dispersal pattern for the eggs. Newly hatched individuals resemble miniature adults and are able to feed independently of any parental care. Early growth involves the formation of chambers behind the living chamber in which the *Nautilus* resides (Greenfest-Allen *et al*. 2010). Young are thought to descend from the shallower depths (~100 m) of spawning (Carlson *et al*. 1992) to deeper water where their life style mirrors that of adults, reaching sexual maturity after 7–8 years (Saunders and Landman 2010). Due to the entire process of maturation occurring on the same reef, dispersal is limited and genetic differences between individuals on different reefs are gradually accentuated.

The COI gene region is now becoming more widely recognised for this role in genetically distinguishing differences. As a DNA barcoding gene (Hebert *et al*. 2003*a*, 2003*b*), COI is applied in an approach designed to differentiate between morphologically similar species (Barrett and Hebert 2005). The utility of DNA barcoding as an identification tool is growing (COI is the gene fragment commonly used to demonstrate this variation in animals, ITS is the sequence used for identifying fungi and both *mat*K and *rbc*L sequences are used in plant identification: Hebert *et al*. 2003*a*, 2003*b*). DNA barcoding works by demonstrating that intraspecific genetic variation is exceeded by interspecific genetic variation to such an extent that a separation exists, enabling the assignment of unidentified individuals to their species with a negligible error rate (Doguzhaeva *et al*. 2007; Wiemers and Fiedler 2007).

Variation between *Nautilus* and all other cephalopods is both phenotypically and genotypically distinguishable, to the extent that *Nautilus* DNA samples may not amplify in PCR reactions using primers that work successfully in other cephalopod taxa (Strugnell *et al*. 2006*a*). Unlike the more recent evolution of coleoids (Kröger *et al*. 2011), *Nautilus* appear to have remained relatively unchanged in the last 200 million years (O'Dor and Webber 1991). This has resulted in a range of phenotypic differences: pin-hole eyes, a leathery hood, a cord-like brain, numerous tentacles without suckers and the absence of the ink sac are all regarded as primitive states and are all exclusively present in *Nautilus* (Shigeno *et al*. 2010). These morphological characteristics hold *Nautilus* as being a unique group within the Cephalopoda. Although identification to distinguish *Nautilus* from other cephalopods can be ascertained visually, this is not always the case between species and, to a greater extent, within a species.

In combination with assessing levels and extent of genetic variation, the COI barcoding technique could also prove useful in the correction of inaccurate distribution estimates. A single living *Nautilus* was even reported to have been transported ~2000 km from the Philippine Islands to the coast of Kyushu Island, Japan, by the Kuroshio oceanic current (Tanabe and Hamada 1978; Saunders and Landman 2010). Whilst the capture of a living individual this far from its known distribution is rare, oceanic currents are commonly reported to carry empty *Nautilus* shells away from their living distribution (Wani and Ikeda 2006; House 2010). Kobayashi (1954) first formalised the concept of post-mortem distribution, emphasising that the occurrence of shells was not representative of their living distribution, several thousand miles away. The knowledge regarding this concept has since developed and continued to inform on both ancient and present day influences of nautiloid dispersal (Manda 2008; Mapes *et al*. 2010; Schlögl *et al*. 2011). As a result of post-mortem transportation, the distribution of shells in the Indo-Pacific far exceeds that of living individual's geographical range; for example, *N. pompilius* shells have been collected from multiple locations around South Africa (House 2010). The ability to identify the geographic origin of a shell would help to prevent inaccurate distribution maps and also provide information on ocean currents and topography. This would rely on definite evidence of the shell origin; however, in the absence of this shell, drift cannot contribute to data on geographic range. Subsequently, the only conclusive way to identify populations is by live trapping, as demonstrated in this study.

Identifying and characterising genetic variation within *Nautilus* is of increasing importance due to the ongoing harvesting pressure on the species for the ornamental shell trade. Limited legislative protection exists to regulate the collection of *Nautilus* from the wild and heightened fishing intensity is known to occur in many areas such as the Philippines where no legislation exists to prevent this (Dunstan *et al*. 2010). In contrast, populations in areas such as the Great Barrier Reef are under protective legislation and populations in the Coral Sea are currently covered by a local agreement with fishermen not to target them (further protective measures are under discussion by Australian regulatory authorities at this time (Marine Bioregional Planning – Coral Sea, see http://www.environment.gov.au/coasts/mbp/coralsea/, accessed 2012, verified 7 November 2012)). The long-term effects of shell collection on the genetic variation of *Nautilus* are unknown; however, protective legislation is vital to the conservation of genetic diversity and evolutionary processes (Frankham *et al*. 2004).

With the morphological differences between *Nautilus* and other cephalopods leaving the COI barcoding system almost redundant at that level, its employment for population separation, origin identification, and geographical ranges could

be further developed to benefit our understanding of the evolution and diversity of the species. Here we assess the innovative system of barcoding and its potential to distinguish between populations of the same species, using *N. pompilius* as a case study.

Methods

Samples were collected from seven reefs (Fig. 1). The Rowley Shoals is a group of atoll-like coral reefs south of the Timor Sea, including Clerke Reef (17°19′S, 119°21′E) and Imperieuse Reef (17°35′S, 118°55′E), on the edge of one of the widest continental shelves in the world. Each atoll covers an area of 80–90 km^2 and both Clerke Reef and Imperieuse Reef rise steeply from the surrounding ocean floor. Imperieuse Reef is located 35 km south-west of Clerke Reef and is the most south-westerly of the reefs of the Rowley Shoals. Scott Reef (14°03′S, 121°46′E) is found at the edge of the continental shelf ~265 km off the coast of north-western Australia. Ashmore Reef is located in the Timor Sea (12°11′S, 122°59′E), on the edge of the Australian continental shelf and ~320 km off the Kimberley coast of Australia. East Australian samples were obtained from Osprey Reef (13°53′44″S, 146°33′27″E) and Shark Reef (14°07′59″S, 146°47′52″E) in the Coral Sea, and the Far North Great Barrier Reef (1°39′59″S, 143°58′56″E) in north-western Australia.

Nautilus samples were collected from traps, which have a curved, round or arrowhead design with a diameter of 1500 mm, a height of 600 mm and a mesh size of 50 mm. The traps were set on the reef slope at depths from 100–300 m for 24-h periods as part of a fish-sampling program, and were baited with ~1 kg of pilchards (*Sardinops sagax*) or chicken (*Gallus gallus domesticus*). Tentacle samples, 1–2 cm long, were taken from a labial tentacle from each individual collected. Identification photographs and shell measurements were taken before returning each individual back to the reef edge (at ~20 m). Tentacles were initially preserved in 20% DMSO (dimethylsulfoxide), 100 mM EDTA, saturated NaCl solution and stored at 4°C in the field. The tissue was subsequently washed in TE buffer and placed into 80% alcohol preservative for storage, until required for DNA extraction.

DNA was extracted from the samples using the QIAGEN DNeasy Tissue Kit following the manufacturers' instructions (Qiagen Pty Ltd, Victoria, Australia). Polymerase chain reaction (PCR) amplifications were conducted using 25-μL reaction volumes. Primer sequences previously demonstrated to be effective in amplifying *N. pompilius* COI were utilised, as described by Sinclair *et al*. (2007). The specific PCR protocol used with these samples had an initial 2 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 51°C and 2 min at 72°C, followed finally by 10 min at 72°C. Samples were purified using QIAGEN QIAquick PCR Purification columns following the manufacturers' instructions. Results were visualised on a 1% agarose gel at each stage in the methodology. Amplified products were sequenced at a commercial sequencing facility at DBS Genomics, Durham University, UK.

Any corrections made to the sequences were carried out manually using Chromas v1.45 Freeware (Technelysium Pty Ltd, Australia). Sequences from samples collected in Papua New Guinea, the Philippines, Indonesia, Vanuatu, Fiji and American Samoa (Fig. 1) were sourced from Bonacum *et al*. (2011), downloaded via GenBank (GenBank accession numbers:

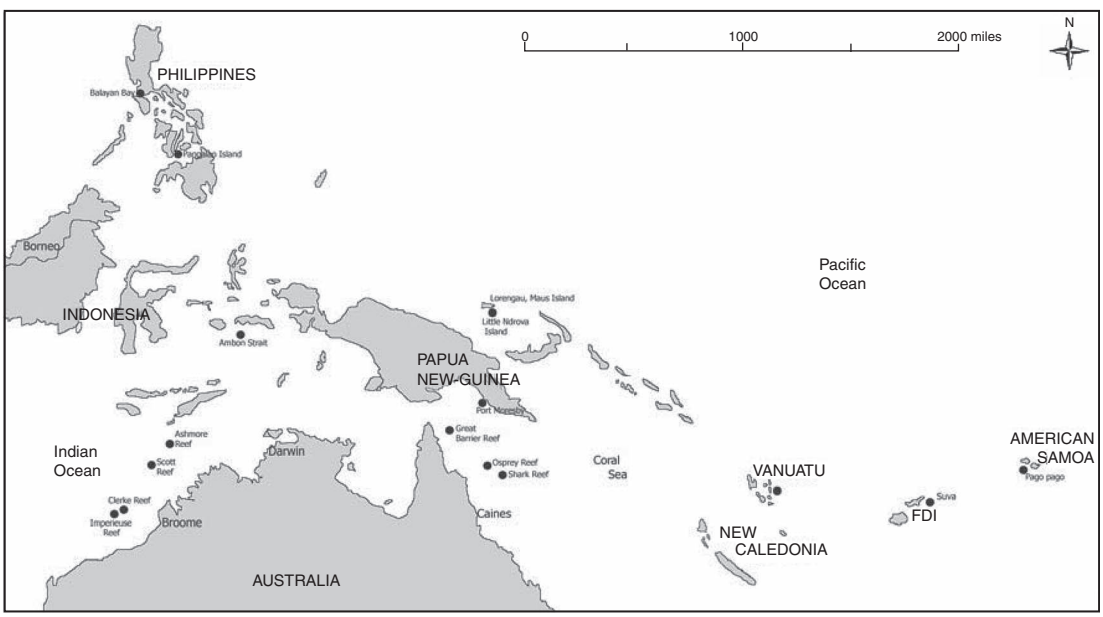

Fig. 1. Geographical locations of the sampling sites for *Nautilus pompilius*. From west Australia: Imperieuse, Clerke, Scott and Ashmore Reefs; east Australia: Shark, Osprey and Great Barrier Reefs; and samples sourced from GenBank: Philippines (Balayan Bay and Pangalao Island), Indonesia (Ambon Strait), Papua New Guinea (Lorengau and Port Moresby), Fiji (Suva), American Samoa (Pago Pago) and Vanuatu. Australian samples were collected from 100–300 m, by attracting individuals into traps using pilchard (*Sardinops sagax*) or chicken (*Gallus gallus domesticus*) baits. Captured individuals were kept in a dark, dedicated refrigerated tank (50 L) for a maximum of 15 h at temperatures of 16–19°C. All individuals were released at night at depths of 20–30 m.

GQ280190–GQ280194, GQ280201–GQ280212, GQ280214–GQ280216, GQ280240–GQ280249) and combined into the data file. The total 128 sequences were aligned using ClustalW in BioEdit v7.0.4.1 Freeware (Ibis Therapeutics, San Diego, CA, USA). Alignment of the sequences required no insertions or deletions (indels). The sections of sequence towards each end of the generated sequences were fully conserved in each individual.

A phylogenetic tree for maximum likelihood was constructed in PAUP* 4.0 Beta 10 Win (Harrison and Langdale 2006), with the TBR (tree-bisection-reconnection) algorithm and rooted on *Nautilus macromphalus* (GenBank Accession DQ472026). The HKY+G model and gamma-alpha shape parameter of 0.301, as indicated by ModelTest V.3.7, were used (Schneider *et al*. 2005). Bootstrap and jacknife values were generated (1000 resampling replicates) to provide statistical support.

Results

DNA was extracted from tentacle snips taken from *N. pompilius* from several sites surrounding Australia. Partial COI sequence information was generated (560 bp) and analysed from a total of 98 samples. In addition, a further 30 *N. pompilius* COI sequences were mined from GenBank, covering accessions from different geographical locations across the species' distribution (Bonacum *et al*. 2011). DNA sequences generated in this study were deposited in GenBank (accession numbers used in this study are shown in Appendix 1). On a GenBank BLAST search, this partial sequence aligned (99.0%) with a *N. pompilius* COI sequence (GenBank accession: AF120628). Similarly, a specimen identification search on the Barcodes of Life Database produced a 99.4% similarity match with voucher sequences from *N. pompilius*.

Alignment of the COI partial sequence data obtained from all 128 samples (412 bp) was carried out and a phylogenetic tree representing maximum likelihood was generated (Fig. 2) and rooted on *N. macromphalus* to assess genetic variation and establish whether geographic origin mirrored genetic separation. Bootstrap and jacknife values were generated and a value greater than 50 indicates that the node is of significance.

Within this tree, the presence of three distinct clades was detected illustrating the biogeographic and evolutionary separation of these accessions. The first clade shows the panmictic nature of the west Australian populations sampled (Ashmore, Imperieuse, Clerke and Scott Reef). These populations show no discrete separation and appear as a single, large panmictic population. Similarly, the Philippine and Indonesian accessions analysed in the dataset fully resolve within this clade. This grouping is strongly supported by both

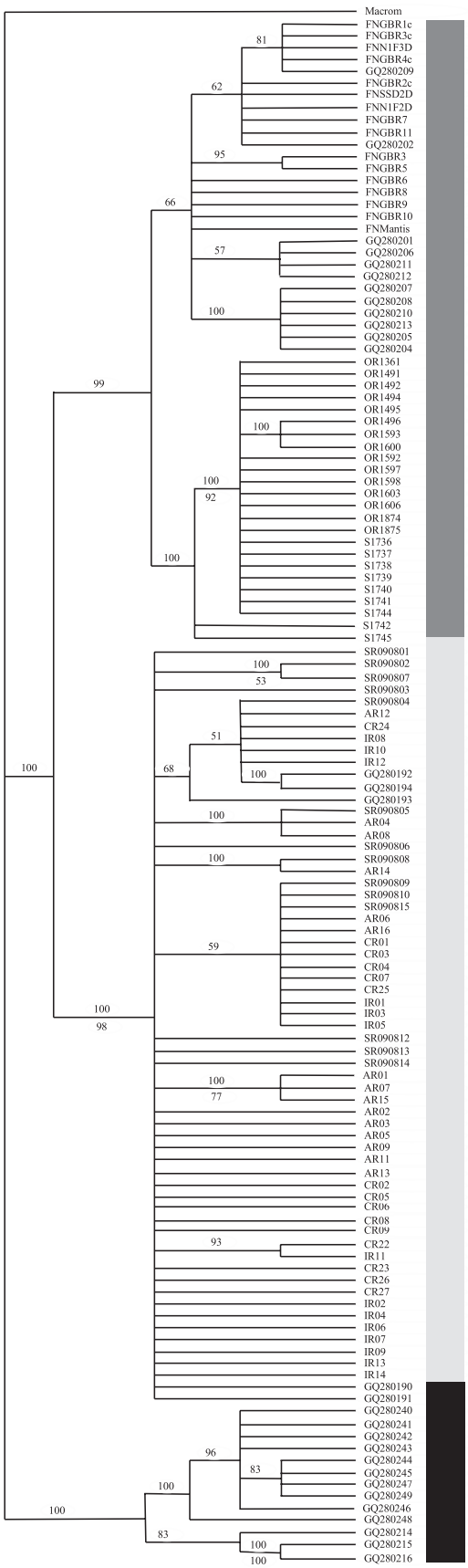

Fig. 2. Phylogenetic analyses of *N. pompilius* COI DNA sequences. Maximum-likelihood analysis generated a consensus tree constructed from the alignment of partial COI sequences of 412 bp from 128 *N. pompilius* sequences, and rooted against *Nautilus macromphalus*. Bootstrap values (1000 resampling replicates) higher than 50% are shown above the branches, Jackknife values are shown below. The dark grey bar indicates accessions from the east Australian clade, the light grey bar indicates accessions from the west Australian clade and the black bar indicates accessions from the western Pacific clade.

bootstrap (100) and jacknife (98) analysis. There is no further hierarchical separation of accessions within this clade into specific population groups; it maintains the panmictic structure with only limited resolution of discrete accessions.

The second independent clade (with strong bootstrap support) represents accessions collected from east Australia (Coral Sea reefs and Great Barrier Reef), in addition to accessions sourced from Papua New Guinea which are also resolved in this clade. There is further structuring within this large clade, however, and accessions from the Coral Sea reefs (Osprey and Shark) represent a sister clade to those accessions from the Great Barrier Reef and Papua New Guinea. This division is also strongly supported with bootstrap analysis (99) and supports previous reports of the divergence between these groups (Sinclair *et al*. 2007, 2011).

Appearing as a third discreet clade are all the western Pacific accessions from Vanuatu, American Samoa and Fiji, which were also strongly supported with bootstrap analysis of 100. Within this clade there was a grouping derived which separated out the accessions from Vanuatu (bootstrap and jacknife values of 100), and those from Fiji and American Samoa (Bonacum *et al*. 2011), which were clustered together again with bootstrap support (96).

A Minimum Spanning Tree (Fig. 3) constructed using Arlequin (ver. 3.5.1.2) illustrates the frequency of discrete haplotypes found within the samples. From the 128 samples analysed in this study, 49 discrete haplotypes were identified. Each of the three discrete clades are represented by different colours, nodes represent discreet haplotypes, with the k value signifying frequency of individuals represented in a particular node (which therefore have identical haplotypes based on the COI sequencing). In total, 12 haplotypes were found within the east Australian/Papua New Guinean clade, 30 haplotypes within the west Australian/Indonesian/Philippine clade and 7 haplotypes within the western Pacific clade (Table 1). Values between nodes illustrate the number of base pair differences between the individual haplotypes. Within the west Australian/Indonesian/Philippine populations, node values are all relatively small (values of 1.0–2.0 bp predominate, with one outlier at 4.0 bp). Within the east Australian/Papua New Guinean populations node values again average 1.0–2.0 bp, with the largest at 6.0 bp. The western Pacific clade, however, has multiple values of 1.0 bp (from Vanuatu accessions), but also some substantially higher at 17.0 and 18.0 bp (American Samoa and Fiji accessions) – this may be an artefact due to the small sample number at each population.

Between the populations of west Australian/Indonesian/Philippines and those of east Australian/Papua New Guinea, the discriminatory node value is much higher than the general internal node size, at 19.0 bp. Similarly, a node value of 30.0 bp separates the western Pacific clade from the east Australian/Papua New Guinea clade. This illustrates the significant separation of these populations in evolutionary terms, highlighted by the fact that no *N. pompilius* individual shares an identical haplotype with an individual from a different clade.

Discussion

The Class Cephalopoda (Phylum Mollusca) is highly successful, but cephalopods, as with many taxa, have not escaped population declines as a result of anthropomorphic

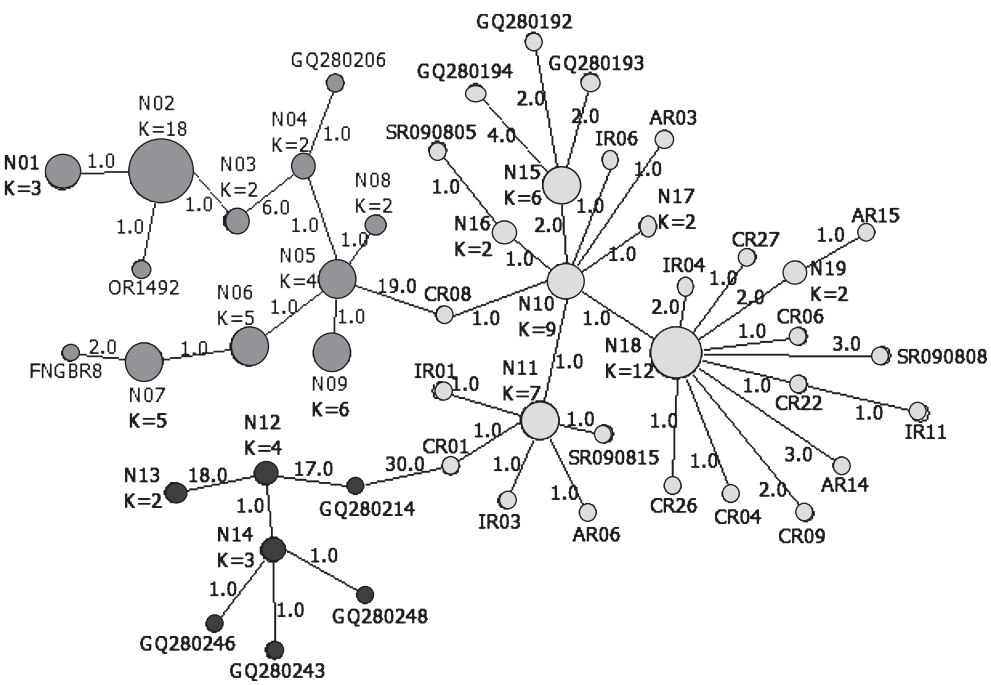

Fig. 3. Minimum Spanning Tree produced using Arlequin v.3.5.1.2. Nodes represent haplotypes, k values signify the frequency of individuals represented in a particular node, values between the nodes demonstrate base pair differences between haplotypes. Node colour corresponds to the three geographical clades: dark grey nodes are east Australian samples, light grey nodes are west Australian and black nodes represent the western Pacific samples.

Table 1. Individual *Nautilus pompilius* accession haplotypes that segregated, based on discrete COI DNA sequence, to form discrete nodes in Arlequin© analysis and MST generation

K values signify the frequency of individuals represented in a particular node

NO1	NO2	NO3	NO4	NO5	NO6	NO7	NO8	NO9	NO10	NO11	NO12	NO13	NO14	NO15	NO16	NO17	NO18	NO19
OR1600	OR1606	S1742	GQ280211	FNGBR9	FNGBR2C	FNGBR1C	FNGBR5	GQ280207	IR07	CR25	GQ280244	GQ280215	GQ280240	IR12	AR08	SR090802	CR02	AR07
OR1593	OR1874	S1745	GQ280212	FNGBR10	FNSSD2D	FNGBR3C	FNGBR3	GQ28008	IR09	CRO7	GQ280245	GQ280216	GQ280241	IR10	AR04	SR090802	CR05	AR01
OR1496	OR1875			FNGBR6	FNN1F2D	FNN1F3D		GQ28010	SR090801	CRO3	GQ280247		GQ280242	IR08			CR23	
	OR1603			FNMANTIS	FNGBR7	FNGBR7		GQ28013	SR090803	IR05	GQ280249			SR090807			IR02	
	OR1597				FNGBR11	FNGBR11		GQ28005	SR090806	AR16				AR12			IR13	
	OR1361							GQ28004	SR090812	SR090809				CR24			IR14	
	OR1491								SR090814	SR090810							AR09	
	OR1598																AR11	
	OR1592																	
	OR1494																	
	OR1495																	
	S1736																	
	S1737																	
	S1738																	
	S1739																	

activity (Moltschaniwskyj *et al*. 2007; Crook *et al*. 2009). The overexploitation of marine resources is an ongoing global concern and the finite supplies of marine organisms are now showing dramatic declines (Tittensor *et al*. 2006). *N. pompilius* is no exception, being the only extant cephalopod with an external shell has been a key factor in its exploitation and consequent decline. The ornamental shell trade targets *Nautilus* species and many populations are being overexploited, leading to population fragmentation and isolation, which contributes to diversification (Sinclair *et al*. 2011).

There is no clear concordance under the current classification of *Nautilus*, where potentially two to five distinct species are all grouped under a single species – *N. pompilius*. There is comparatively little knowledge of their population genetics, growth rates, and related population dynamics, which are essential criteria for sustainably managing *Nautilus* fisheries. Furthermore, the evolutionary division of the different *Nautilus* species is under question and needs to be redefined (Sinclair *et al*. 2007).

Our results show a significant degree of genetic divergence between the three proposed evolutionary clades, indicative of both movement between reefs, and ancient evolutionary history, as proposed by Wray *et al*. (1995). Maximum-likelihood analysis (Fig. 2) illustrates the panmictic nature of the west Australian/Indonesian/Philippines clade, showing no clear separation of the different populations. The west Australian populations (Ashmore, Imperieuse, Clerke and Scott Reefs) are all represented as one large intermixed population, indicating the extent of connectivity between the reefs. The Philippine and Indonesian accessions are also interspersed and resolved within the clade. This would indicate that *Nautilus* from the surrounding seas of the Indonesian archipelago are still, or have been in their recent evolutionary history, sympatrically distributed with the *Nautilus* surrounding west Australia.

The east Australian/Papua New Guinea clade demonstrates an increase in separation within the clade and appears to divide into two: the first half encompasses samples from Papua New Guinea and the far north Great Barrier Reef, and the second half contains samples from Osprey Reef and Shark Reef. Within the clade containing accessions from Papua New Guinea and the Great Barrier Reef, over 80% of Papua New Guinean samples are clustered together within the clade; the remaining are resolved within the Great Barrier Reef accessions (Fig. 2). This may reflect the recent evolutionary dispersal of accessions from Papua New Guinea moving down towards the east coast of Australia and becoming part of the intrinsic population there. The Osprey/Shark Reef grouping demonstrates a level of similarity indicative of that of a panmictic population (no population separation). When this is considered in terms of the topography of these locations, which, although physically quite close to each other, are separated by water deeper than 1000 m, they would be expected to show patterns indicative of isolated, self-contained populations. Their separation, however, in evolutionary timescales is such that they may not have been isolated from each other for long enough for such trends to have been established within the populations to date. Although it is evident that Papua New Guinea, and Osprey, Shark and far north Great Barrier Reefs form an individual clade, their phylogenetic structure would indicate that there is less

connectivity within this geographical clade than that found between the populations from west Australia, suggesting little or no current migration between sites.

The third clade incorporates samples solely from Bonacum *et al*. (2011); despite emerging as one separate clade, it illustrates a division with 100% statistical support for a divide between the Vanuatu accessions, and those from Fiji and American Samoa. This would indicate that, although their evolutionary distribution has resulted in a higher level of relatedness to each other than to the rest of the dataset, there appears to be no current migration between populations.

All three clades are shown to be more closely related to each other than they are to the outgroup used in this study, *N. macromphalus.*

The overall phylogeny presented here is a result of ancient distribution and current dispersal patterns, creating a measurable genetic divergence dependent on the degree of separation between populations. Genetic divergence between populations can be avoided with gene flow promulgated by dispersing individuals and multidirectional gene flow (Chesser 1991). The phylogenetic results for west Australia are indicative of individuals moving between populations, whereas the phylogenetic divide within the east Australian clade indicates otherwise. The dispersal ability of the species is determined by the topology between the discussed reefs; the lower depth limit of *Nautilus* is ~800 m (Saunders and Wehman 1977; Kanie *et al*. 1980; Wani 2004), with long-term habitat depth suggested to be limited to 300–500 m due to cameral flooding (Saunders and Landman 2010). Water depths surrounding the west Australian reefs show no potential inhibitory effects to movement of *N. pompilius*, which supports the topology of the maximum-likelihood analysis. During sampling, Clerke Reef had a surrounding depth of 390 m, therefore an individual *Nautilus* could easily travel the 35-km distance to Imperieuse Reef (O'dor *et al*. 1993; Wray *et al*. 1995), which is surrounded by ocean of depth ~320 m. There are also surrounding coral patches, thus there is the likelihood that these areas act as suitable transit 'corridors,' connecting the larger reefs (Genin *et al*. 1986; Rypien *et al*. 2008).

This topology does not apply to the east Australian clade, where Osprey Reef and the Great Barrier Reef are separated by a distance of ~250 km and by depths of more than 1000 m. Although this distance has been successfully travelled before by *Nautilus* (Tanabe and Hamada 1978; Saunders and Landman 2010), it is not a regular occurrence; their physiology suggests that the depths were too great to allow benthic travel, which would result in passage through large expanses of open water, thus dramatically reducing their survival rate due to predation (Saunders and Landman 2010). The genetic consequence of their inability to travel large distances over great depths is demonstrated in their phylogenetic separation within the clade. Although one individual is unlikely to travel the maximum dispersal distance of the west Australian reefs (determined by the length of connecting reefs in this area: Wieters *et al*. 2008), dispersal along partial distances of connecting reefs by individuals is sufficient to prevent the inbreeding problems commonly associated with isolated populations (Madsen *et al*. 1996; Kuhls *et al*. 2007; Rypien *et al*. 2008; Caputo *et al*. 2009; Griffiths *et al*. 2009; Santos *et al*. 2009; Trinkel *et al*. 2010). This potentially inhibits the development of any significant differences occurring between reefs.

The results presented here support the work of Wray *et al*. (1995) in demonstrating the presence of three distinct clades within *Nautilus* evolution. We expand on this hypothesis to reveal further separation in their more recent evolutionary history. Despite this separation, populations within each clade continue to show greater similarity to each other than they do to populations of another clade, demonstrating that the process of diversification has been occurring for longer between the clades than it has within them.

Recent work (Bonacum *et al*. 2011) suggests that the living *Nautilus* lineage originated around New Guinea, potentially only two million years ago, and one lineage of *Nautilus* voyaged from New Guinea to the more easterly archipelagos of New Caledonia, Fiji and Samoa, whereas another travelled to Australia, the Philippines and the South China Sea. Samples from potential progenitor populations in Papua New Guinea and the Philippines have allowed clearer elucidation of evolutionary divergence further back along evolutionary time. The varied lineage of each *N. pompilius* clade, and the current separation into smaller discrete populations, has resulted in genetic sequences unique to specific evolutionary clades. Results show that highly conserved regions have remained unchanged, still enabling species identification; however, variable regions are no longer generic across the species.

As an identification tool, DNA barcoding using the COI sequence has proved successful in distinguishing between clades but migration between populations has prevented population-level (individual reefs) identification of accessions. COI could therefore prove effective in reducing the scope for the plausible geographic origins of a sample found through post-mortem transportation. Although the tissue of the living animal is often no longer located within the retrieved shell, a sample could be taken from the remaining siphuncle tissue within the shell (Strugnell *et al*. 2006*b*). DNA barcoding would, at this present time, be unable to distinguish between an unprotected specimen taken from Papua New Guinea, and a protected specimen taken from the Great Barrier Reef, due to both populations belonging to the same geographic clade.

In earlier work, Dunstan *et al*. (2010) indicated that market forces are driving the ongoing development of the *Nautilus* fishing industry and called for an assessment of *Nautilus* species by the IUCN, potentially to have it categorised as globally endangered. Here we present data generated by DNA barcoding that supports the inherent vulnerability of *N. pompilius* populations to fishing pressure due to the discrete evolutionary history of each of the three identified clades and the current populations they represent. If these current populations in these areas were targeted by commercial fishermen, the risk of losing a unique genetic resource (potentially representing unique species and subspecies) is extreme.

Future work

Future work could expand on the use of barcoding to determine the origins of empty shells through the use of other barcoding sequences to assess whether a higher level of variation is

displayed between populations. This could incorporate the development of population-specific identifiers for discrete sections of the COI sequence, potentially through the use of single nucleotide polymorphisms as population-specific markers. This will require investment in the initial start-up process, it will provide a useful mechanism by which to address questions such as identity establishment for samples of unknown provenance to determine whether they have been illegally fished – thereby helping to corroborate and enact any protective legislation on *Nautilus*.

Further research is needed into the effects of fishing efforts on the overall structure of the current *Nautilus* populations and how this impacts the genetic and evolutionary diversity across the distribution. Such work will determine whether and where a sustainable fishery for *Nautilus* could be established while maintaining the range of genetic variation within the *Nautilus* group as a whole.

The continuing development of new, more powerful molecular technologies has opened up the genome of the world's flora and fauna to unprecedented scrutiny. By harnessing this technology, as has been done with DNA barcoding projects, we can utilise it to help understand the diversity of life around us. The range of life found in the world's oceans is dropping through a range of factors, not least anthropogenic, and as a priority, we must strive to understand what we can within as short a time as possible. Unique species such as *Nautilus* represent a flagship, iconic lifeform about which we know so little, yet DNA barcoding has helped to show the evolutionary pathways of dispersal in this ancient species, has shown the degree of genetic variation contained within the species, and may even be a deciding factor in reclassifying *N. pompilius* into discrete species or subspecies.

Acknowledgements

This work was funded in part by The Centre for Wildlife Conservation, Faculty of Science and Natural Resources, University of Cumbria, United Kingdom. The Department of Fisheries, Government of Western Australia, provided logistical support. The authors thank Tony, Liz, Euan and Douglas for their help in recording all the tissue samples used in the study. The authors sincerely thank their colleagues Dr Christine McPhie and Professor Graham Pegg for their constructive comments and assistance in the preparation of this manuscript.

References

Barrett, R. D. H., and Hebert, P. D. N. (2005). Identifying spiders through DNA barcodes. *Canadian Journal of Zoology* **83**, 481–491. doi:10.1139/z05-024

Bonacum, J., Landman, N. H., Mapes, R. H., White, M. M., White, A. J., and Irlam, J. (2011). Evolutionary radiation of present-day *Nautilus* and *Allonautilus. American Malacological Bulletin* **29**, 77–93. doi:10.4003/006.029.0221

Bonnaud, L., Ozouf-Costaz, C., and Boucher-Rodoni, R. (2004). A molecular and karyological approach to the taxonomy of *Nautilus. Comptes Rendus Biologies* **327**, 133–138. doi:10.1016/j.crvi.2003.12.004

Caputo, V., Giovannotti, M., Nisi Cerioni, P., Splendiani, A., Marconi, M., and Tagliavini, J. (2009). Mitochondrial DNA variation of an isolated population of the Adriatic brook lamprey *Lampetra zanandreai* (Agnatha: Petromyzontidae): phylogeographic and phylogenetic inferences. *Journal of Fish Biology* **75**, 2344–2351. doi:10.1111/j.1095-8649.2009.02413.x

Carlson, B. A., McKibben, J. N., and DeGruy, M. V. (1984). Telemetric investigation of vertical migration of *Nautilus belauensis* in Palau. *Pacific Science* **38**, 183–188.

Carlson, B., Awai, M., and Arnold, J. (1992). Hatching and early growth of *Nautilus belauensis* and implications on the distribution of the genus. *The Nautilus* **1**, 587–592.

Chesser, R. K. (1991). Influence of gene flow and breeding tactics on gene diversity within populations. *Genetics* **129**, 573–583.

Crook, R. J., Hanlon, R. T., and Basil, J. A. (2009). Memory of visual and topographical features suggests spatial learning in *Nautilus* (*Nautilus pompilius*). *Journal of Comparative Psychology* **123**, 264–274. doi:10.1037/a0015921

Doguzhaeva, L. A., Summesberger, H., Mutvei, H., and Brandstaetter, F. (2007). The mantle, ink sac, ink, arm hooks and soft body debris associated with the shells in Late Triassic coleoid cephalopod *Phragmoteuthis* from the Austrian Alps. *Palaeoworld* **16**, 272–284. doi:10.1016/j.palwor.2007.08.002

Dunstan, A., Alanis, O., and Marshall, J. (2010). *Nautilus pompilius* fishing and population decline in the Philippines: a comparison with an unexploited Australian *Nautilus* population. *Fisheries Research* **106**, 239–247. doi:10.1016/j.fishres.2010.06.015

Frankham, R., Ballou, J. D., and Briscoe, D. A. (2004). 'A Primer of Conservation Genetics.' (Cambridge University Press: Cambridge, UK.)

Genin, A., Dayton, P. K., Lonsdale, P. F., and Spiess, F. N. (1986). Corals on seamount peaks provide evidence of current acceleration over deep-sea topography. *Nature* **322**, 59–61. doi:10.1038/322059a0

Greenfest-Allen, E., Landman, N. H., and Saunders, W. B. (2010). *Nautilus* and *Allonautilus:* annotated bibliography of references published since 1987. In '*Nautilus:* the Biology and Paleobiology of a Living Fossil'. (Eds W. B. Saunders, and N. H. Landman.) pp. xxxix–lxxvii. (Springer: New York.)

Griffiths, A. M., Koizumi, I., Bright, D., and Stevens, J. R. (2009). A case of isolation by distance and short-term temporal stability of population structure in brown trout (*Salmo trutta*) within the River Dart, southwest England. *Evolutionary Applications* **2**, 537–554. doi:10.1111/j.1752-4571.2009.00092.x

Harrison, J. C., and Langdale, J. A. (2006). A step by step guide to phylogeny reconstruction. *The Plant Journal* **45**, 561–572. doi:10.1111/j.1365-313X.2005.02611.x

Hebert, P. D. N., Cywinska, A., Ball, S. L., and DeWaard, J. R. (2003*a*). Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London. Series B. Biological Sciences* **270**, 313. doi:10.1098/rspb.2002.2218

Hebert, P. D. N., Ratnasingham, S., and de Waard, J. R. (2003*b*). Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London. Series B. Biological Sciences* **270**, 96–99. doi:10.1098/rsbl.2003.0025

Hewitt, G. (2004). Genetic consequences of climatic oscillations in the Quaternary. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **359**, 183–195. doi:10.1098/rstb.2003.1388

Hindar, K., Jonsson, B., Ryman, N., and Ståhl, G. (1991). Genetic relationships among landlocked, resident, and anadromous brown trout, *Salmo trutta* L. *Heredity* **66**, 83–91. doi:10.1038/hdy.1991.11

Holland, C. H. (1987). The nautiloid cephalopods: a strange success. *Journal of the Geological Society* **144**, 1–15. doi:10.1144/gsjgs.144.1.0001

House, M. R. (2010). Geographic distribution of *Nautilus* shells. In '*Nautilus:* the Biology and Paleobiology of a Living Fossil'. (Eds W. B. Saunders, and N. H. Landman.) pp. 53–64. (Springer: New York.)

Jereb, P., and Roper, C. F. E. (2005). 'Cephalopods of the World: Chambered Nautiluses and Sepioids (Nautilidae, Sepiidae, Sepiolidae, Sepiadariidae, Idiosepiidae, and Spirulidae).' (Food and Agriculture Organization of the United Nations: Rome.)

Kanie, Y., Fukuda, Y., Nakayama, H., Seki, K., and Hattori, M. (1980). Implosion of living *Nautilus* under increased pressure. *Paleobiology* **6**, 44–47.

Kobayashi, T. (1954). A contribution towards Paleo-Flumenology, science of the oceanic currents of the past, with a description of a Miocene *Aturia* from central Japan. *Japanese Journal of Geology and Geography* **25**, 35–56.

Kröger, B., Vinther, J., and Fuchs, D. (2011). Cephalopod origin and evolution: a congruent picture emerging from fossils, development and molecules. *BioEssays* **33**, 602–613.

Kuhls, K., Keilonat, L., Ochsenreither, S., Schaar, M., Schweynoch, C., Presber, W., and Schonian, G. (2007). Multilocus microsatellite typing (MLMT) reveals genetically isolated populations between and within the main endemic regions of visceral leishmaniasis. *Microbes and Infection* **9**, 334–343. doi:10.1016/j.micinf.2006.12.009

Madsen, T., Stille, B., and Shine, R. (1996). Inbreeding depression in an isolated population of adders *Vipera berus*. *Biological Conservation* **75**, 113–118. doi:10.1016/0006-3207(95)00067-4

Maloof, A. C., Porter, S. M., Moore, J. L., Dudas, F. O., Bowring, S. A., Higgins, J. A., Fike, D. A., and Eddy, M. P. (2010). The earliest Cambrian record of animals and ocean geochemical change. *Bulletin of the Geological Society of America* **122**, 1731–1774. doi:10.1130/B30346.1

Manda, Š. (2008). Palaeoecology and palaeogeographic relations of the Silurian phragmoceratids (Nautiloidea, Cephalopoda) of the Prague Basin (Bohemia). *Bulletin of Geosciences* **83**, 39–62. doi:10.3140/bull.geosci.2008.01.039

Mapes, R. H., Landman, N. H., Cochran, K., Goiran, C., de Forges, B. R., and Renfro, A. (2010). Early taphonomy and significance of naturally submerged *Nautilus* shells from the New Caledonia region. *Palaios* **25**, 597–610. doi:10.2110/palo.2009.p09-109r

Maravelias, C. D. (1999). Habitat selection and clustering of a pelagic fish: effects of topography and bathymetry on species dynamics. *Canadian Journal of Fisheries and Aquatic Sciences* **56**, 437–450. doi:10.1139/f98-176

Mitchell, H. (1975). Sea-surface topography around Australia. *Surveys in Geophysics* **2**, 117–129. doi:10.1007/BF01447940

Moltschaniwskyj, N. A., Hall, K., Lipinski, M. R., Marian, J. E. A. R., Nishiguchi, M., Sakai, M., Shulman, D., Sinclair, B., Sinn, D., and Staudinger, M. (2007). Ethical and welfare considerations when using cephalopods as experimental animals. *Reviews in Fish Biology and Fisheries* **17**, 455–476. doi:10.1007/s11160-007-9056-8

Neumann, G. (1960). On the effect of bottom topography on ocean currents. *Ocean Dynamics* **13**, 132–141.

Nunes, F., Norris, R., and Knowlton, N. (2009). Implications of isolation and low genetic diversity in peripheral populations of an amphi-Atlantic coral. *Molecular Ecology* **18**, 4283–4297. doi:10.1111/j.1365-294X.2009.04347.x

O'dor, R. K., and Webber, D. M. (1991). Invertebrate athletes: trade-offs between transport efficiency and power density in cephalopod evolution. *The Journal of Experimental Biology* **160**, 93–112.

O'dor, R., Forsythe, J., Webber, D., Wells, J., and Wells, M. (1993). Activity levels of *Nautilus* in the wild. *Nature* **362**, 626–628. doi:10.1038/362626a0

Palumbi, S. R. (1994). Genetic divergence, reproductive isolation, and marine speciation. *Annual Review of Ecology and Systematics* **25**, 547–572. doi:10.1146/annurev.es.25.110194.002555

Rypien, K. L., Andras, J. P., and Harvell, C. (2008). Globally panmictic population structure in the opportunistic fungal pathogen *Aspergillus sydowii*. *Molecular Ecology* **17**, 4068–4078. doi:10.1111/j.1365-294X.2008.03894.x

Santos, S., Hrbek, T., Farias, I. P., Schneider, H., and Sampaio, I. (2006). Population genetic structuring of the king weakfish, *Macrodon ancylodon* (Sciaenidae), in Atlantic coastal waters of South America: deep genetic divergence without morphological change. *Molecular Ecology* **15**, 4361–4373. doi:10.1111/j.1365-294X.2006.03108.x

Santos, X., Brito, J. C., Caro, J., Abril, A. J., Lorenzo, M., Sillero, N., and Pleguezuelos, J. M. (2009). Habitat suitability, threats and conservation of isolated populations of the smooth snake (*Coronella austriaca*) in the southern Iberian Peninsula. *Biological Conservation* **142**, 344–352. doi:10.1016/j.biocon.2008.10.030

Saunders, W. B., and Landman, N. H. (2010). '*Nautilus*: the Biology and Paleobiology of a Living Fossil.' (Springer Verlag: New York.)

Saunders, W. B., and Spinosa, C. (1979). *Nautilus* movement and distribution in Palau, western Caroline Islands. *Science* **204**, 1199–1201. doi:10.1126/science.204.4398.1199

Saunders, W. B., and Wehman, D. A. (1977). Shell strength of *Nautilus* as a depth limiting factor. *Paleobiology* **3**, 83–89.

Schlögl, J., Chirat, R., Balter, V., Joachimski, M. M., Hudackova, N., and Quillévéré, F. (2011). Aturia from the Miocene Paratethys: An exceptional window on nautilid habitat and lifestyle. *Palaeogeography, Palaeoclimatology, Palaeoecology* **308**, 330–338. doi:10.1016/j.palaeo.2011.05.037

Schneider, S., Roessli, D., and Excoffier, L. (2005). Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1**, 47–50.

Shigeno, S., Takenori, S., and Boletzky, S. V. (2010). The origins of cephalopod body plans: a geometrical and developmental basis for the evolution of vertebrate-like organ systems. In 'Cephalopods – Present and Past'. (Eds K. Tanabe, Y. Shigeta, T. Sasaki and H. Hirano.) pp. 23–34. (Tokai University Press: Tokyo.)

Silva, I. C., Mesquita, N., and Paula, J. (2010). Lack of population structure in the fiddler crab *Uca annulipes* along an East African latitudinal gradient: genetic and morphometric evidence. *Marine Biology* **157**, 1113–1126. doi:10.1007/s00227-010-1393-9

Sinclair, B., Briskey, L., Aspden, W., and Pegg, G. (2007). Genetic diversity of isolated populations of *Nautilus pompilius* (Mollusca, Cephalopoda) in the Great Barrier Reef and Coral Sea. *Reviews in Fish Biology and Fisheries* **17**, 223–235. doi:10.1007/s11160-006-9030-x

Sinclair, W., Newman, S., Vianna, G., Williams, S., and Aspden, W. (2011). Spatial subdivision and genetic diversity in populations on the east and west coasts of Australia: the multi-faceted case of *Nautilus pompilius* (Mollusca, Cephalopoda). *Reviews in Fisheries Science* **19**, 52–61. doi:10.1080/10641262.2010.533794

Smith, M. R., and Caron, J. B. (2010). Primitive soft-bodied cephalopods from the Cambrian. *Nature* **465**, 469–472. doi:10.1038/nature09068

Strugnell, J., Jackson, J., Drummond, A. J., and Cooper, A. (2006*a*). Divergence time estimates for major cephalopod groups: evidence from multiple genes. *Cladistics* **22**, 89–96. doi:10.1111/j.1096-0031.2006.00086.x

Strugnell, J., Norman, M., and Cooper, A. (2006*b*). DNA from beach-washed shells of the ram's horn squid, *Spirula spirula*. *Bulletin of Marine Science* **78**, 389–391.

Suzuki, T., Fukuta, H., Nagato, H., and Umekawa, M. (2000). Arginine kinase from *Nautilus pompilius*, a living fossil. *The Journal of Biological Chemistry* **275**, 23884. doi:10.1074/jbc.M002926200

Tanabe, K., and Hamada, T. (1978). Keeping record of a drifted living *Nautilus pompilius* specimen caught off Kagoshima Bay and its significance. *Kaiyo Kagaku* **10**, 1011–1017.

Thornhill, D. J., Mahon, A. R., Norenburg, J. L., and Halanych, K. M. (2008). Open-ocean barriers to dispersal: a test case with the Antarctic Polar Front and the ribbon worm *Parborlasia corrugatus* (Nemertea: Lineidae). *Molecular Ecology* **17**, 5104–5117. doi:10.1111/j.1365-294X.2008.03970.x

Tittensor, D. P., Worm, B., and Myers, R. A. (2006). 'Macroecological Changes in Exploited Marine Systems.' (University of Chicago Press: Chicago, IL.)

Trinkel, M., Funston, P., Hofmeyr, M., Hofmeyr, D., Dell, S., Packer, C., and Slotow, R. (2010). Inbreeding and density-dependent population growth in a small, isolated lion population. *Animal Conservation* **13**, 374–382. doi:10.1111/j.1469-1795.2009.00344.x

Wani, R. (2004). Experimental fragmentation patterns of modern *Nautilus* shells and the implications for fossil cephalopod taphonomy. *Lethaia* **37**, 113–123. doi:10.1080/00241160410006420

Wani, R., and Ikeda, H. (2006). Planispiral cephalopod shells as a sensitive indicator of modern and ancient bottom currents: new data from flow experiments with modern *Nautilus pompilius. Palaios* **21**, 289. doi:10.2110/palo.2004.p04-92

Wiemers, M., and Fiedler, K. (2007). Does the DNA barcoding gap exist? A case study in blue butterflies (Lepidoptera: Lycaenidae). *Frontiers in Zoology* **4**, doi:10.1186/1742-9994-4-8

Wieters, E. A., Gaines, S. D., Navarrete, S. A., Blanchette, C. A., and Menge, B. A. (2008). Scales of dispersal and the biogeography of marine predator–prey interactions. *American Naturalist* **171**, 405–417. doi:10.1086/527492

Wray, C. G., Landman, N. H., Saunders, W. B., and Bonacum, J. (1995). Genetic divergence and geographic diversification in *Nautilus. Paleobiology* **21**, 220–228.

Appendix 1. GenBank accession numbers used in this study

Sampling location and sample reference	GenBank accession no.
Far North Great Barrier Reef, east Australia	
FNGBR1c	JQ862293
FNGBR2c	JG862294
FNSSD2D	JN227635
FNN1F2D	JQ862295
FNGBR3	JQ862296
FNGBR3c	JQ862297
FNN1F3D	JQ862298
FNGBR4c	JQ862299
FNGBR5	JQ862300
FNGBR6	JQ862301
FNGBR7	JQ862302
FNGBR8	JQ862303
FNGBR9	JQ862304
FNGBR10	JQ862305
FNGBR11	JQ862306
FNMantis1	JQ862307
Osprey Reef, east Australia	
OR1361	JQ862308
OR1491	JQ862309
OR1492	JQ862310
OR1494	JQ862311
OR1495	JQ862312
OR1496	JQ862313
OR1592	JQ862314
OR1593	JQ862315
OR1597	JQ862316
OR1598	JQ862317
OR1600	JN227630
OR1603	JQ862318
OR1606	JQ862319
OR1874	JQ862320
OR1875	JQ862231
Scott Reef, west Australia	
SR090801	JQ890081
SR090802	JQ890082
SR090803	JQ890083
SR090804	JQ890084
SR090805	JQ890085
SR090806	JQ890086
SR090807	JQ890087
SR090808	JQ890088
SR090809	JQ890089
SR090810	JQ890090
SR090812	JQ862322
SR090813	JQ862323
SR090814	JQ862324
SR090815	JQ862325
Ashmore Reef, west Australia	
AR01	JN227639
AR02	JN227640
AR03	JN227641
AR04	JN227642
AR05	JN227643
AR06	JN227644
AR07	JN227645
AR08	JN227646
AR09	JN227647
AR11	JN227648

(*continued next page*)

Appendix 1. (*continued*)

Sampling location and sample reference	GenBank accession no.
AR12	JQ429507
AR13	JQ429508
AR14	JQ429509
AR15	JQ429510
AR16	JQ429511
Clerke Reef, west Australia	
CR01	JN227649
CR02	JN227650
CR03	JN227651
CR04	JN227652
CR05	JN227653
CR06	JN227654
CR07	JN227655
CR08	JN227656
CR09	JN227657
CR22	JN227658
CR23	JQ429526
CR24	JQ429527
CR25	JQ429528
CR26	JQ429529
CR27	JQ429530
Imperieuse Reef, west Australia	
IR01	JN227659
IR02	JN227660
IR03	JN227661
IR04	JN227662
IR05	JN227663
IR06	JN227664
IR07	JN227665
IR08	JN227667
IR09	JQ429555
IR10	JQ429556
IR11	JQ429557
IR12	JQ429558
IR13	JQ429559
IR14	JQ429560
Shark Reef, east Australia	
S1736	JQ862326
S1737	JQ862327
S1738	JQ862328
S1739	JQ862329
S1740	JQ862330
S1741	JQ862331
S1742	JQ862332
S1744	JQ862333
S1745	JQ862334
Vanuatu	
GQ280240	GQ280240
GQ280241	GQ280241
GQ280242	GQ280242
GQ280243	GQ280243
GQ280244	GQ280244
GQ280245	GQ280245
GQ280246	GQ280246
GQ280247	GQ280247
GQ280248	GQ280248
GQ280249	GQ280249
Ambon Strait, Indonesia	
GQ280190	GQ280190

(*continued next page*)

Appendix 1. (*continued*)

Sampling location and sample reference	GenBank accession no.
GQ280191	GQ280191
Pangalao Island, Philippines	
GQ280192	GQ280192
Balayan Bay, Philippines	
GQ280193	GQ280193
GQ280194	GQ280194
Port Moresby, Papua New Guinea	
GQ280201	GQ280201
GQ280202	GQ280202
Little Ndrova Island, Papua New Guinea	
GQ280206	GQ280206
GQ280207	GQ280207
GQ280208	GQ280208
GQ280209	GQ280209
GQ280210	GQ280210
GQ280211	GQ280211
GQ280212	GQ280212
GQ280213	GQ280213
Lorengau, Manus Island, Papua New Guinea	
GQ280204	GQ280204
Komuli Island, Papua New Guinea	
GQ280205	GQ280205
Pago Pago, American Samoa	
GQ280214	GQ280214
Suva, Fiji	
GQ280215	GQ280215
GQ280216	GQ280216