Evolutionary biology of the exploited cephalopod,

Nautilus pompilius

Rachel C. Williams

Lancaster University

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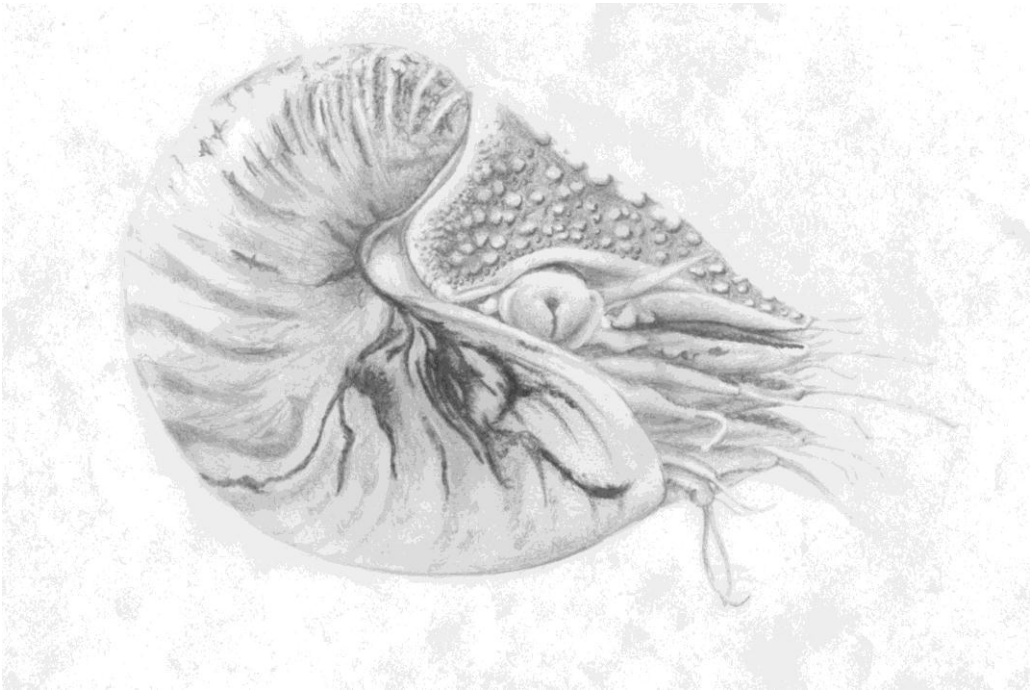
A thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

DECLARATION

I declare that this thesis is my own work and has not been submitted for a degree at any other university. Any section of the thesis that has been published has been clearly identified.



"Few organisms have been as well known to the layman, but as poorly known to science, as the chambered nautilus"

— Saunders and Landman (2010)

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Author: Rachel Clare Williams, BSc (Hons)
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ABSTRACT

Commercial species worldwide are experiencing significant population declines. Marine research is needed to understand species biology in an attempt to sufficiently manage levels of exploitation. *Nautilus pompilius* is unsustainably fished for the ornamental shell trade and large-scale population losses in the Philippines have been reported. We generated data that are intended to inform legislation to protect *N. pompilius* from overexploitation. Specifically, we investigated the evolutionary divergence and genetic structure between populations sampled from across the Indo-Pacific. In addition to research into the genetic structure of *N. pompilius* populations we also addressed the pharmacological responses of neurotransmitters widely used across the animal kingdom.

We collected data from the mitochondrial locus cytochrome c oxidase sub unit I (COI) and a novel microsatellite-enriched genomic library to describe the relatedness of *N. pompilius* populations sampled from across their range. Gene flow between west Australia and the Philippines was modelled using approximate Bayesian computation (ABC) analysis. Initial experiments to determine phylogenetically taxon specific usage of widely used neurotransmitters were conducted by testing for the presence and absence of targeted receptors using specific drugs. Demonstrating sufficient responses to these drugs will confirm the scope for further research into the physiological role played by receptors in *N. pompilius,* thus allowing us to determine taxonomic relationships at this

fundamental level. Finding relatively high protein sequence homologies in cross species comparisons would be informative both on the evolution of nautiloids, and the functional role of receptors across taxa.

Results of maximum likelihood phylogenetic analyses show that *N. pompilius* is divided into three distinct clades, with additional subdivision occurring within two of these clades. Genetic structure is high between Great Barrier Reef individuals, and those of Osprey Reef and Shark Reef in the Coral Sea (F_{ST}=0.312, 0.229 respectively). Interestingly, low genetic structure was shown between west Australia and the Philippines (F_{ST}=0.015), despite the large geographic scale from which these samples were collected. Further analyses of genetic data collected from west Australia and Philippine samples using ABC indicate that the observed genetic similarity is not the result of current gene flow and that individuals sampled from these regions are indeed genetically isolated. This similarity is explained by large effective population sizes over time suggesting a minor role for genetic drift. The population structure of east Australia and the west Pacific reflects oceanic topographic features. Recorded drug/tissue responses warrant further exploration into this area of their evolution, enabling us to compare biological signalling pathways among taxa and understand the unique nature of *Nautilus* at this fundamental level.

Our data demonstrate widespread substructure and isolation among populations of *N. pompilius*. Furthermore, we show that local extinction of *N. pompilius* in the Philippines is possible due to their low levels of migration. These findings reduce gaps in our knowledge of *N. pompilius* biology that currently inhibit their conservation classification. Results support the case to gain CITES protection for *N. pompilius.*

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INTRODUCTION

1.1 THE EVOLUTION OF CEPHALOPODS

1.1.1 HISTORIC CLASSIFICATION OF CEPHALOPODS

The first reference to ammonites made in literature is likely contained in Gaius Plinius Secundus's (23–79 A.D.) book, *Naturalis Historia*. His reference to Egyptian gods was interpreted literally by medieval scientists and the fossils were termed "Ammon's horns" by Georgius Agricola (1494-1555). The name "ammonites" was coined by French natural historian Bruguière (1792) and encompassed all fossils previously known as Ammon's horns. Although dated 1792, ammonite descriptions were published earlier, in 1789 (Dodge 1947), a date generally accepted as the first use of Linnaean binominal nomenclature in ammonitology (Shevyrev 2006). Despite the inclusion of "ammonite" in the Index of the Rejected Names in Zoology (Starobogatov 1983), it is now a term widely accepted by both scientists and laypeople to describe a group of fossil cephalopods.

The cephalopod lineage can be traced back over 500 million years (O'Dor & Webber 1991), during which they have developed into the third most speciose class of Mollusca (Yokobori *et al.* 2007). Cephalopods reached the peak of their taxonomic diversity, abundance and geographic distribution during the Cambrian period (Teichert 1988). Early cephalopods occupied the top of the food chain in marine Ordovician environments (Kröger & Yun-Bai 2009). Knowledge of the extent and duration of this dominance remains incomplete, but the literature is in agreement that the majority of cephalopods were extinct by the end of the Cretaceous, leaving nautiloids as the sole surviving group (Teichert 1988; Jereb & Roper 2005; Kröger & Yun-Bai 2009; Kashiyama *et al.* 2010; Kröger 2011).

1.1.2 *DIVERGENCE WITHIN CEPHALOPODA: NAUTILOIDS AND COLEOIDS*

The class Cephalopoda now includes the most complex of the invertebrates (Kröger *et al.* 2011), and they are distributed in all oceans excluding only the Black Sea (Jereb & Roper 2005). Members of this class inhabit every marine microhabitat, from surface waters to intertidal rocky shores to the deep sea, and are ecologically important in both tropical reefs and arctic habitats (Boyle & Rodhouse 2005; Jereb & Roper 2005).

Current classification divides living cephalopods into two subclasses: the Coleoidea (squids, cuttlefishes and octopuses), and the Nautiloidea (*Nautilus* spp. Linaeus and *Allonautilus* spp. Ward and Saunders). Time estimates for the divergence within Cephalopoda and the appearance of coleoids have been regularly re-assigned within the Palaeozoic era, based on technological advancements (Jereb & Roper 2005; Strugnell *et al.* 2006a; Bergmann *et al.* 2006; Warnke *et al.* 2011; Allcock *et al.* 2011; Kröger *et al.* 2011). The reduction and internalisation, or complete loss, of the shell in coleoids is noticeably different between the two subclasses (Teichert 1988; Allcock *et al.* 2011; Kröger *et al.* 2011). This is an evolutionary event thought to be unique (Kröger *et al.* 2011), leaving nautiloids as the only extant cephalopods with an external shell (Jereb & Roper 2005; Sasaki *et al.* 2010). The evolution and consequent loss or internalisation of the shell in coleoid cephalopods has led to the poor preservation of this subclass (Strugnell *et al.* 2005). The combination of the almost total absence of soft parts (Morris 1989) and the rapid decay in most sedimentary environments has resulted in a scarcity of well-fossilised specimens (Strugnell *et al.* 2005; Shigeno *et al.* 2010). The fossil history of cephalopods before shell loss remains rich due to their phragmocone having been commonly preserved in the rock record (Kröger 2011). Previous to the accessibility of molecular analysis as a tool, hypotheses regarding cephalopod origins have relied heavily on the anatomical analysis of extant cephalopods (Owen 1832) and fossil analysis (Kashiyama *et al.* 2010). Unfortunately the preservation of hard parts is not

absolute; transport, physical abrasion and chemical damage can result in fossils that are of varying use (Morris 1989) and consequently the specifics of the early evolution of cephalopods have sparked controversy (Donovan 1964; Lipps & Sylvester 1968).

In reconstructing cephalopod taxonomy, the discovery of soft parts preserved in the Burgess Shale held significance on a scale that had not previously been seen (Morris 1989). Due to the morphological similarity of the earliest undoubted cephalopods and their soft bodied reconstructions, the ancestral state of cephalopods was thought of as one resembling a benthic mollusc with a high conical shell (Kröger 2011). The fossils led to the suggestion that nautiloids evolved from a non-mineralised, coleoid-like ancestor related to the nectocaridids (Smith & Caron 2010). Kröger *et al.* (2011) disputed the conclusion that *Nectocaris* represents a cephalopod at all; hypothesising that the specimen is most likely not a mollusc but a representative of an independent lineage within the Lophotrochozoa, whose mode of life will have been remarkably similar to cephalopods.

1.1.3 *Taxonomic distinction of Nautiloids*

It is through their rich fossil history that *Nautilus*, often referred to as a living fossil, have become of great significance in the clarification and arrangement of ancient nomenclature (Barnes 1984). There is very little to dispute that as the most primitive extant cephalopod, *Nautilus* appear to have remained relatively unchanged for the last 200 million years (O'Dor & Webber 1991). Unfortunately there is a scarcity of fossil nautiloids in Caenozoic sediments, in comparison to the Palaeozoic and Mesozoic nautiloids and ammonoids, possibly due to lower population densities at this time (Wani *et al.* 2008). The first *Nautilus* fossil was collected in 1979 and is believed to be an early Pleistocene specimen (Wani *et al.* 2008). Whilst this fossil solely represents hard parts, it has enabled predictions regarding the evolution of their entire physiology.

Pinhole eyes, a leathery hood, a cord-like brain, numerous tentacles without suckers and the absence of the ink sac are all seen as primitive states (Shigeno *et al.* 2010), and are all present in *Nautilus*. The lowest number of chromosomes among cephalopods (52), suggests that the chromosome characteristics of *Nautilus* (small number and small in size) are the ancestral state for cephalopods (Bonnaud *et al.* 2004). Their primordial state was supported upon the discovery that *Nautilus* hemocyanin genes lack introns (Lieb *et al.* 2004). Multiple tentacles however, were dismissed as a primitive state in cephalopods and results concluded that this was in fact a derived character (Kröger 2011).

The numerous tentacles of *Nautilus* are classified into three groups (Owen 1832): (i) one pre- and one post-ocular tentacle located in front of and behind each eye, (ii) a variable number of labial tentacles, (iii) 19 pairs of digital tentacles. These tentacles differ from those of other cephalopods by lacking suckers or hooks, and instead have adhesive structures found on the digital tentacles. The "glue" found here has been shown to consist mainly of neutral mucopolysaccharides (von Byern *et al.* 2012). Each digital tentacle consists of an extendable, muscular cirrus enclosed in a protective sheath (Fig. 1), and the cirri have adhesive annular ridges on one side (Saunders & Landman 2010). In males the labial tentacles are modified as the spadix, a complex of three tentacles enclosed by a sheath, on the right ventral side (Sasaki *et al.* 2010). Like Octopus, *Nautilus* cirri have the same muscle arrangement and are capable of creating changes in length and bending movement (Saunders & Landman 2010). Adhesive detachment is also thought to be operated manually via muscle contraction of the adhesive ridges and then tentacle retraction (von Byern *et al.* 2012).

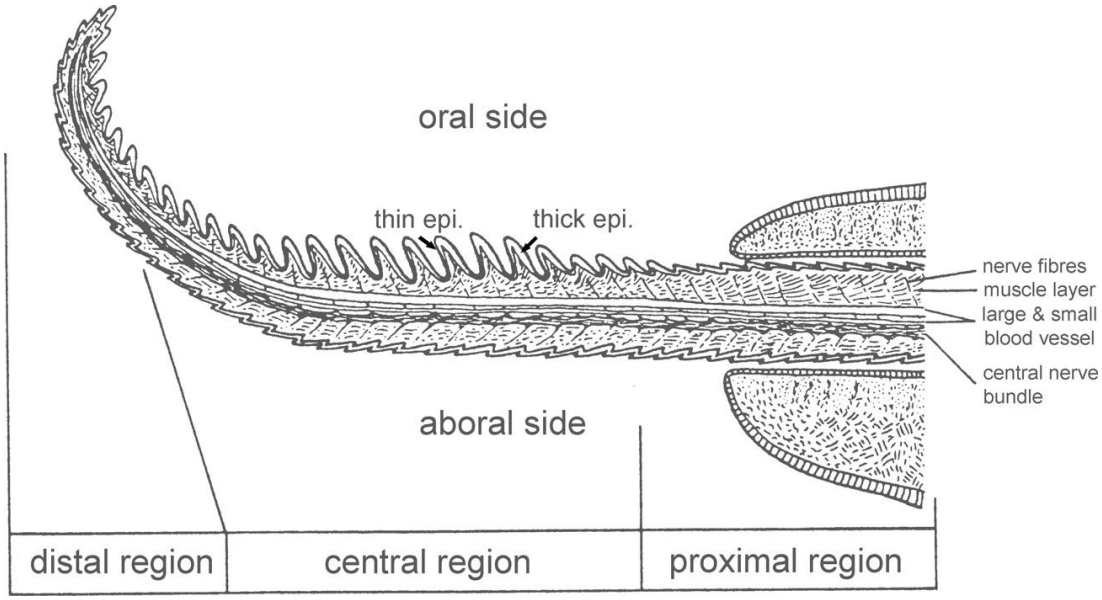


Figure 1 The longitudinal structure of a long digital tentacle of *Nautilus* (taken from Saunders & Landman 2010).

Physiological differences between the sub-classes can have a significant impact on results when considering the group as a whole; Wood & O'Dor (2000) found that including Nautiloidea in their analyses masked the correlation between age at maturity and size at maturity that was seen when they were excluded. They concluded that due to the differences between nautiloids and coleoids, it made biological and statistical sense to exclude them. Testing the effect of the exclusion of nautiloids from the dataset in physiological studies illustrates the differences within Cephalopoda.

1.2 *THE STUDY SPECIES: NAUTILUS POMPILIUS*

1.2.1 *GENERAL ECOLOGY OF NAUTILUS POMPILIUS*

Recognised species of *Nautilus* now include: *Nautilus pompilius* [Linnaeus 1758], *Nautilus belauensis* [Saunders 1981], *Nautilus macromphalus* [Sowerby 1848] and *Nautilus stenomphalus* [Sowerby 1848]. There also remain four questionable species: *Nautilus repertus* [Iredale 1944], *Nautilus ambiguous* [Sowerby 1848], *Nautilus alumnus* [Iredale 1944] and *Nautilus moretoni* [Willey 1896]. In 1997, a genus descendant from *Nautilus* was defined (Ward & Saunders 1997); *Allonautilus* consists of species

previously known as *Nautilus scrobiculatus* [Lightfoot 1786] and *Nautilus perforatus* [Conrad 1848].

As one of 11 species and seven variants of living *Nautilus* that have been proposed, our study species of *N. pompilius* is conclusively accepted (Saunders & Landman 2010). Found across the Indo-Pacific, populations are located from the Philippines across to the western Pacific (Bonacum *et al.* 2011). One factor thought to be contributing to its success with the fishing industry (Dunstan *et al.* 2010) is its large distribution; *N. pompilius* have the widest distribution of the *Nautilus* species (Jereb & Roper 2005).

The full life cycle of *N. pompilius* is not yet completely understood, but knowledge on the juvenile stage has been greatly increased with successful implementation of modern aquarium techniques (Saunders & Landman 2010). *N. pompilius* is iteroparous, yielding fewer than 10 eggs per batch which take up to a year to develop (Barord & Basil 2014). Sex determination or the development of a social structure is another area of *N. pompilius* ecology which is not fully understood. The recorded sex ratio of individuals in east Australia (in animals mature enough to distinguish sex) was 83:17 male:female (Dunstan *et al.* 2011a). A high male sex ratio has also been recorded in the Philippines (Haven 1977), Palau (Saunders & Spinosa 1978) and Papua New Guinea (Saunders *et al.* 1991).

As in all cephalopods, *N. pompilius* movement is facilitated through jet propulsion. Cephalopods have retained this basic mechanism for locomotion for over 500 million years (O'Dor & Webber 1991). In determining its origin, the presence of a funnel in the soft-bodied fossils from Burgess Shale (Morris 1976), in the lower and middle Cambrian (Morris 1989), indicated that jet propulsion evolved in cephalopods before the presence of a shell (Smith & Caron 2010). Plectronocerid nautiloids from the Upper Cambrian had

small orthoconic or cyrtoconic shells, which were not adapted for jet-powered swimming (Mutvei *et al.* 2007). The appearance of the shell was seen in Cambrian cephalopods, the first of their kind to evolve a solution to buoyancy control, that is, to achieve neutral buoyancy while carrying a partly gas-filled shell tending to have positive buoyancy. The relative buoyancy of the shell must be adjusted to accomplish this, and the history of cephalopods exhibits a great variety of responses to this challenge (Teichert 1988).

The nautiloid system of pressurising water in the mantle cavity results in the energy cost of transport being dramatically lower for *Nautilus* than for squid (O'Dor *et al.* 1990). Energy costs can be removed further by movement into deeper waters where a meal can be sufficient for months (O'Dor *et al.* 1990). This is a useful adaptation for reported scavengers (Ward & Wicksten 1980) where feeding may not occur regularly or in abundance. Dissections of *Nautilus* caught in baited traps show stomach contents of chicken, suggesting carnivorous and opportunistic feeding habits. They use paired rhinophores to track and locate the food (Basil *et al.* 2000, 2005), making vertical migrations to do so (Dunstan *et al.* 2011b), therefore utilising their ability to cope with sudden changes in depth. This is controlled by buoyancy adjustment within the shell. *Nautilus* hatch with less than seven septa formed, and the animal remains in the outer chamber forming new septa and additional chambers before reaching full maturity with approximately 28 septa after seven to eight years (Westermann *et al.* 2004).

1.2.2 *THE UNIQUE PHYSIOLOGY OF THE NAUTILUS SHELL*

The *Nautilus* shell, referenced in the work of Aristotle, has been well described throughout literature (e.g. Stenzel 1964). The two-layered, mother of pearl-lined, spiral-coiled shell is known to both the scientist and the layperson. The internal subdivision is commonly recognised and is present from hatching, demonstrating a series of successive chambers, termed phragmocone (Pita *et al.* 2002). The closely spaced septa are characteristic of Late Cambrian nautiloids (Mutvei *et al.* 2007). A calcareous septum creates a new chamber and is produced behind the animal as it grows, with the animal remaining in the open larger end of the shell as the chambers seal behind it (Landman *et al.* 1989). The complex spiral geometry found within the *Nautilus* shell has never been found in any other natural object (Pita *et al.* 2002).

The word complex is often used to describe the properties of organisms that demonstrate evolutionary progression (McShea 1991). Within Animalia, *Nautilus* are regarded as relatively simple and the term within this context therefore refers to their morphological complexity (the physical system of the organism), and not that of their evolution (McShea 1991). In agreement with Darwin's theory of increasing complexity (Darwin 1859), ammonites demonstrated increasing anatomical complexity over long periods of time (Boyajian & Lutz 1992). Although records show no differential survival between simple and complex forms (Boyajian & Lutz 1992), *Nautilus* survival has been attributed to the preservation of the shell structure (Pita *et al.* 2002).

Air, water and cameral fluid are manipulated in proportion via osmosis, facilitating depth changes of hundreds of metres (Denton & Gilpin-Brown 1966; Greenwald *et al.* 1980). This shell structure (Fig. 2) however, is known to influence movement and dispersal of *Nautilus* (Sinclair *et al.* 2007). When artificially tested using empty shells, implosion data showed variable strength between individuals, with smaller shells

withstanding a greater amount of pressure than that of mature adults (Saunders & Wehman 1977). Shells of 20-300 mm in diameter withstood the hyperbaric chambers' limit of 13.8 MPa (equivalent to a depth of 1360 m). These results were in support of earlier findings, in which the authors incorrectly inferred that juveniles live in deep water (Wehman 1976). More recent work has shown that the mature shell wall is stronger than that of juveniles, with the siphuncle known to rupture at a slightly higher pressure than the last septum (Hewitt & Westermann 2010). Fragmentation studies using live animals showed that the internal arrangement of the *Nautilus* shell can withstand pressure of 8.05 MPa (785m) before the risk of implosion (Kanie *et al.* 1980a).

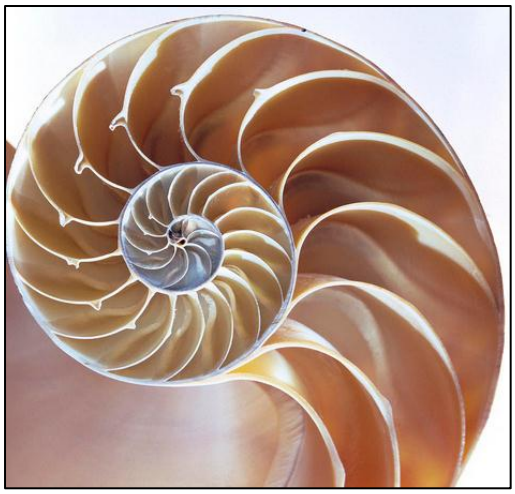


Figure 2 A cross section though a *Nautilus* shell. This shows the internal chambers separated by septa, and shows the path of the siphuncle (photo from Lawry 2014).

The weakest component within the shell is determined by the septal strength index (SSI). The SSI estimates maximum living depth by comparing the ratio of septal thickness to curvature in the septa of fossil taxa to the ratio of these parameters in *Nautilus* (Westermann 1973). Discrepancies in the validity of the SSI were used to state that as a measurement it did not yield reliable strength or depth estimates; claims were made that the SSI's unreliability was accountable to its inability to consider complexities

or mechanical failure in morphologically complex cephalopod shells (Chamberlain & Chamberlain 1985). The experiments used to disprove SSI and justify these statements, however, were rebutted as not relevant or applicable to do so (Westermann 1985) and SSI is still used to explain *Nautilus* architecture (Hewitt & Westermann 2010).

1.2.3 *NAUTILUS POMPILIUS CONSTRAINTS ON MOVEMENT*

Nautilus typically remain close to the reef for protection and travel between reefs along the ocean floor (O'Dor *et al.* 1993), their range is typically between depths of 130 m and 700 m (Dunstan *et al.* 2011b) with risk of implosion limiting their vertical distribution in the water column. This behaviour is similar to that of early plectronocerids, whose lateral movement would have been limited to crawling or moving slowly on the sea-floor (Mutvei *et al.* 2007). Vertical movements were probably common, although their siphuncular epithelium had limited osmotic capacity because small chambers contained low volumes of cameral liquid to be replaced by gas (Mutvei *et al.* 2007).

Modern *Nautilus* has been shown to make a vertical migration daily (Dunstan *et al.* 2011b), this behaviour is also displayed by many mid-water animals to avoid predation from visually cued predators (Robison 2003). With their shell as their sole form of defence, they are vulnerable to predatory attacks. Octopus attacks leave visible marks on the shells, caused by their beak in an attempt to break through (Tucker & Mapes 1978; Saunders *et al.* 1991). Teleost attacks have been witnessed and *Nautilus* were recorded retreating into their hood and showing no defence or escape response (Saunders *et al.* 2011). This deters individuals from moving between reefs separated by water that is deeper than their physiology allows them to cross on the sea floor. As such, gene flow is restricted resulting in documented diversification between populations, the pattern of which appears to be occurring along geographic boundaries (Sinclair *et al.* 2007, 2011; Bonacum *et al.* 2011). Low levels of dispersal are furthered by the absence of a juvenile

stage (Saunders & Landman 2010). Constraints on dispersal ability within marine species can exert a negative influence on the genetic structure of a population (Hewitt 2004; Santos *et al.* 2006; Wieters *et al.* 2008; Nunes *et al.* 2009; Silva *et al.* 2010).

1.3 *CONSERVATION OF EXPLOITED POPULATIONS*

1.3.1 *THE EFFECTS OF HARVESTING ON POPULATIONS*

The *N. pompilius* shell is now the cause of their decline as species are being heavily targeted for the ornamental shell trade. Catch from Palawan fisheries in the Philippines has been reported as over 100,000 *Nautilus* a year (Dunstan *et al.* 2010). The long-term effects of fishing on populations, or their ability to recover, are unknown (De Angelis 2012). With the low fecundity and long developmental time of *k*-selected species, (Carlson *et al.* 1984; Landman & Cochran 2010), it is predicted that population recovery is not fast enough to cope with this fishing pressure over long periods of time. In support of this, an 80% decline in catch per unit effort (CPUE) between 1980–2010 has been reported from these Philippine fisheries (Dunstan *et al.* 2010). When questioned, 81% of respondents stated that *Nautilus* are now harder to catch than in previous years, however 88% thought there was no risk of *Nautilus* "disappearing" if they continued to fish for them (Dunstan *et al.* 2010).

The dramatic declines in CPUE from fisheries in Palawan demonstrate the damage experienced by populations as a result of the shell trade. Investigating CPUE fishery reports between the start of fishing efforts to either current or last recorded CPUE (if fishing was discontinued) for *Nautilus* in the Palawan and Balabac regions of the Philippines showed declines in 4 out of 5 areas. The economic return of these fisheries has been estimated to remain viable for only the next 10-20 years, therefore becoming unprofitable in the lifetime of the current generation of fisherman (Dunstan *et al.* 2010). Population assessments were undertaken using baited remote underwater video

systems to compare abundance in the Philippine Islands, American Samoa, Fiji and the Great Barrier Reef (Barord *et al.* 2014). There were significantly fewer *Nautilus* found at the Philippines Islands site, most likely as a result of the fisheries found in this area. All sites have historical records of *Nautilus* populations, but as the only site with a fishing industry, the declining numbers seem unlikely to naturally regenerate in the Philippine Islands.

1.3.2 *GENETIC CONSEQUENCES OF POPULATION DECLINES*

Harvest-induced evolution has been well documented (Sasaki *et al.* 2008; Allendorf & Hard 2009; Enberg *et al.* 2009) and *Nautilus* will be no exception to this phenomenon. Harvesting selects against traits that would be advantageous under natural conditions, for example selecting for larger individuals despite smaller individuals having increased fitness (Carlson *et al.* 2007). While methods of *Nautilus* collection do not follow trait based regulations because fishermen will collect any individual caught in their trap, the very nature of using traps induces selection criteria. Small individuals, such as juveniles, can swim into the traps (Fig. 3) to feed and then descend through the gaps and back out again.

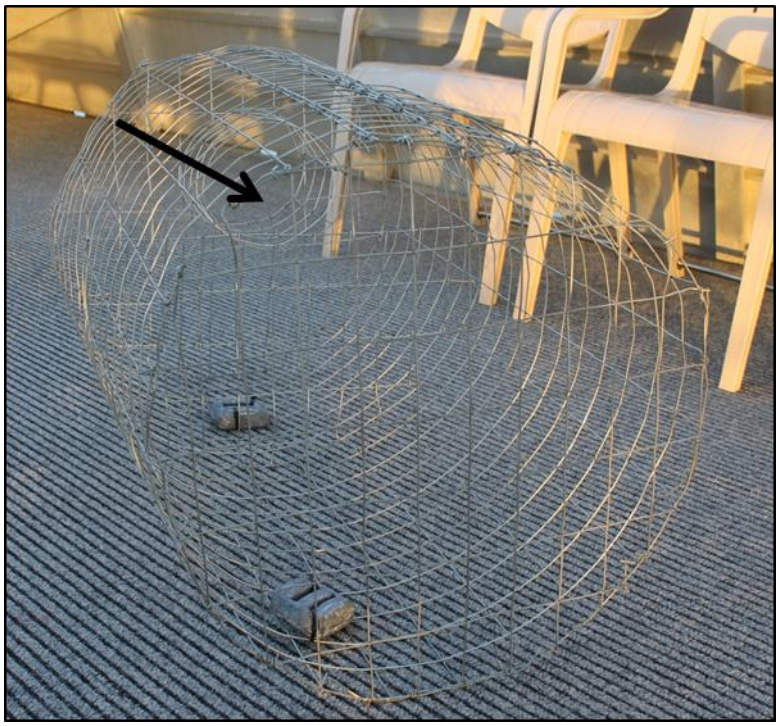


Figure 3 A *Nautilus* trap. The trap is baited and suspended next to the reef at approximately 200 m. Traps are open ended to allow individuals to swim out if the trap could not be retrieved. Point of entry indicated on far side of trap.

The entrance dimensions of the trap are set to the approximate size of a mature *Nautilus* in the collection area, preventing anything larger from entering the trap and feeding on either the bait or the *Nautilus*. This therefore creates an opportunity to prevent the capture of *Nautilus* showing the greatest variation from the mean. While the phenotypic traits can be retrieved, the recovery of behavioural traits and genetic diversity remains difficult (Law 2000). The extent of variation within the *N. pompilius* diet is unknown and thus hypothetically, individuals failing to demonstrate a preference in food variation that would result in trap avoidance, will continue to breed. Trap happy (McGarvey *et al.* 2012) *Nautilus* have been shown from catch and release studies (Dunstan *et al.* 2011a). These individuals that show a preference for trap bait will be removed from the gene pool. Genetic loss, such as the loss of specific alleles and associated traits, imposed by a population bottleneck is difficult to recover (Law 2000).

Variation in allele frequency occurs naturally; mutation is the driving force of evolution through genetic drift and allele fixation. Genetic drift is the change in allele or genotype frequency due to chance alone, and is responsible for fixation of neutral mutation. Fixation due to drift will occur in any population of a finite size, although this takes longer in a larger population (Hedrick 2011). In large populations, mutation, selection and migration have essentially deterministic effects, and the effects of chance are generally minimal, except for neutral alleles. This is unlike a small population where the role of chance predominates, leaving the effects of selection typically reduced or even eliminated (Frankham *et al.* 2004). Chance introduces a stochastic element into the evolution of small populations, and as such they become inbred at a faster rate than seen in large populations (Groombridge *et al.* 2000). Genetic drift can result in the loss of genetic diversity and fixation of alleles within populations, with consequent reduction in the ability to evolve (Allendorf *et al.* 2010). It occurs due to random sampling of gametes and can result in the diversification of allele frequencies among replicate populations from the same original source. It can also result in genetic drift overpowering natural selection, which can ultimately cause all but one allele to be lost (Frankham *et al.* 2010).

One mechanism for genetic drift is a population bottleneck (Frankham *et al.* 2010). A population bottleneck is a sharp reduction in population size, either short or long term. The event results in the loss of alleles (especially rare ones), reduced genetic diversity and random changes in allele frequencies (Allendorf 1986; Arif *et al.* 2010). This event is likely to be occurring in current *N. pompilius* populations in the Philippines due to their reduction in numbers. The effective population size remaining after a bottleneck event cannot contain the diversity present in the pre-bottleneck population (Hedrick 2011), thus the evolutionary potential of a population is reduced (Frankham *et al.* 2010; Santos *et al.* 2012). Inbreeding is inevitable in any population but will take longer to occur in a larger one. It can lead to reduction in heterozygosity, to reduced reproduction and

survival (inbreeding depression), and to the increased risk of extinction (Frankham *et al.* 2010). Loss of genetic diversity arises predominantly from sustained reduction in population size rather than single generation bottlenecks (Allendorf *et al.* 2010). Management can increase population size but genetic diversity cannot recover as quickly (Groombridge *et al.* 2000). The reported loss of numbers in Philippine *N. pompilius* populations (Dunstan *et al.* 2010; Barord *et al.* 2014) suggests that detrimental effects of inbreeding may manifest in future generations.

1.3.3 *PROTECTION AND RECOVERY: THE ROLE OF LEGISLATION IN OCEAN DIVERSITY*

Ideally, management plans for conservation units are put into place before a loss in diversity, but in reality this is not always possible. Marine reserves are a hugely successful way to both preserve and restore biodiversity (Halpern 2003). It has been shown that marine protected areas (MPAs) can serve as an anchor for large-scale conservation, securing the future of marine conservation (Agardy 1994), Australia has been acknowledged as a country that recognises and protects their biodiversity, enforcing protection such as Great Barrier Reef Marine Park. Created in 1975 through the Great Barrier Reef Marine Park Act, and now approximately the size of Germany, this covers 344,400 km^2 in area (Australian Government 2006, 2014a). No-take zones are increasing and as an MPA, its outcomes can be used as guidance around the world (Fernandes *et al.* 2005). The Coral Sea, east Australia, has also been recognised as a remote ocean ecosystem, documented for its unique physical, ecological and heritage values. The management to protect the Coral Sea Conservation Zone is currently being updated as it has now been combined with the Coral Sea Commonwealth Marine Reserve (Australian Government 2014b). This will help to conserve the diversity seen there, and help to maintain its unspoilt ecosystems.

1.3.4 *NEGATIVE IMPACTS OF MARINE PROTECTED AREAS*

As a negative side to MPAs, they can lead to overfishing outside the protected boundaries. Attempts to concentrate fishing efforts on the edge of marine protected areas rely on the increased population sizes spilling over the boundary. It has been shown, even for species with limited adult mobility, that fishing the edge of a protected area leads to stocks within the reserve becoming reduced, and lowers the ability of reserve stock to replenish areas outside of the protected boundaries (Kellner *et al.* 2007; Gaines *et al.* 2010). As Agardy (1994) highlights, "No MPA is an island"; if the area around an MPA is allowed to decline then this limits the future of the protected area.

For an ecosystem-based approach to be sufficient for the species which it aims to benefit, we need to fundamentally understand species ecology and therefore establish species movement patterns and their effect on population dynamics. Understanding the movement patterns of vulnerable species is important for providing appropriate functional protection through *in-situ* means (Grüss *et al.* 2011). For example, it would be ineffective to target migratory species with an MPA.

1.3.5 *COMMUNITY INVOLVEMENT IN LOCAL PROTECTION*

All legislation requires enforcement and this can be difficult on large scales such as the Great Barrier Reef. Community based marine protected areas (CB-MPA) have been demonstrated as a way to tackle this and the efforts of this have been documented in the Philippines (Christie *et al.* 2002; Hind *et al.* 2009; Weeks *et al.* 2010). The role played by MPAs for humans must be acknowledged to increase the chances of success for an MPA. Successful feedback (i.e. increased productivity through stock regeneration) is imperative for long term success in such schemes (Cicin-Sain & Belfiore 2005). Individuals within communities involved in the over 400 CB-MPA around the Philippines felt that only 20-25% were a success (Crawford *et al.* 2000). Failure to see this can

result in the rejection of the scheme and so management must be designed to maximise improvement at each site (Pollnac *et al.* 2001; Pomeroy *et al.* 2005). CB-MPA could be an effective way to conserve *N. pompilius* populations in the Philippines. Nautiloids would benefit from multiple levels of protection; having both local and international protection will increase the chances of preventing their further decline.

1.3.6 *NAUTILUS POMPILIUS PROTECTION*

Despite the extensive exploitation, *Nautilus* and *Allonautilus* remain unprotected by CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) and therefore large scale shell trade is unregulated. *Nautilus* are offered local protection; seen as species of concern in Australia, no commercial fisheries exist, and this is also the present fishery status in Indonesia. The Philippines also have restrictions but these are reportedly unenforced (Aguiar 2000). Individuals located in marine protected areas (MPAs) are offered protection but unfortunately this does not extend across their range. This local protection appears insufficient; The Law Enforcement Management Information System (LEMIS 2010) indicated that US declarations from 2005-2010 exceeded 789,000 *Nautilus* products (see De Angelis 2012), comprising whole shells to shell products such as jewellery and buttons.

In 2008, the US Fish and Wildlife Service were asked by both the Humane Society of the United States and Humane Society International to consider submitting a proposal for the inclusion of *Nautilus* and *Allonautilus* in CITES Appendix II. An initial assessment concluded that the biological and international trade data were insufficient to assess *N. pompilius* for CITES listing (De Angelis 2012). As a result, there have been no official proposals to list *Nautilus* in the CITES Appendices (Pers. Comm. Patricia De Angelis 2013, US Fish & Wildlife Service).

Efforts to address the gaps in knowledge preventing their protection, such as research on population declines, show overwhelming support for their immediate protection (Dunstan *et al.* 2010; Barord *et al.* 2014). Local population declines were attributed to fishing, suggesting that although international protection is needed, local management may also be beneficial for both *Nautilus* and local communities. While *Nautilus* life history traits make them vulnerable to overharvesting, present knowledge on their movement also means that damage to populations is localised to areas targeted by fishing (Barord *et al.* 2014). Local assessments and appropriate management plans could enable a way to facilitate the continuation of fishing in communities without driving populations to local extinction. Catch limits and rotating fishing efforts to different geographic regions could potentially enable species recovery while attempting to limit the impact on the local communities.

Initial listing on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species would highlight the vulnerability of *Nautilus* on a global scale. This would analyse their status and population trends to enable action for conservation. The status of *Nautilus* species was reviewed by IUCN in 2002. They were not included on the Red List as a result of information lacking on population demographics and fishery impact (IUCN 2014). The marine realm currently comprises less than 12% of the Red List species. The IUCN Species Programme Marine Biodiversity Unit are reported to be conducting the first global review of threats to extinction, and will include "selected invertebrates" (IUCN 2014).

1.3.7 *THESIS RATIONALE*

It has been proposed that the sustained decline in numbers qualifies *N. pompilius* to be classified as endangered on the IUCN Red List (Dunstan *et al.* 2010) but this recognition has yet to be put into action. The rates of decline designate their vulnerability equal to that of the tiger (*Panthera tigris*) (IUCN 2014). Unfortunately *Nautilus* do not receive the same publicity as tigers, an additional factor contributing towards their lack of protection. Cephalopods covered in the media are commonly the more charismatic coleoids and consequently, *Nautilus* remain relatively unknown to most people. Whilst a *Nautilus* shell is instantly recognisable to so many, very few know anything about the animal inside them. General ecology is still in an age of discovery about these ancient sea creatures, and so the field in some sense is still catching up to coleoid research.

The effects of the shell trade on population numbers should be communicated beyond the researchers in this field to aid *Nautilus* protection. Generating data to gain CITES protection will not only prevent the on-going loss of numbers, but also raise awareness and start a conversation regarding their current situation. Purchases could potentially be reduced, as has been witnessed in the ivory trade; first world appetite decreased with the realisation of inevitable elephant slaughter to fulfil ornamental enjoyment. This was concurrent with their upgraded CITES protection (Keller 1992). For shells, it is often unrealised that those sold in shops have not been washed up on beaches. It is through a combination of insufficient protection and common misunderstanding, that fishing and their consequent decline continues. This reiterates that it is vitally important to communicate our scientific knowledge thus far. The research within this thesis is designed to address knowledge gaps in *N. pompilius* biology, evolution and population dynamics that could contribute to their protection.

1.4 *PROJECT AIMS*

The thesis aims to increase knowledge on the biology of *N. pompilius* and the movement of populations. This will feed back into assessing the evolutionary impacts across the distribution, caused by localised fishing.

1.4.1 *RESEARCH QUESTIONS AND PROJECT OBJECTIVES*

1. Investigate the evolutionary relatedness of *N. pompilius* populations around Australia and in the western Pacific using a partial fragment of the cytochrome c oxidase sub unit I (COI) gene sequence:

- Does the molecular work support the theory that *Nautilus* evolved into three evolutionary clades from a progenitor population in the Philippines?

- If evidence has been found to support three evolutionary clades, are we now seeing evolutionary and spatial separation within these clades or evidence of gene flow?

- If evidence is found to reject the theories of evolutionary clades, what are the patterns of genetic similarity between populations?

2. Develop a microsatellite enriched genomic library to genotype *Nautilus pompilius* populations:

- Can microsatellite markers be isolated from *Nautilus* samples?

- Will the primers developed be of high enough quality to facilitate population analysis?

3. Investigate the population structure of *N. pompilius* samples from across their distribution using microsatellite markers:

- Is there genetic structure between reefs surrounding Australia, the Philippines and in the western Pacific?

- Is genetic similarity observed between sampling areas a result of gene flow?

- Do populations appear to show genetic effects of over-exploitation?

4. Investigate the potential to assess relatedness with other taxa by comparing the biologically important trait of signalling pathways, this will enable us to establish how unique *Nautilus* are at this fundamental level:

- Does *Nautilus* tissue react to drugs known to activate receptors in higher order animals?

- Can we establish the presence or absence of specific receptors based on tissue responses?

- Does this work provide sufficient results to warrant further investigation into nautiloid receptors and their evolution comparable with other taxa?

1.4.2 *THESIS STRUCTURE*

The data chapters within this thesis have been presented in the form of manuscripts for publication:

- **Chapter Two: DNA Barcoding in *Nautilus pompilius*: evolutionary divergence of an ancient species in modern times.** This chapter presents the first data chapter and begins from an evolutionary perspective, looking at the evolutionary relatedness of *N. pompilius* populations. Here, the conserved cytochrome c oxidase subunit I was used to construct phylogenetic trees based on maximum likelihood. This chapter builds on previous research, incorporating sequences generated from

sample collection to data in the literature. The chapter includes samples from west Australia to give a wider picture across their distribution. This documents the use of barcoding sequences such as COI and their use in science and more specifically conservation.

- **Chapter Three: Isolation and characterisation of microsatellite loci for the ancient cephalopod, *Nautilus pompilius.*** This presents the steps taken to expand on Chapter Two by using a finer scale marker. The utilisation of finer markers and greater depth of analyses is discussed, in combination with the development of such markers. The chapter discusses the application of microsatellites for species conservation.

- **Chapter Four: The genetic structure of *Nautilus pompilius* populations surrounding Australia and the Philippines.** This chapter documents the use of the markers developed in Chapter Three to investigate population structure and gene flow. The work builds on that in Chapter Two by expanding the sample set to incorporate individuals from the progenitor population in the Philippines. The study uses approximate Bayesian computation analysis to investigate the likelihood of evolutionary scenarios.

- **Chapter Five: Pilot Study: Comparative effects between drugs on tissue reactivity in *Nautilus pompilius.*** The final data chapter presents a pilot study investigating the biological signalling pathways of *N. pompilius*. This work aims to identify specific receptors in nautiloids, and to establish their relatedness with other taxa. To first determine the presence or absence of receptors, reactions of tissues in contact with specific drugs were recorded and then mixed models used to conclude whether these reactions were statistically significant. The chapter also

contains proposed changes to the experimental design for implementation when conducting the work in the future.

- **Chapter Six: Final Discussion.** This Chapter summarises Chapter Two to Chapter Five and assesses the wider applications of the research. The importance of marine research and the relevance of the presented thesis to the future of *Nautilus* conservation is discussed.

CHAPTER TWO:

DNA BARCODING IN *NAUTILUS POMPILIUS* (MOLLUSCA, CEPHALOPODA): EVOLUTIONARY DIVERGENCE OF AN ANCIENT SPECIES IN MODERN TIMES

Nautilus pompilius, Osprey Reef, Coral Sea
Photo taken by Lani Tuckfield

The data in this chapter have been published in INVERTEBRATE SYSTEMATICS:

Williams RC, Newman SJ, Sinclair W (2012) DNA Barcoding in *Nautilus pompilius* (Mollusca: Cephalopoda): evolutionary divergence of an ancient species in modern times. Invertebrate Systematics, **26**, 548–560.

2.1 *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 individuals. 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*: (i) populations sourced from west Australia, Indonesia and the Philippines; (ii) consists of samples collected from east Australia and Papua New Guinea; (iii) comprises western Pacific samples 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 individuals, 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.

2.2 *INTRODUCTION*

The collation of evidence from fossils and molecular data demonstrates that the major classes of molluscs diverged in the early Cambrian Period (Smith & Caron 2010; Maloof *et al.* 2010; Kröger *et al.* 2011). Nautiloids mark the first appearance of cephalopods as a separate molluscan entity over 500 million years ago (Jereb & 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 1758) 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 sub unit 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. It has been inferred 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 inability to travel below approximately 800 m without imploding due to increasing water pressure (Saunders & Wehman 1977; Kanie *et al.* 1980a; Wani 2004), and their vulnerability to predators when travelling through open water, restricts the movement of *N. pompilius* 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 & Spinosa 1979), however they are able to adapt to adjusting temperatures and can move from 6°C – 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 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).

Whilst physical separation can create a degree of diversity within the species, it also results in small isolated populations, with no reproductive 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 discrete units attached to benthic substrate, and the resulting zygote is one of the largest known invertebrate eggs, taking approximately 11 months to hatch (Saunders & 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 (~100m) 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 & Landman 2010). When mature, males have larger shells and a greater overall weight (Saunders & Spinosa 1978). 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.* 2003a; b), COI is applied in an approach designed to differentiate between morphologically similar species (Barrett & 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, the internal transcribed spacer (ITS) is the region used for identifying fungi and both *mat*K and *rbc*L chloroplast genes are used in plant identification (Hebert *et al.* 2003a; 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 (Wiemers & Fiedler 2007; Brandstaetter *et al.* 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 which work successfully in other cephalopod taxa (Strugnell *et al.* 2006a). 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 & 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 cephalopods. Whilst 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 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 approximately 2000 km from the Philippine Islands to the coast of the Kyushu Island, Japan, by the Kuroshio oceanic current (Tanabe & Hamada 1978). 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 & 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 both ancient and present day influences of nautiloid dispersal (Manda 2008; Mapes *et al.* 2010b; 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 on-going 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 is enforced 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 the Coral Sea Commonwealth Marine Reserve (Australian Government 2014b). The long term effects of shell collection on the genetic variation of *N. pompilius* 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.

2.3 *METHODS*

Samples were collected from a total of seven reefs (fig.1). The Rowley Shoals is a group of atoll-like coral reefs south of the Timor Sea, including Clerke (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 to 90 km^2 and both Clerke and Imperieuse Reef rise steeply from the surrounding ocean floor. Imperieuse Reef is located 35 km southwest of Clerke Reef and is the most south westerly of the reefs of the Rowley Shoals. Ashmore Reef is located in the Timor Sea (12°11'S 122°59'E), on the edge of the Australian continental shelf and approximately 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, the Far North Great Barrier Reef (1°39 59'S, 143°58 56'E) and Scott Reef (14◦03'S, 121◦46'E), found at the edge of the continental shelf approximately 265 km off the coast of north western Australia.

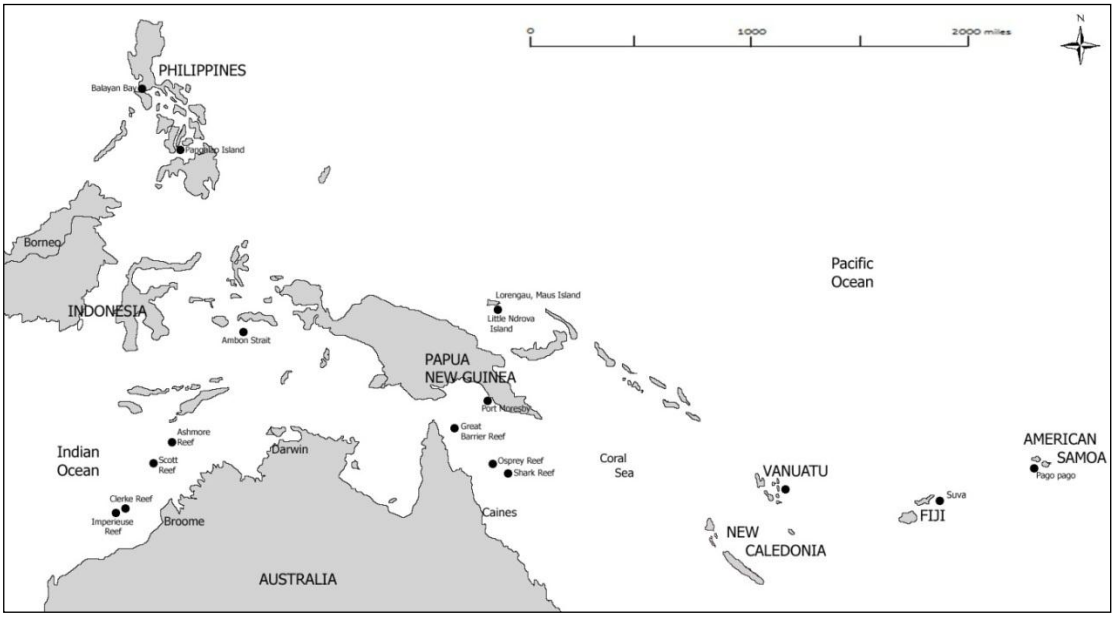


Figure 1 Sampling site locations for *Nautilus pompilius*. From west Australia: Imperieuse, Clerke, Scott and Ashmore Reefs; east Australia: Shark, Osprey and Great Barrier Reefs; and samples sourced from GenBank: Philippines (Balayan Bay and Pangalao Island), Indonesia (Ambon Strait), Papua New Guinea (Lorengau and Port Moresby), Fiji (Suva), American Samoa (Pago pago) and Vanuatu. Australian samples were collected from 200-300 m, by attracting individuals into traps using pilchards (*Sardinops sagax*) or chicken (*Gallus gallus domesticus*). Captured individuals were kept in a dark, dedicated refrigerated tank (50 L) for a maximum of 15 hours at temperatures between 16–19°C. All individuals were released at night at depths of 20–30 m.

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 of approximately 200 m for 24 hour periods, and were baited with approximately 1 kg of pilchards (*Sardinops sagax*) or chicken (*Gallus gallus domesticus*). Tentacle samples, 1-2 cm in length, were taken from a labial tentacle from each individual collected. Sample collection is non-lethal and ethical clearance was granted by both the University of Cumbria Ethics Council and Central

Queensland University Animal Ethics Committee. Photos and shell measurements were taken before returning each individual back to the reef edge (at ~20 m). Tentacles were initially preserved in 20% DMSO (dimethylsulphoxide), 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 98 samples using the QIAGEN DNeasy Tissue Kit following the manufacturers' instructions (Qiagen Pty. Ltd., Victoria, Australia). Polymerase chain reaction (PCR) amplifications were conducted using 25µl 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 Accessions: 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, CA., USA). Alignment of the sequences required no insertion/deletion events (indels). The sections

of sequence toward 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 & Langdale 2006), with the tree-bisection-reconnection (TBR) 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 jackknife values were generated (1,000 resampling replicates) to provide statistical support.

2.4 *RESULTS*

DNA was extracted from tentacle snips taken from *N. pompilius* from 15 sites surrounding Australia. Partial COI sequence information was generated (560bp) and analysed from a total of 98 samples. In addition, a further 30 *N. pompilius* COI sequences were mined from GenBank, covering 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 I). 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 (412bp) 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 jackknife values were generated.

Within this tree, the presence of three distinct clades (genetic clusters) was detected illustrating the biogeographic and evolutionary separation of these sampling locations. One clade shows the panmictic nature of the west Australian populations sampled (Ashmore, Imperieuse, Clarke and Scott Reef). These populations show no discrete separation and appear as a single, large panmictic population. Similarly, the Philippine and Indonesian samples analysed in the data set fully resolve within this clade. This grouping is strongly supported by both bootstrap (100) and jackknife (98) analysis. There is no further hierarchical separation of samples within this clade into specific population groups; it maintains the panmictic structure with only limited resolution of discrete samples.

A second independent clade (with strong bootstrap support) represents samples collected from east Australia (Coral Sea reefs and Great Barrier Reef), in addition to samples sourced from Papua New Guinea which are also resolved in this clade. There is further structuring within this large clade however and samples from the Coral Sea reefs (Osprey and Shark) represent a sister clade to those 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).

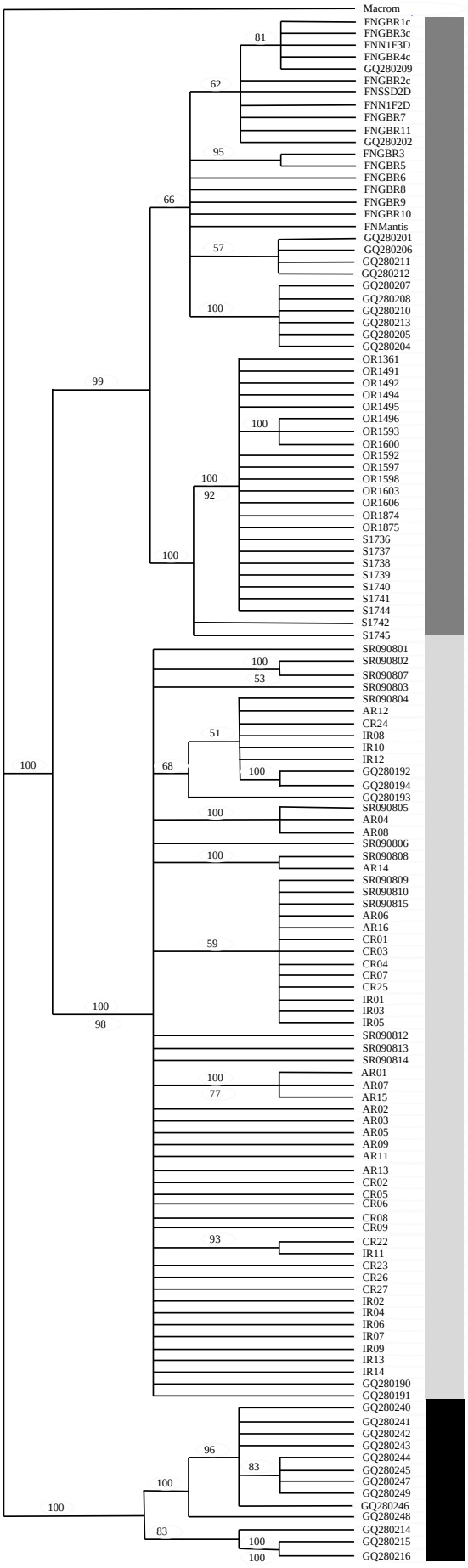


Figure 2 Phylogenetic analyses of *Nautilus pompilius* COI DNA sequences. Maximum likelihood analysis generated a consensus tree constructed from the alignment of partial COI sequences of 412 bp from 128 *N. pompilius* sequences, and rooted against *Nautilus macromphalus*. Bootstrap values (1000 re-sampling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

Appearing as a third discrete clade are all the western Pacific samples 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 samples from Vanuatu (bootstrap and jackknife 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 (v. 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 discrete haplotypes, with the k value signifying frequency of individuals represented in a particular node (which therefore have identical haplotypes based on the COI sequencing). A total of 12 haplotypes were found within east Australian/Papa New Guinean clade, 30 haplotypes within 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 samples), but also some substantially higher at 17.0 and 18.0 bp (American Samoa and Fiji samples) – this may be an artefact due to the small sample number at each population.

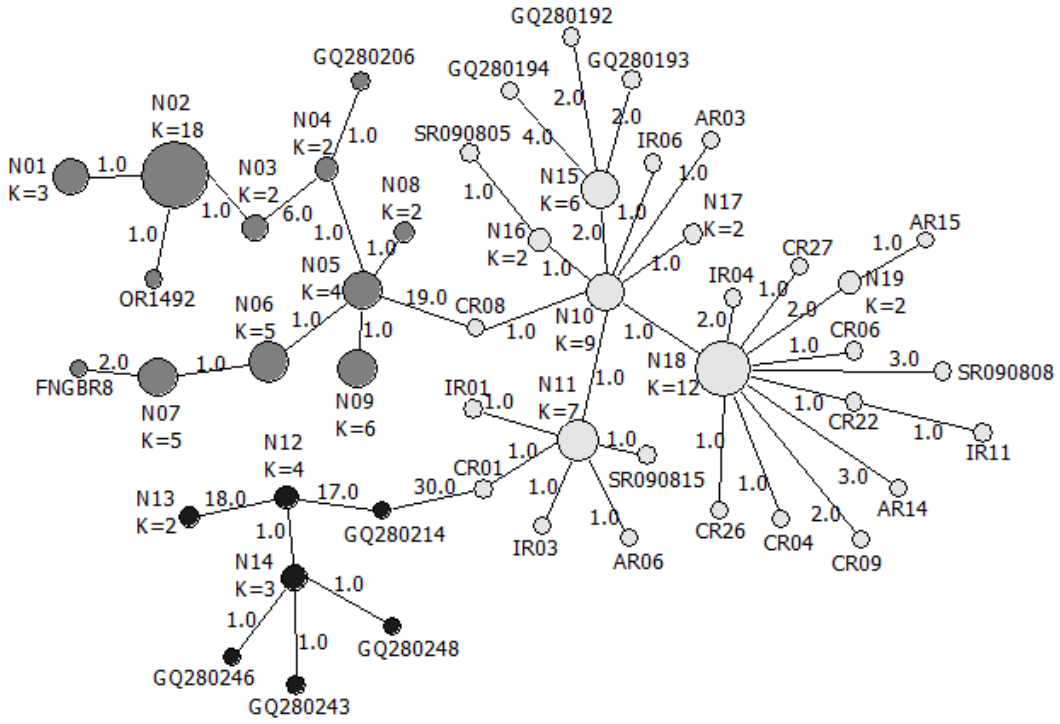


Figure 3 Minimum Spanning Tree. Nodes represent haplotypes, k values signify the frequency of individuals represented in a particular node, values between the nodes demonstrate base pair differences between haplotypes. Node colour corresponds to the three geographical clades: dark grey nodes are east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

Table 1 Corresponds to Fig. 3 and identifies which samples are found in the nodes labelled N-01 - N-197, of Fig. 3, where K > 1. K values signify the frequency of individuals represented in a particular node.

N01	N02	N03	N04	N05	N06	N07	N08	N09	N10	N11	N12	N13	N14	N15	N16	N17	N18	N19
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	GQ280208	IR09	IR05	GQ280245	GQ280216	GQ280241	IR10	AR04	SR090807	CR05	AR01
OR1496	OR1875			FNGBR6	FNN1F2D	FNN1F3D		GQ280210	SR090801	CR07	GQ280247		GQ280242	SR090804			CR23	
	S1736			FNMantis	FNGBR7	FNGBR4C		GQ280213	SR090803	SR090809	GQ280249			AR12			IR02	
	S1737				FNGBR11	GQ280209		GQ280205	SR090806	SR090810				CR24			IR13	
	S1738							GQ280204	SR090812	CR03				IR08			IR14	
	S1739								SR090814	AR16							AR09	
	S1740								GQ280190								AR11	
	S1741								GQ280191								AR02	
	S1744																AR13	
	OR1603																SR090813	
	OR1597																AR05	
	OR1361																	
	OR1491																	
	OR1598																	
	OR1592																	
	OR1494																	
	OR1495																	

Between the populations of west Australian/Indonesian/Philippines and those of east Australian/Papua New Guinean, 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.

2.5 *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 activity (Moltschaniwskyj *et al.* 2007; Crook *et al.* 2009). The overexploitation of marine resources is an on-going 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 their exploitation and consequent cause of decline. The ornamental shell trade targets *Nautilus* species and many populations are being overexploited (Dunstan *et al.* 2010; De Angelis 2012), 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 samples are also interspersed and resolved within the clade. This would indicate that *N. pompilius* from the surrounding seas of the Indonesian archipelago are still, or have been in their recent evolutionary history, sympatrically distributed with *N. pompilius* surrounding west Australia.

The east Australian/Papua New Guinea clade demonstrates increased separation within the clade and appears to divide into two: the first half encompasses samples from Papa New Guinea and the Far North Great Barrier Reef, and the second half contains samples from Osprey and Shark Reef. Within the clade containing samples from Papa New Guinea and the Great Barrier Reef, over 80% of Papa New Guinean samples are clustered together within the clade, the remaining are resolved within the Great Barrier Reef samples (Fig. 2). This may reflect the recent dispersal of individuals 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 demonstrate a level of similarity indicative to that of a panmictic population (no population separation). When this is considered in terms of the topography of these locations, although physically quite close to each other, they are separated by water in excess of 1000 m deep (Dunstan *et al.* 2011b) and 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. Whilst it is evident that Papa 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 samples, and those from Fiji and American Samoa. This would indicate that although their ancient distribution has resulted in a higher level of relatedness to each other than to the rest of the data set, 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 & Wehman 1977; Kanie *et al.* 1980a; Wani 2004), with long-term habitat depth suggested to be limited to 130-700 m due to cameral flooding (Saunders & Landman 2010; Dunstan *et al.* 2011b). Water depths surrounding the west Australian reefs show no potential inhibitory effects to *N. pompilius* movement, 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 approximately 250 km and by depths in excess of 1000 m. Whilst this distance has been successfully travelled before by *N. pompilius* (Tanabe & Hamada 1978), 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 expanse of open water, thus dramatically reducing their survival rate due to predation (Saunders & 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 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; Pleguezuelos *et al.* 2009; Griffiths *et al.* 2009; Caputo *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. This inferred that one lineage of *Nautilus* colonised from New Guinea to New Caledonia, Fiji and Samoa, while another moved towards Australia, the Philippines and the South China Sea. Samples from potential progenitor populations in the Philippines (Wray *et al.* 1995) 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 individuals. 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.* 2006b). 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 market forces are driving the on-going development of the *Nautilus* fishing industry and called for an assessment of *Nautilus* species by the IUCN, potentially to have it categorized as globally endangered. Here we present data generated by DNA barcoding which 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 the current populations in these areas were targeted by commercial fishermen, the risk of losing a unique genetic resource (potentially representing unique species and sub-species) is extreme.

2.5.1 *Future Work*

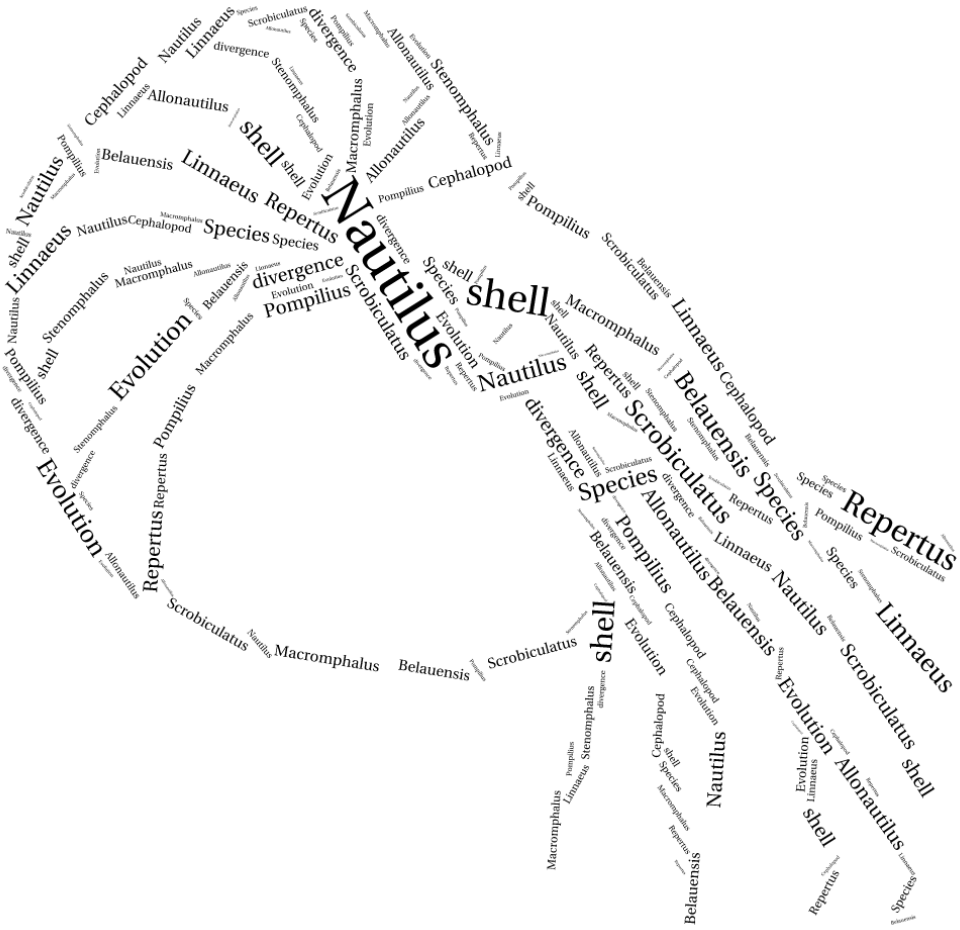
Future work could expand on the use of barcoding to determine the origins of empty shells through the use of other barcoding sequences to assess whether a higher level of variation is displayed between populations. This could incorporate the development of population-specific identifiers for discrete sections of the COI sequence, potentially through the use of single nucleotide polymorphisms as population-specific markers. Whilst 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. This could 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 genomes 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 life-form which we know so little about. DNA Barcoding, however, 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 re-classifying *N. pompilius* into discrete species/sub-species.

CHAPTER THREE:

ISOLATION AND CHARACTERISATION OF MICROSATELLITE LOCI FOR THE ANCIENT CEPHALOPOD, *NAUTILUS POMPILIUS*



The data in this chapter have been published in CONSERVATION GENETICS RESOURCES:

Williams RC, Dawson DA, Horsburgh GJ, Sinclair W (2015a) Isolation and characterisation of microsatellite loci for the ancient cephalopod, *Nautilus pompilius*. Conservation Genetics Resources, **7**, 107-111

3.1 *ABSTRACT*

Microsatellites are some of the most variable fragments of DNA in the genome. The observable variation between individuals can be used to understand connectivity between populations and determine management units for species undergoing decline. Many species are under significant extractive pressure and *Nautilus pompilius* is no exception, being overfished for the ornamental shell trade. Understanding the fine scale population structure of *N. pompilius* would assist in effective management when legal protection is implemented. A microsatellite-enriched genomic library was created from a single *N. pompilius* individual and clones/fragments sequenced using Sanger and Illumina MiSeq sequencing. Forty-four markers were tested in four individuals and 30 markers selected for further testing in a population of unrelated individuals from Imperieuse Reef, west Australia. Observed levels of heterozygosity in the Imperieuse Reef population ranged from 0.17 to 1.00 with a mean of 17.2 alleles per locus. No groups of loci displayed evidence of significant linkage disequilibrium. Tests showed that 21 markers were in Hardy-Weinberg equilibrium and of high enough quality to be used to study population structure in *N. pompilius*.

3.2 *INTRODUCTION*

Microsatellites are tandem repeats of one to six base pair motifs and are found throughout the genome. The term microsatellite was the product of a succession of individual discoveries; a fraction of sheared DNA found in the 1960s showed a satellite peak in density gradient centrifugation, seen as a distinct buoyant dense entity. This was identified as large centromeric tandem repeats, and shorter tandem repeats named minisatellites. Microsatellite was used to term the later discovery of tandem iterations of simple sequence repeats (Ellegren 2004).

The distribution and frequency of microsatellites is variable in prokaryotic (Kassai-Jáger *et al.* 2008) and eukaryotic genomes, in addition to coding and noncoding regions (Tóth *et al.* 2000). Also referred to as short tandem repeats (STRs) or simple sequence repeats (SSRs), they are passed on from generation to generation through Mendelian inheritance. They are among the most variable forms of DNA sequence (Weber 1990), with variation detected as differences in the number of motif repeats rather than polymorphisms in the primary sequence. This variation is characterised by high levels of heterozygosity and multiple alleles (Ellegren 2004).

Due to the enormity of genome sequences, specific gene sequence comparison may not always lead to detectable differences. Microsatellites are classified, among DNA repeat variation analyses, as areas of the genome where individual differences are more abundant (Ellegren 2004). High levels of polymorphism are predominantly attributable to high mutation rates. Mathematical models are used to calculate mutation rates, and the mutation model commonly used for microsatellite analysis is the stepwise mutation model (Kimura & Ohta 1978). This assumes that, with equal probability, a mutation will result in one repeat unit increase or decrease in allele size. Their high abundance and high levels of polymorphisms have made them popular in population studies and

forensics (Kelkar *et al.* 2010). Distinguishable loci with co-dominant alleles, microsatellites provide similar information to allozymes but are generally neutral and more variable (Queller *et al.* 1993).

The use of microsatellites now extends far beyond the presence or absence of a difference between individuals. Analysis now enables the inference of historic and current population dynamics. If a question requires Mendelian markers, microsatellite loci and subsequent analysis are often a more applicable approach.

3.2.1 *INFERENCE FROM A COALESCENT FRAMEWORK*

Neutral markers can be used to study the spatial distribution of alleles used to study local gene flow. The investigation into metapopulation dynamics can be used for both control and conservation. For example, Fountain *et al.* (2014) used microsatellite loci to look at the local extinctions and colonisations of bed bugs *(Cimex lectularius).* The lifespan of subpopulations is limited by pest control causing frequent local extinctions, and human facilitated dispersal allows the colonisation of vacant areas. The use of approximate Bayesian computation analysis (ABC) allowed the reconstruction of such events in a coalescent framework. ABC aims to obtain the posterior distribution (the probability of the returned parameter values after simulations) of parameters using simulations under a demographic model of interest. The models allow explanation of how these data were formed, whilst allowing flexibility in demographic models for which there are no explicit likelihood functions (Marjoram & Tavaré 2006). Parameter values for simulated data sets are drawn from prior distributions (a distribution that contains the expected returned parameter values, while still allowing for uncertainty). If simulated data are similar to the observed data then the corresponding parameters are accepted (Sousa *et al.* 2012). Parameter values that generate data sets closest to the observed data are taken as an approximation of the posterior distribution

$P(\theta|d(D_{sim},D_{obs})<\delta)$ where *Dsim* and *Dobs* are the simulated and observed data, and δ represents the tolerance level (Sousa *et al.* 2012). ABC methods do not use the observed data directly, and instead implement the use of chosen summary statistics for comparison of real and simulated data. Its use in Fountain *et al.* (2014) allowed the elucidation of individual movement and consequent mechanisms of colonists that can be used to facilitate effective pest control such as bed bugs.

Combining microsatellites and ABC analyses can be utilised for conservation as well as eradication. Guillemaud *et al.* (2010) inferred the route of invasive species using such techniques; ABC provided information on the history of an invasion process but also on the origin and construction of the genetic composition of the invading population. Identifying patterns of invasion allows inference to prevent future invasive species and their effect on natives.

3.2.2 *ESTIMATING EFFECTIVE POPULATION SIZE*

The Bayesian movement in population genetics provides an alternative to a direct approach when the demographic history of a species through census data is impossible. Inferring the past demography from an observed distribution of genetic variation in a population is an area of interest in applied ecology, evolutionary biology and conservation biology (Beaumont 1999). Understanding past variations in population sizes can help to understand the influence of past climatic fluctuations on current population sizes and current distributions of species (Girod *et al.* 2011).

Knowledge of current population sizes can be an effective tool in active conservation. Small populations are in danger of inbreeding, and a long term loss of quantitative genetic variation can limit future evolutionary change (Franklin & Frankham 1998; Lynch & Lande 1998). Calculating effective population sizes can influence management

of both captive and wild populations. Inferring time specific changes in effective population sizes of the African elephant (*Loxodonta africana*; Okello *et al.* 2008) showed that due to the short duration of intense persecution, overall allelic diversity was not detectably affected. Results indicated that it had only been temporarily impacted due to individuals sired before the impacts of poaching then becoming reproductively active. Calculating current effective population sizes allowed calculation of the population threshold to maintain long-term evolutionary potential.

3.2.3 *DETECTION AND PREVENTION OF INBREEDING*

Certain ecological traits leave some species more susceptible to inbreeding; small dispersal distance and overlapping home ranges, a previous bottleneck event or barriers to gene flow can all contribute to relatively high levels of inbreeding. Ariani *et al.* (2013) used the software BOTTLENECK (Cornuet & Luikart 1996) to investigate evidence of a recent genetic bottleneck in populations of an endangered and endemic species of sand lizard *(Liolaemus lutzae)* found in Brazil. BOTTLENECK computes, for each locus and population sample, the distribution of the heterozygosity expected from the observed number of alleles under the assumption of mutation-drift equilibrium. This distribution is generated via the coalescent process under two possible mutation models, infinite-alleles-model (Kimura & Crow 1964) and stepwise-mutation-model (Kimura & Ohta 1978). Populations of *L. lutzae* were tested for evidence of a mode shift in allele frequency distribution and for heterozygosity excess, both of which occur briefly after a bottleneck (Cornuet & Luikart 1996). The microsatellite data were combined with mitochondrial DNA sequence data and results showed the species to be in a critical state. This informed conservation plans to extend protection to a wider range, to connect fragmented beaches, to restock the most critical populations from those with higher levels of diversity and to begin captive breeding while habitat restoration is

implemented. The genetic picture shown from these analyses allowed the severity of the situation to be assessed with potentially enough time to prevent extinction.

3.2.4 *Extraction of information from ancient DNA*

Prevention of a critical state or even of the loss of a species is not always feasible. Molecular techniques now allow amplification of DNA from ancient DNA (Allentoft *et al.* 2009). Microsatellites can be analysed using this DNA from ancient specimens. Allentoft *et al.* (2011) used low copy number ancient DNA templates of approximately 600 to 5000 years of age to develop microsatellite markers for an extinct taxon, the giant moa (*Dinornis robustus*).

Both single nucleotide polymorphisms (SNPs) and microsatellite markers can be used in such cases. The process of extraction is more successful for both when the DNA has been preserved in lower temperatures, because it increases the longevity of the DNA. In the presence of poorly preserved DNA, microsatellites require better preservation than SNPs. With post mortem hydrolytic DNA damage however, distinguishing between a damaged site and a true polymorphism when using SNPs can be difficult. This does not apply with microsatellite data because allele lengths are recorded and not base pair composition (Allentoft *et al.* 2011). Microsatellite variation requires a fewer number of amplified loci to retrieve a large amount of information, whereas variation in any given SNP is limited to four states.

Ambiguous results in this area (Zischler *et al.* 1995) are being reduced with standardised procedures and next generation sequencing (Cooper & Poinar 2000; Rizzi *et al.* 2012). As molecular work becomes more accessible, the enormity of genetic data provided can be extremely valuable, but much of it can also be surplus to immediate requirements. For parentage analysis, small scale markers are still adequately efficient.

3.2.5 *THE USE OF MOLECULAR MARKERS TO ESTIMATE RELATEDNESS*

Kinship can be established using a variety of markers; allozymes, restriction fragment length polymorphisms (RFLPs), and randomly amplified polymorphic DNA (RAPDs) have all been used successfully (Fig. 1). Allozymes can have too little variation to successfully estimate relatedness (Queller *et al.* 1993), but microsatellites are a reliable choice because they have distinguishable loci with co-dominant alleles.

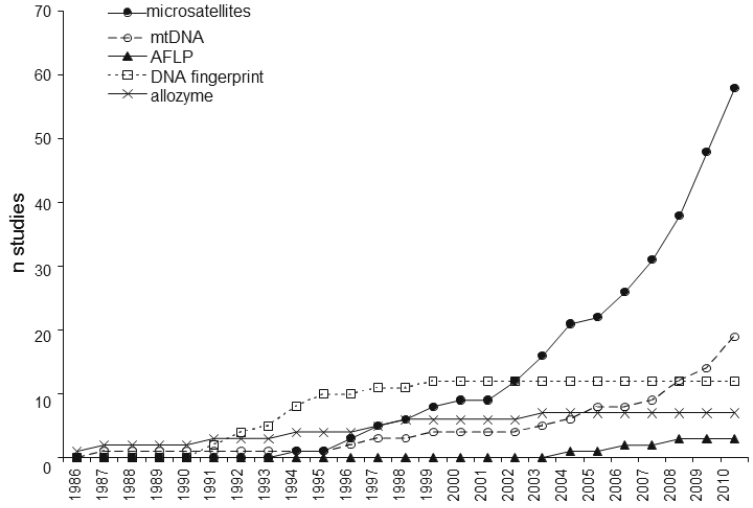


Figure 1 Cumulative number of studies using molecular marks since 1986. The five most frequently used methods have been considered (taken from Witzenberger & Hochkirch 2011).

Despite a general preference for microsatellites, a study of their use in kinship determination in comparison with SNPs in a wild sockeye salmon (Oncorhynchus nerka) population found a higher and more accurate assignment of parentage using 80 SNPs rather than 11 microsatellite loci; Hauser *et al.* (2011) found that assignment success was always higher with SNPs, despite having 80 vs 192 independent alleles. They also found an average error rate of 2% per genotype, while SNPs had a lower error rate of 0.34% per genotype.

To analyse the DNA they used the software Colony2 and Cervus3. Colony2 (Wang & Santure 2009; Jones & Wang 2010) implements a maximum likelihood method to assign sibship and parentage jointly, using individual multilocus genotypes at a number of codominant or dominant marker loci. It can be used in estimating full- and half-sibling relationships, assigning parentage, inferring mating systems (polygamous/monogamous) and for analysing reproductive skew in both diploid and haplo-diploid species. Cervus3 (Marshall *et al.* 1998; Kalinowski *et al.* 2007) uses likelihood to assign parentage; it uses likelihood ratios that are calculated allowing for the possibility that the genotypes of parents and offspring may be mistyped, and it determines via simulation the level of confidence in the parentage it assigns. Analyses in the study differed in the number of offspring assigned to parent pairs and single parents, but Colony2 had better performance overall. They concluded this was most likely due to Colony2 using not only parent-offspring relatedness but also relatedness among putative offspring.

If parentage can be assigned, hidden behaviour such as mating can be inferred. This was achieved when multiple paternities were suspected in squid *Loligo forbesi;* high levels of variation are needed when looking into a single colony or series of embryos and the development of microsatellite markers were variable enough to confirm their hypotheses (Shaw & Boyle 1997). Unfortunately they could not determine the genotype of the female, but techniques have since developed and this is now unlikely to be an issue with good quality DNA.

3.2.6 *THE IMPORTANCE OF MOLECULAR TECHNIQUES IN CAPTIVE BREEDING*

The relatedness of a population must be understood to develop viable captive breeding populations. A loss of fitness has been shown to occur in many species in a captive environment, explanations for which include relaxed natural selection, domestic

selection (adaptation to a captive environment), heritable epigenetic effects and inbreeding among close relatives (Christie *et al.* 2012).

Domestic selection promotes the fixation of alleles which can be deleterious in nature (Lynch & Hely 2001). Without genetic management, it has been proposed that ultimate genetic transformations could eventually lead to natural populations that cannot sustain themselves, unless the selection pressures of a captive environment are designed to resemble those in the wild (Lynch & Hely 2001). These deleterious genetic consequences have been shown to occur relatively quickly; Araki *et al.* (2007) measured lifetime productive success of two generations of steelhead trout (*Oncorhynchus mykiss*) that had been reared in captivity and then bred in the wild after release. Microsatellites were used to create a three generation pedigree and showed that the effects of domestication reduce subsequent reproductive capabilities by ~40% per captive reared generation. The authors conclude that repeatedly supplementing wild populations with captive-reared individuals for reproduction of captive-reared progenies should be reconsidered. As techniques improved, it was shown that captive breeding was having an effect after just one generation and the decline in fitness was attributed to domestic selection (Christie *et al.* 2012). Breeding programmes must understand which traits are under selection and manage them appropriately.

Domestic selection can be circumvented with fewer logistical issues in a more highly managed, recreational population. Waldbieser and Bosworth (2012) developed a microsatellite marker panel for parentage and kinship analyses in channel catfish (*Ictalurus punctatus*) to help aid the human consumption of catfish within the industry of US aquaculture. Commercial catfish farmers were reported to use mass selection to improve the growth of the catfish. Results recommended management that would

incorporate molecular techniques and help to implement a more efficient pedigree-based selection program.

Such a prescribed degree of control cannot always be implemented with individuals taken from the wild to help the survival of the species. The ability to determine relatedness was utilised in a conservation action plan for Anegada iguanas (*Cyclura pinguis*) in the British Virgin Islands. Informative microsatellite markers were used for pairwise relationship analysis, mean kinship calculations and parentage analysis. This allowed the genetic investigation into relatedness of six individuals selected as founders for a captive population. Witzenberger & Hochkirch's (2011) review of molecular studies on the genetic consequences of captive breeding found that there appeared to be a minimum number of founders (15) and a minimum captive population size (100) to minimise the loss of genetic diversity. Not all conservation efforts can feasibly follow these guidelines, which can bring the purpose of specific conservation efforts into question. If fitness will decline to such an extent that individuals cannot be returned to the wild, are captive populations actually better than none at all?

Ideally, research can be conducted, and management implicated, in an *in situ* environment. The genetic information can then be used on an entire population and not a subset of it. It is for this purpose that the described microsatellites have been developed. This study aimed to isolate polymorphic microsatellite markers in *Nautilus pompilius* that can be used to determine the population structure in the overexploited cephalopod. Establishing the connectivity of populations around Australia and the Philippines will help to understand the movement of individuals between populations across a range of distances.

3.3 *METHODS*

N. pompilius were sampled from Imperieuse Reef (IR), west Australia (17˚35'S, 118˚55'E) under an Australian Fisheries Management Authority (AFMA) Scientific Permit. Individuals were caught in traps that were baited with ~1 kg of chicken (*Gallus gallus domesticus*) and positioned on the reefs at a depth of ~200 m (Sinclair *et al.* 2011). Traps were set at dusk and collected at dawn. Sample collection was non-lethal; a 1-2 cm long labial tentacle biopsy was collected before each individual was released on the reef wall at a depth of ~40 m. Tentacles were placed immediately into a 20% DMSO (dimethylsulfoxide), 100 mM EDTA, saturated NaCl solution and stored at 4°C in the field. Samples were later washed in TE buffer and placed into absolute ethanol for storage (Sinclair *et al.* 2011).

Genomic DNA was extracted from tentacle tissue using a Qiagen DNeasy tissue kit (Qiagen Pty. Ltd.). DNA concentration was quantified using a fluorimeter (Fluostar Optima) and its quality assessed by electrophoresis on a 1% agarose gel. The library was created from one individual (IR38) by digesting its DNA with the restriction enzyme *Mbo*I and enriching the *Mbo*I-fragments for the dinucleotide microsatellite motifs $(CA)_n$, $(GA)_n$, $(TC)_n$, $(TG)_n$ (following Armour *et al.* 1994). Transformed colonies (n=192) were then Sanger sequenced using BigDye Terminators v3.1 (Applied Biosystems) in both directions, to allow the construction of a consensus sequence. An Illumina paired-end library was also created using 1 µg of the IR38 dinucleotide-enriched genomic DNA. The SureSelect Library Prep Kit, ILM (Agilent Technologies Inc.) protocol was followed and DNA sequencing was conducted using a MiSeq Benchtop Sequencer (Illumina). Primer sets were designed from 44 microsatellite sequences (39 Sanger and five MiSeqs; EMBL accession numbers HG918068-HG918111) using PRIMER3 v4.0.0 (Rozen & Skaletsky

1999). Primer sets were then tested on four individuals from four different sampling locations (the Philippines, Ashmore Reef, Great Barrier Reef and Osprey Reef).

Multiplex sets were designed based on allele length using dyes 6FAM, HEX, VIC and P ET (Fig. 2). Products were amplified in 2 µl multiplex PCR reactions, including 1.0 µl DNA (air dried), 1 µl primer mix (fluoro-labelled forward and reverse primer(s) at 0.2 µM) and 1 µl Qiagen Multiplex Master mix. Multiplexes were amplified under the following profile: 95°C for 15 min, followed by 44 cycles of 94°C for 30 s, 56°C for 90 s, 72°C for 90 s and finally 72°C for 10 mins. PCR products were genotyped on an ABI 3730 48-well capillary DNA Analyser (Applied Biosystems Inc.) using LIZ GS500 size standard (Applied Biosystems Inc.). Alleles were scored using GENEMAPPER v3.7 software (Applied Biosystems Inc.). Thirty markers that could be scored reliably were tested in 24 individuals from the IR population (Table 1). Markers that were indistinct or did not amplify consistently were discounted from use in further analysis at this stage (Table 2).

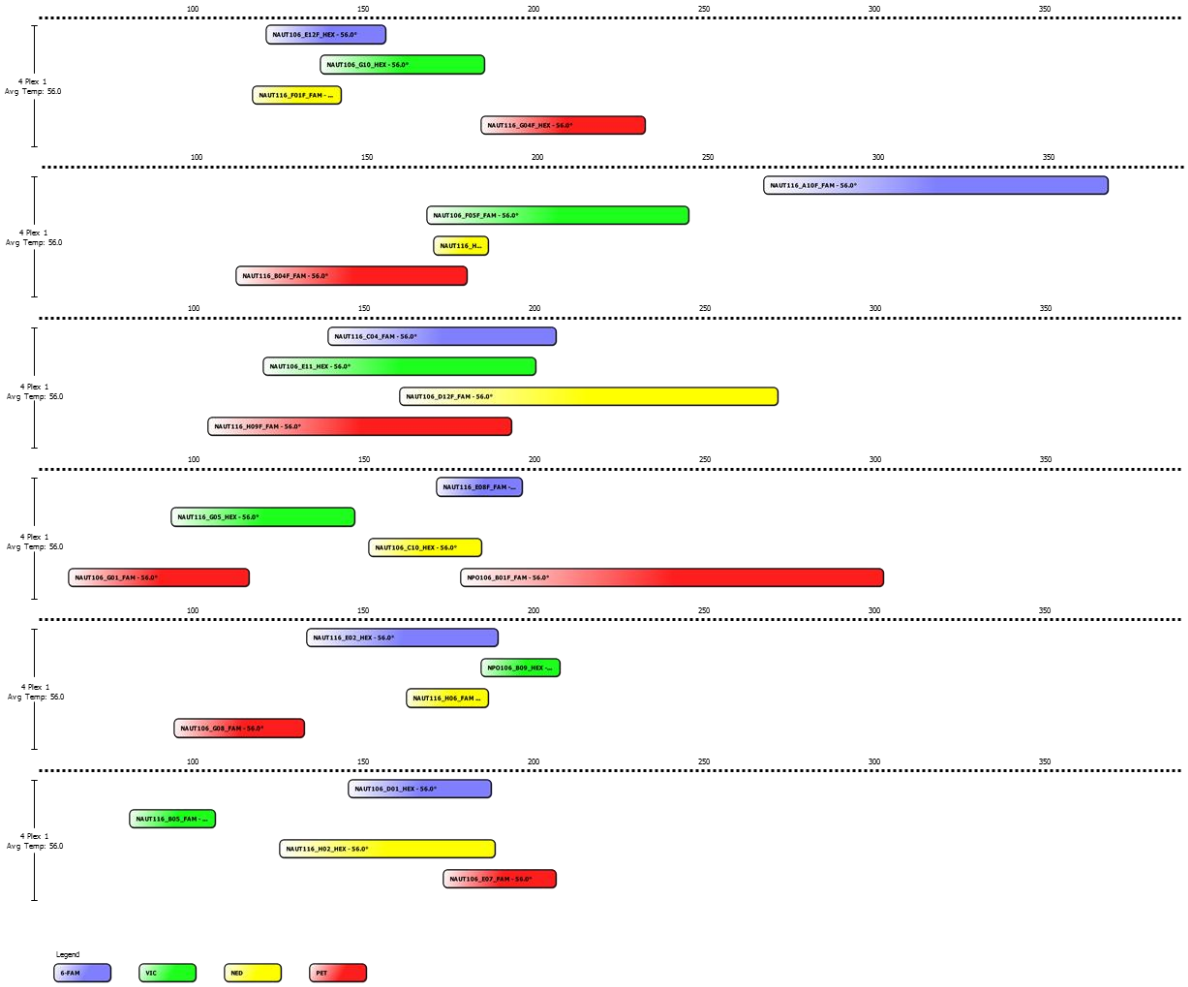


Figure 2 Multiplex combinations designed using Multiplex Manager. For time and cost efficiency, the maximum number of microsatellites should be amplified in each reaction. Labelling the forward primer with a different fluorescent dye enables targeted areas to overlap and still be distinguished. Markers with the same dyes cannot overlap. Here, 26 loci were combined into six reactions. Colours represent the fluorescent dyes, and the bar length represents the allele size that is targeted with that primer set. Allele size shown in base pairs (*x*-axis).

Approximately 60% of individuals were re-extracted and re-genotyped across the selected loci. Alleles not matching between reruns were identified using Microsatellite Toolkit and scoring inaccuracies rectified. All individuals genotyped were confirmed as unrelated using SPAGEDI (Hardy & Vekemans 2002). Observed and expected heterozygosities were calculated using CERVUS v3.0.3 (Kalinowski 2005). Allelic drop out was investigated using MicroChecker (Van Oosterhout *et al.* 2004). Tests for deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD)

were calculated in GENEPOP v4.2 software (Raymond & Rousset 1995; Rousset 2008). To correct *p*-values in multiple tests, the False Discovery Rate (FDR; Verhoeven *et al.* 2005) was applied to linkage disequilibrium *p*-values.

3.4 *RESULTS*

DNA extractions had a 100% extraction success rate and no amplification error was detected; all rerun mismatches were accountable to scoring error and were corrected on inspection. Electrophoresis showed the DNA quality to be good for all samples except for those from the Philippines, where quality observed was lower. DNA concentration did not reflect this and no difference was seen in amplification rates between the Philippines and the Australian samples (Fig. 3).

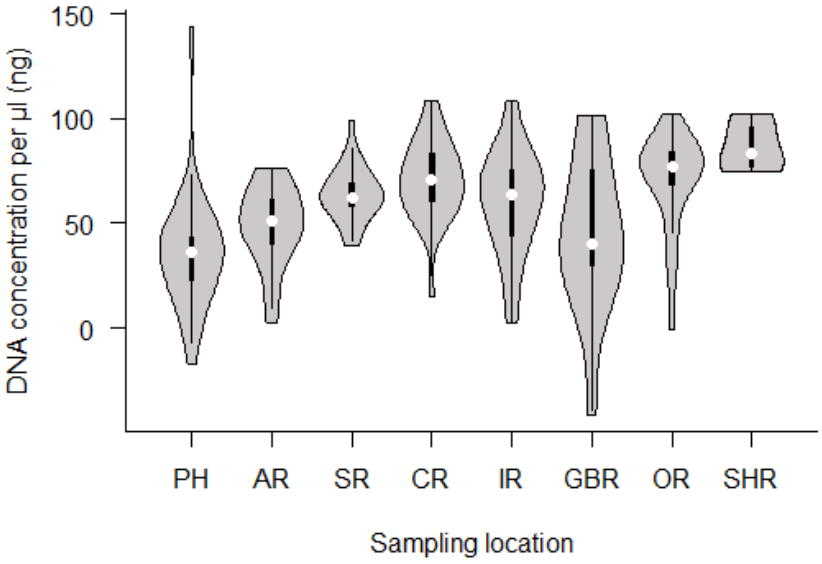


Figure 3 DNA concentration of samples from each location. Quantification was performed using a fluorimeter (Fluostar Optima), values represent nanograms of DNA found in one micro litre. PH, Phillipines; AR, Ashmore Reef; SR, Scott Reef; CR, Clerke Reef; IR, Imperieuse Reef; GBR, Great Barrier Reef; OR, Osprey Reef; SHR, Shark.

Microsatellites were successfully isolated from *N. pompilius.* From the 40 markers tested, 39 amplified to the designed primers, and 21 were in Hardy-Weinberg equilibrium (Table 1, 2). There was no evidence of large allele drop out and no loci were consistently deficient in heterozygotes. A shortage of heterozygote genotypes was found in Npom08 in Osprey Reef and Scott Reef populations which may have resulted from scoring errors. Loci showed no evidence for null alleles (freq>0.1).

Table 1 Characterisation of 30 *Nautilus pompilius* microsatellite loci in the Imperieuse Reef population, west Australia

Locus	Clone name and EMBL accession number	Primer sequences and fluoro label (5'- 3')	Multiplex set	Repeat motif	Expected and observed allele size of library individual (bp)	n	# Alleles obs.	Allele size range (bp)	H_{obs}	H_{exp}	HWE *p*-value <0.05	Est. f(null)
Npom01	Naut106_B01	F: [6FAM]ACCAAATCAACATAATCTGGTGTG	–	(GA)28	221	31	31	184–298	0.92	0.98	0.00*	0.02
	HG918068	R: CCTTAAATTTGCTTACCATGTGC			221, 221							
Npom02	Naut106_B09	F: [VIC]GCCCAAATGGATAAATACAAGC	6	(CA)14	191	31	19	180–226	0.74	0.85	0.17	0.06
	HG918069	R: TCAAATCTCGCCATCACATC			188, 209							
Npom03	Naut106_C10	F: [PET]ACGGAGCCGAAACCACTAC	1	(GA)17	177	31	18	159–184	0.90	0.91	0.34	0.00
	HG918070	R: ACACGTGGAATTTCCTTTGC			167, 179							
Npom04	Naut106_D01	F: [PET]GTGGTTTATTGGTGGGAAGG	2	(GT)15	162	22	20	150–212	0.86	0.94	0.16	0.03
	HG918071	R: CCGCTCTTCTGTCAACAGTG			152, 167							

Npom05	Naut106_D12	F: [6FAM]TTTACTTTGGAGAACTTTCGCTAATC	3	(GA)39	209	31	25	166–267	1.00	0.97	0.11	-0.03
	HG918072	R: GTGGTGACCTTCAACGGTTC			205, 205							
Npom06	Naut106_E07	F: [NED]GATCACGCAGCAAAGTCG	5	(CA)11	191	31	10	179–202	0.79	0.72	0.95	0.05
	HG918073	R: GAGCGAGCGGTACGATTC			187, 191							
Npom07	Naut106_E11	F: [PET]TTCATCCGTTAATTCGTATTGC	3	(CA)20	147	34	24	124–196	0.97	0.94	0.01*	-0.03
	HG918074	R: CTCCCAAATTCTTATACGTCAGC			153, 157							
Npom08	Naut106_E12	F: [PET]TTGGCTAAATCGACGAAACATAG	5	(GA)24	127	29	12	115–160	0.17	0.91	0.00*	0.68
	HG918075	R: GCCGATTCACAATAACAGTGG			137, 137							
Npom09	Naut106_F05	F: [VIC]TTCCAACAAATGCCTTCAAAC	5	(CA)17	188	31	29	176–239	0.96	0.97	0.63	0.00
	HG918076	R: GCTTGGAATAGCTGCACAGAG			185, 206							
Npom10	Naut106_G01	F: [6FAM]GGTCGACGCTGGAACAAT	4	(GT)17	85	31	16	67–102	0.94	0.92	0.39	-0.01
	HG918077	R: TGTCACGACTACGGAATAGGTG			81, 85							
Npom11	Naut106_G08	F: [NED]CCTATTTCAGTGAGTGGAGCTG	1	(CT)20	124	31	8	116–128	0.79	0.78	0.76	0.00
	HG918078	R: TTCATGTGGCTGATGTAAGAAAG			122, 124							
Npom12	Naut106_G10	F: [NED]AGGACAATTAGACTGACGTCTGAAG	4	(GT)17	148	31	31	124–205	0.97	0.96	0.54	-0.01
	HG918079	R: GATGGCGTGCTTATTGGTG			156, 171							

Npom13	Naut116_A10	F: [6FAM]ATTGCCGTCTCGATGTGC	5	(CT)14	297	31	31	272–365	1.00	0.98	1.00	-0.02
	HG918080	R: GCCTCCAGACAGTCGAACC			295, 299							
Npom14	Naut116_B04	F: [6FAM]CAATGGACGTACACCAGAGG	–	(CT)21	142	31	16	118–176	0.25	0.93	0.00*	0.57
	HG918081	R: AAGACAAACTTCCCTCCGAAT			144, 144							
Npom15	Naut116_B05	F: [6FAM]AAGCACGTACAGGTGTGCAG	2	(GT)10	99	25	7	87–102	0.79	0.82	0.99	0.01
	HG918082	R: TGTTATTTCGCTCTAGCAACAGTC			94, 98							
Npom16	Naut116_C04	F: [VIC]GACTATTTGCGTAGAAACACAAGG	1	(CA)17	154	31	29	137–209	0.94	0.96	0.84	0.00
	HG918083	R: CGTTAATCCTAATCCAACTATCTCG			156,202							
Npom17	Naut116_E02	F: [VIC]TCCCACCAGTTTACATACAATAGC	3	(CA)15	179	31	24	134–209	0.84	0.95	0.18	0.05
	HG918084	R: GAAACCTGGAATTCTGATTATGC			164,178							
Npom18	Naut116_E08	F: [6FAM]TAGCATGCTCGCTGGTTATG	6	(GT)11	182	31	8	177–191	0.92	0.82	0.91	-0.06
	HG918085	R: CTTGAGTACCAACCAAGACCAAG			177,183							
Npom19	Naut116_F01	F: [6FAM]TATAGGGATGCGTCGTCACC	–	(GA)8	132	31	8	123–139	0.17	0.83	0.00*	0.66
	HG918086	R: CCCTAAGGCTTTATGAAGTCAGC			129,129							
Npom20	Naut116_G04	F: [NED]CGGACAGACCTAATGCAATG	3	(CT)33	208	31	26	175–232	1.00	0.96	0.96	-0.03
	HG918087	R: CAAATGAAACTCGGCAGAAAC			189,223							

Npom21	Naut116_G05	F: [VIC]TGATGGACCGGACTAGGAA	4	(GA)16	123	31	11	99–143	0.88	0.86	0.04*	-0.03
	HG918088	R: CGCCAATGACCAGTCAGA			113,122							
Npom22	Naut116_H02	F: [HEX]CTGGTTACTATCATTATGGTTTCTCG	2	(CA)36	186	31	20	134–185	1.00	0.96	0.00*	-0.03
	HG918089	R: CGACATCGTCCTGCATTTAG			127,127							
Npom23	Naut116_H06	F: [6FAM]CTGTCCTGGCTGCTAACCTAC	–	(CA)10	178	31	4	168–178	0.26	0.52	0.01*	0.32
	HG918090	R: CTTCGATTCATCAGAACCTAATACC			174,176							
Npom24	Naut116_H09	F: [6FAM]CAAAGAATTTGAAGCTCGAACAG	5	(CA)25	146	31	26	114–189	0.88	0.95	0.11	0.03
	HG918091	R: TTTCCACAAATCGTGTCTTGAG			132,144							
Npom25	Naut116_H12	F: [PET]GTGCTTTACTGACAGTTACATATCGTG	6	(GT)9	183	31	5	176–184	0.75	0.63	0.72	-0.12
	HG918092	R: CCTAGACCATCGATGCAAGC			185,187							
Npom26	Naut2904	F: [6FAM]AACCCGGTACAGGAGCAAC	1	(CT)11	162	31	9	138–162	0.92	0.83	0.99	-0.62
	HG918093	R: ACGAGCGAAGAACCACTTTG			148,160							
Npom27	Naut5395	F: [PET]AAAGTTTCCCGGCTTCTTTG	4	(CA)10	147	31	15	144–188	0.92	0.84	0.71	-0.07
	HG918094	R: CGGAGGATTAATATTGATTATTTGTTG			147,175							
Npom28	Naut5442	F: [NED]AAACAGTTCGGTGCATCCTC	6	(GT)10	167	25	13	169–194	0.76	0.89	0.65	0.07

	HG918095	R: CATCCAGCAAATCAGTCGTG			175,190							
Npom29	Naut5466	F: [VIC]CTGCAGCAAAGTAGGCTGTG	2	(CT)12	152	31	16	136–184	0.92	0.94	0.95	0.00
	HG918096	R: AAGTGGCCATGGGTATTTTG			146,154							
Npom30	Naut7474	F: [HEX]ATCAAACGCTCGGATGTAAAC	–	(GT)10	100	31	6	89–113	0.56	0.74	0.01*	0.13
	HG918097	R: ACGCATTCGTCTCTATTCTGC			94,98							

Loci sequenced using Sanger (*Npom01–Npom25),* Miseq (*Npom26–Npom30*); n, total individuals genotyped; # Alleles obs, total alleles observed; bp, base pairs; library individual, the individual genotype from which the library was developed; H_{obs}, observed heterozygosity; H_{exp}, expected heterozygosity; HWE, Hardy Weinberg equilibrium; *, significant deviation from HWE ($p<0.05$); Est. f(null), estimated null allele frequency; -, excluded from multiplex. Primer melting temperatures 59-61°C

Table 2 Details of 14 *Nautilus pompilius* microsatellite loci rejected after initial amplification due to difficulty in scoring following testing in the Imperieuse Reef population.

Locus	Clone name and EMBL accession number	Primer sequences and fluoro label (5'- 3')	Repeat motif	Expected allele size (bp)
Npom31	Naut106_B03	F: [HEX]CGACTCAAGGGACTACATTCG	(CT)32	250
	HG918098	R: AAACAGTAACGCCTAAACACCAC		
Npom32	Naut106_B12	F: [6FAM]CGACTGCTGAACCTACAAAGC	(GA)30	170
	HG918099	R: GCGGGAGAACAAATCAAGAC		
Npom33	Naut106_C07	F: [6FAM]CGGAATTTAGCACGGTGAC	(CA)12	148
	HG918100	R: AGCGGTGTTGGGAAGAATAC		
Npom34	Naut106_C11	F: [6FAM]TTCCTTAAATTTGCCACCGTA	(GA)16	259
	HG918101	R: ATCACTTAAACCATCAGTTACGACA		
Npom35	Naut106_D05	F: [6FAM]AAATTTCCTTGGTCGATATACGG	(GA)33	169
	HG918102	R: TTCTGCCAGTCTATCCTGACG		
Npom36	Naut106_E08	F: [HEX]CCGAAGCTGAAAGAATTTGC	(CT)37	183
	HG918103	R: TGGCTATGCCCATATTAGCC		
Npom37	Naut106_E10	F: [6FAM]TCTAATGCCACCAAATGAAGTC	(GA)29	203
	HG918104	R: TGTTTGTTCTGCCATCATCC		
Npom38	Naut106_F07	F: [HEX]AGAAAGACCCGATACGCAAC	(GA)32	282
	HG918105	R: TCTGCTATTTCGCAGCGTAG		
Npom39	Naut116_A09	F: [HEX]CGTTCACTGTTGGCCATACTT	(GT)7	92
	HG918106	R: GCCGACAGGCCTCTACTCT		
Npom40	Naut116_B02	F: [HEX]GCGCAAAGTGAAAGCTGAC	(GT)8	141
	HG918107	R: CCCTGACCCTTTCACATACTTC		
Npom41	Naut116_C06	F: [HEX]AGCCCTTCAGCCTACATTCTG	(CA)9	227
	HG918108	R: ATAATCGGTCGTCGCATTTG		
Npom42	Naut116_E12	F: [HEX]CGACTAAATTGCTTCAGCAGAC	(GA)10	146
	HG918109	R: CTCCAGGTAGGCGCTCAG		
Npom43	Naut116_G08	F: [HEX]CTTGTAGGGCAAAGTTTGTGG	(GT)9	323

	HG918110	R: GATCATAGGCAACTCAACAGACAC		
Npom44	Naut116_H07	F: [HEX]GGATAATTGAAATTCTGGAGTTGG	(GT)10	190
	HG918111	R: AACCGTAAGTTATCCCGCAAC		

F, forward; R, reverse; bp, base pairs. Primer melting temperatures 59-61°C

Relatives within the study (half siblings and full siblings) were removed from the dataset. Departure from HWE was not detected consistently across populations; Npom02, Npom11, Npom15, Npom21 showed deviation in the Great Barrier Reef samples but in no others. Values could not be calculated for Npom24 and Npom25 in Osprey Reef samples because the loci were monomorphic in this population. Some groups of loci displayed linkage disequilibrium within one single population (Table 3). No loci displayed linkage disequilibrium in multiple populations suggesting that there is no evidence for physical linkage between loci based on the few individuals genotyped.

Table 3 Linkage disequilibrium between loci. Loci listed that showed linkage disequilibrium between loci with a population.

Location	Loci 1	Loci 2
Scott Reef	N106_B09	Naut5395
Clarke Reef	N116_B05	N106_G08
	Naut5395	N116_H09
	Naut5395	N106_G08
Great Barrier Reef	Naut5466	N116_H09
	N116_B05	N116_H09
	N116_E08	N116_H12
	N116_H12	N116_B05
	N116_G05	N106_G08
	N106_G08	N116_H09
	N116_E08	N116_E02
	N116_E08	Naut5466
	N116_G05	N116_E02

Observed levels of heterozygosity ranged from 0.17 to 1.00 with a mean of 17.2 alleles per locus. Influence of results by kinship structure was excluded as a possibility through the use of Queller and Goodnight (1989). All individuals with a relatedness of 0.5 were removed from the data set before analysis. Test results, amplification consistency and ease of scoring determined primers taken forward to analysis.

3.5 *DISCUSSION*

We present the first set of microsatellite markers isolated from *N. pompilius* (Cephalopoda). The markers were designed with the aim to investigate population structure in *N. pompilius*. Published data supports qualification for *Nautilus* as "endangered" on the IUCN red list (Dunstan *et al.* 2010), yet data deficiency currently inhibits the true classification of the species. Previous research has shown genetic isolation occurring between three evolutionary clades across the *N. pompilius* range (Sinclair *et al.* 2007, 2011; Bonacum *et al.* 2011; Williams *et al.* 2012). *Nautilus* are being unsustainably overfished across much of their range for the ornamental shell trade and their numbers are declining dramatically (Dunstan *et al.* 2010; De Angelis 2012; Barord *et al.* 2014). Investigating diversity between and within these isolated populations will highlight populations with an immediate requirement for greater levels of enforceable protection.

The use of genetic data for molluscs has been notoriously problematic; heterozygote deficiency has been present in work from the start of such molecular techniques (Mallet *et al.* 1985; Borsa *et al.* 1991; Chacón *et al.* 2013). Here, heterozygote deficiency was not encountered, but we did observe a large number of alleles per locus that impacted on the study. Due to the markers being developed to investigate population structure, loci with a high frequency of alleles had to be removed from the analysis to prevent them from

hiding genetic similarities and differences. High variability was counterproductive to the project because if each population had every combination of alleles, then differences could not be detected. Though DNA quality was good, amplification resulted in a lot of "noise" that made scoring subjective. Only loci that could be easily scored will be used in subsequent population analysis.

Microsatellites were chosen as a fine scale marker to build on the already established mitochondrial information. As well as being highly variable, their choice as a popular marker is aided by the fact that they are relatively inexpensive as a molecular technique. One consideration that must be taken into account is the potential for microsatellite loci to show size homoplasy. As a result, alleles are identical in state without being identical by descent and can make the evolutionary processes leading to species ancestry difficult to infer (Estoup *et al.* 2002). Due to allele classification being determined by fragment size, high mutation rates can result in size homoplasy. This can affect structural results because populations can indicate gene flow and similarity in analyses, when this is actually a result of convergent evolution (Adams *et al.* 2004).

This adds to the on-going drive to use more encompassing markers in research. As accessibility to hardware increases, costs per unit drop and techniques simultaneously improve, genome wide association scans are now possible. As the size of the bioinformatics task decreases, we are moving towards this to answer ecological questions. Initial costs may be higher, but they calculate as better value for money due to the extent of data returned. The range of software discussed here for microsatellite analysis has yet to be developed for whole genome work. This will reduce the enormity of whole genome data handling, leaving few boundaries to inhibit the movement towards whole genome work becoming standard practice.

For the work discussed here, microsatellites can be used to efficiently investigate population differentiation. To develop the research and gain a greater understanding of *N. pompilius* at a molecular level, the next step towards rectifying their data deficiency would logically be at the genome level.

CHAPTER FOUR:

THE GENETIC STRUCTURE OF NAUTILUS POMPILIUS POPULATIONS SURROUNDING AUSTRALIA AND THE PHILIPPINES

The data within this chapter have been accepted for publication:

Williams, RC, Jackson, BC, Duvaux, L, Dawson, DA, Burke, T, Sinclair, W (2015b) The genetic structure of *Nautilus pompilius* populations surrounding Australia and the Philippines. Molecular Ecology. **24**, 3316-3328

4.1 *ABSTRACT*

Understanding the distribution of genetic diversity in exploited species is fundamental for conservation. Low levels of genetic variation in cryptic deep-water species may be accountable to their diverse dispersal mechanisms. Genetic structure and potential gene flow among populations must be assessed to designate appropriate protection. *Nautilus pompilius* is unsustainably fished in the Philippines for the ornamental shell trade and have limited legislative protection despite dramatic declines. Here, we describe the population structure of *N. pompilius* around Australia and the Philippines using a set of 14 microsatellite markers. Despite their relative geographic proximity, Great Barrier Reef individuals were shown to be genetically isolated from Osprey Reef and Shark Reef in the Coral Sea (F_{ST}=0.312, 0.229 respectively). Conversely, despite larger geographic distances between the Philippines and west Australian reefs, samples displayed low genetic structure (F_{ST}=0.015). Approximate Bayesian computation analysis indicated that this was not a result of gene flow occurring between the Philippines and west Australia. Large average effective population sizes over time shown at both locations suggest a minor role for genetic drift and explain the observed genetic similarity. The absence of migration indicates it is unlikely the Philippines would be repopulated should fishing in this area continue towards extinction. These data contribute to the case for legislative protection for *N. pompilius* and help to rectify the paucity of information currently inhibiting their conservation classification. When used to inform effective management plans, understanding population structure can facilitate sustainable harvesting and prevent local extinctions, thereby preserving the diversity of genetically distinct stocks.

4.2 *INTRODUCTION*

Throughout the world, many commercial marine species are experiencing significant population declines (Hutchings 2000; Worm *et al.* 2006; Neubauer *et al.* 2013; Watson *et al.* 2013). Low success rates seen in their recovery (Hutchings 2000) suggest gaps in scientific knowledge that must be resolved for effective conservation (Sale *et al.* 2005). Nautiloids (*Nautilus* spp. Linaeus and *Allonautilus* spp. Ward and Saunders) are heavily overfished for the ornamental shell trade. The long term effects of fishing on populations, or their ability to recover, are unknown (De Angelis 2012). An 80% decline in catch per unit effort from 1980–2010 has been reported from Philippine fisheries. This decline qualifies *Nautilus pompilius* to be classified as endangered on the IUCN Red List (Dunstan *et al.* 2010) and yet the current data deficiency leaves no legislative mechanisms in place to inhibit species overexploitation and decline. Understanding migration and its effect on population dynamics is important to provide appropriate protection to populations through *in-situ* means such as marine protected areas (Grüss *et al.* 2011).

Current movement and migration of cephalopods has been monitored through tracking devices, but results have been variable (see Semmens *et al.* 2007). As the only cephalopod with an external shell, this approach has been utilised to track *Nautilus* by fitting ultrasonic tags to the shell (Dunstan *et al.* 2011b). In coleoids, juveniles are often too small to be tagged and tag placement in adults can be difficult, capture rates fluctuate and are often reliant on fishermen reporting catches (Sauer *et al.* 2000). Improvements in technology with concomitant decreases in device size and cost will aid progress in this area. Acoustic tracking has been used to monitor smaller scale movement and migration patterns with the transmitter placed inside the ventral mantle (Stark *et al.* 2005). Chemical tags (incorporating compounds such as fluorescent chemicals, elements and isotopes into calcified tissues) enable tagging at all life stages for several weeks or more,

and on large numbers simultaneously, but result in the death of the animal (Semmens *et al.* 2007).

If specific individuals cannot be tracked then molecular techniques allow the detection of gene flow among populations through patterns of shared genetic variation (Levin 2006; Cowen & Sponaugle 2009). For example, hypothesised differentiation of *N. pompilius* has been confirmed with the use of cytochrome c oxidase sub unit I (COI), which separated populations into three evolutionary clades (Wray *et al.* 1995; Sinclair *et al.* 2007, 2011; Bonacum *et al.* 2011; Williams *et al.* 2012). This observed population structure was proposed to be the result of movement from an ancestral population in the Philippines (Wray *et al.* 1995). Historic expansions of nautiloid distribution will have been restricted by at least three biogeographic barriers (Crick 1993): water depth, distance between adjacent shelf seas and temperature. These restrictions are also relevant to modern *Nautilus* and *Allonautilus,* whose movement is limited to a certain extent by their morphology. The internal arrangement of the *Nautilus* shell means their deepest position in the water column is limited by their risk of shell implosion. As a consequence, the maximum depth at which an individual would be encountered is considered to be approximately 800 m (Saunders & Wehman 1977; Kanie *et al.* 1980b). They are typically found between depths of 130 and 700 m (Dunstan *et al.* 2011b), remaining close to the reef for protection. These limitations create a barrier to movement and despite small geographic distances, genetic differentiation occurs (Sinclair *et al.* 2007), furthered by the absence of a juvenile larval stage to aid dispersal (Saunders & Landman 2010).

Population genetic analyses can estimate movement among populations (Pearse & Crandall 2004). Inference of demographic history over long time scales can answer questions regarding long term gene flow between and distribution of genetic diversity

among populations (Semmens *et al.* 2007), and therefore can aid design of specific genetic management. Population structure is often defined by an interaction of environmental factors and their influence on individual movement (Doubleday *et al.* 2009; Bestley *et al.* 2013). For vulnerable species, establishing population structure facilitates sustainable harvesting, prevents local extinctions and preserves the diversity of genetically distinct stocks (Carvalho & Hauser 1994). It is assumed that population sub-structure is inversely related to dispersal potential, although structuring in species with high dispersal potential is commonly seen (Palumbi 2004). Oceans are considered to have few physical barriers to gene flow and so widely separated areas can remain connected, making absolute vicariance rare in a marine environment (Palumbi 1994; Mirams *et al.* 2011). Population structure and speciation have however been attributed to oceanic features, such as salinity (Rocha 2003; Lessios *et al.* 2003), depth and temperature (Zardi *et al.* 2007).

Oceanic features with the potential to act as a barrier to movement can result in species responses ranging from panmixia (Lessios *et al.* 2003) to complete separation (Baums *et al.* 2012). These responses can differ *post-mortem*; *Nautilus* shells have been found thousands of kilometres outside of their genus distribution (House 2010). Waves, currents and transient organisms can all influence their distance and direction of travel (Mapes *et al.* 2010a). Separating *post-mortem* drift from distribution expansion is important in understanding species ecology. Molecular techniques and statistical analyses are valuable tools when aiming to achieve this. Their ability to determine species movement can be used in various areas of conservation. For example to evaluate whether marine protected areas are providing sufficient protection (i.e. to assess that species movement does not extend beyond protected boundaries; Grüss *et al.* 2011), they can be used to trace the origin of catch (Hobson 1999), or they be used to ensure that illegal catch is being not sold incorrectly to the consumer (Griffiths *et al.* 2013).

We aim to use molecular methods to increase knowledge regarding the genetic structure of *N. pompilius* populations surrounding Australia and the Philippines. Current fishing activity could lead to the local extinction of *N. pompilius* in areas of the Philippines (Barord *et al.* 2014), and so understanding population connectivity will help to establish the impact that fishing is having elsewhere across the distribution. These data will contribute towards rectifying the information deficiency currently inhibiting the legal classification of *N. pompilius*, and will provide information that can be utilised to inform effective management of exploited populations.

4.3 *MATERIALS AND METHODS*

4.3.1 *SAMPLE COLLECTION*

Australian samples were collected from seven reefs in the Indo-Pacific Ocean (fig. 1) on an Australian Fisheries Management Authority Scientific Permit (Permit number: 1002548). West Australian (WA) samples were taken from four reefs: Clerke Reef, Imperieuse Reef, Ashmore Reef and Scott Reef. East Australian (EA) samples consist of Osprey Reef and Shark Reef in the Coral Sea, and the Far North Great Barrier Reef. Samples from the Philippines (PH) were collected from three locations: Tinitian, Roxas and Palawan. Collections were made under a Gratuitous Permit from the Department of Agriculture in the Republic of the Philippines.

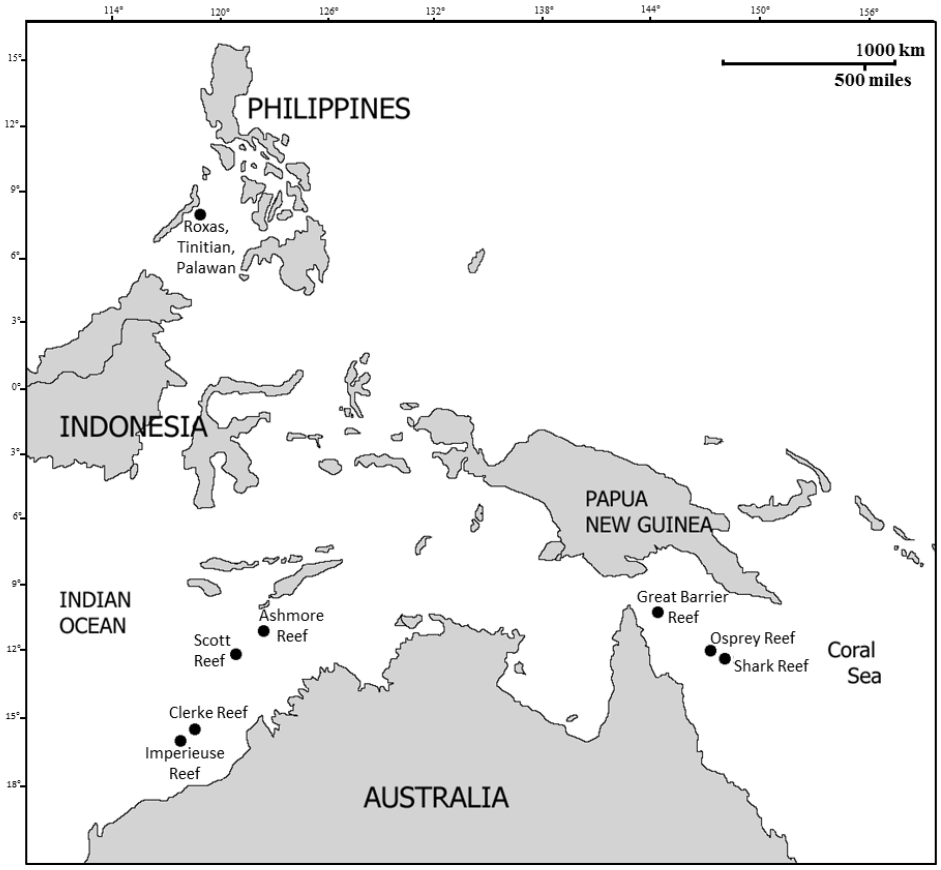


Figure 1 *Nautilus pompilius* distribution; locations sampled are indicated. A total of 215 *N. pompilius* were sampled from eight collection sites (range 8–45 individuals per location).

N. pompilius were caught using traps positioned on the reefs at a depth of ~200 m (Sinclair *et al.* 2011) baited with ~1 kg of uncooked chicken (*Gallus gallus*), set at dusk and collected at dawn. Sample collection is non-lethal; a 1–2 cm long labial tentacle sample was collected before each individual was released. Tentacles were immediately placed into a 20% DMSO (dimethylsulfoxide), 100 mM EDTA pH 8, saturated $NaCl_2$ solution and stored at 4°C in the field. Samples were later washed in TE buffer (1 M Tris-HCl pH 7.5; 0.5 M EDTA pH 8; Sambrook *et al.* 1989) and placed into absolute ethanol for storage at room temperature (Sinclair *et al.* 2011).

4.3.2 *MICROSATELLITE GENOTYPING AND VALIDITY*

DNA was extracted using Qiagen DNeasy tissue kits (QIAGEN Ltd, Manchester, UK). DNA concentration was quantified using a fluorometer (Fluostar Optima) and its quality assessed with electrophoresis on a 1% agarose gel. Fourteen polymorphic *N. pompilius* microsatellite loci (Williams *et al.* 2015a) were selected based on good results from quality checks, and used to genotype all 215 individuals sampled (n = PH 27; AR 29; SR 30; CR 32; IR 31; GBR 13; OR 45; SHR 8). PCR amplification was performed in 2 µl PCR reactions, including 10 ng air-dried DNA, 0.2 µM reverse primer, 0.2 µM forward fluorescent primer (6FAM, HEX, VIC or PET labelled) and 1 µl Qiagen Multiplex Master mix. Multiplexes were amplified under the following profile: 95°C for 15 min, followed by 44 cycles of 94°C for 30 s, 56°C for 90 s, 72°C for 90 s and finally 72°C for 10 min. PCR products were analysed on an ABI 3730 48-well capillary DNA analyser (Applied Biosystems Inc.) using LIZ GS500 size standard (Applied Biosystems Inc.). Allele sizes were assigned using the GENEMAPPER v3.7 software (Applied Biosystems Inc.).

Relatedness between individuals was estimated with SPAGEDI (Hardy & Vekemans 2002) using Queller and Goodnight's (1989) measure of relatedness. Relatives were removed and departure from Hardy-Weinberg equilibrium (HWE; $P < 0.05$) and linkage disequilibrium (LD) were calculated using GENEPOP (Raymond & Rousset 1995; Rousset 2008). LD was assessed using 1000 iterations per population and *P*-values corrected using the False Discovery Rate (FDR; Verhoeven *et al.* 2005). Corrections were made on a population-by-population basis to avoid overinflating the number of tests corrected for (using the FDR for multiple comparisons can lead to an increased chance of a type I error). Each microsatellite locus was assessed to estimate the presence of null alleles and identify scoring errors due to stutter using MICRO-CHECKER (Van Oosterhout *et al.* 2004). Null allele frequency per locus was estimated using CERVUS v3.0 (Kalinowski 2005). To assess genotyping error rate, 60% of samples were re-extracted and re-

genotyped across all loci. Error rates per reaction were calculated according to Hoffman & Amos (2005).

4.3.3 *POPULATION STRUCTURE*

Three Bayesian clustering methods were used to determine the most likely number of genetic clusters within the data set: STRUCTURE (Pritchard *et al.* 2000), TESS (Durand *et al.* 2009) and GENELAND (Guillot *et al.* 2005). The software STRUCTURE was run with an admixture model with no prior information on the sampling locations (Table 1).

Table 1 Settings for structural analyses run in STRUCTURE, TESS and GENELAND.

	Burn in	MCMC	K tested	Runs
STRUCTURE	150,000	250,000	1-10	15
STRUCTURE_EA	150,000	250,000	1-8	15
STRUCTURE_WA	150,000	250,000	1-8	15
TESS	100,000	500,000	2-10	15
GENELAND	1000	500,000	1-10	20

East Australian (EA) and west Australian (WA) clades run independently in STRUCTURE; MCMC, Markov chain Monte Carlo steps; K tested, the number of populations tested. The number of populations tested was set to allow further subdivision within the sampling sites. For STRUCTURE, the top ten runs with the highest probability of the estimated natural logarithm were used. GENELAND thinning set to 100.

To avoid the influence of kinship on inferred structure, all individuals with a relatedness of 0.5 were removed from the data set before analysis (Queller & Goodnight 1989). Plotting the natural logarithm of the posterior probability (PP) of K given these data over multiple runs determined the predicted number of clusters (Fig. 2), and this was compared with ΔK (Evanno *et al.* 2005) determined in STRUCTURE HARVESTER v.0.6.93 (Earl & VonHoldt 2011). Independent runs for all data sets were averaged in CLUMPP v.1.1.2 (Jakobsson & Rosenberg 2007) using the *Greedy* algorithm with 10,000 repeats to develop a consensus value for K. Graphical representation was produced in DISTRUCT

v.1.1 (Rosenberg 2003). Bayesian clustering of TESS was run without admixture and K was inferred from the modal value of the replicate with the highest likelihood. A correlated allele frequency model was used in GENELAND and the burn in length was based on the appearance of the posterior density log as suggested by the manual. The number of proposed clusters was selected from the highest average log PP (Guillot *et al.* 2009).

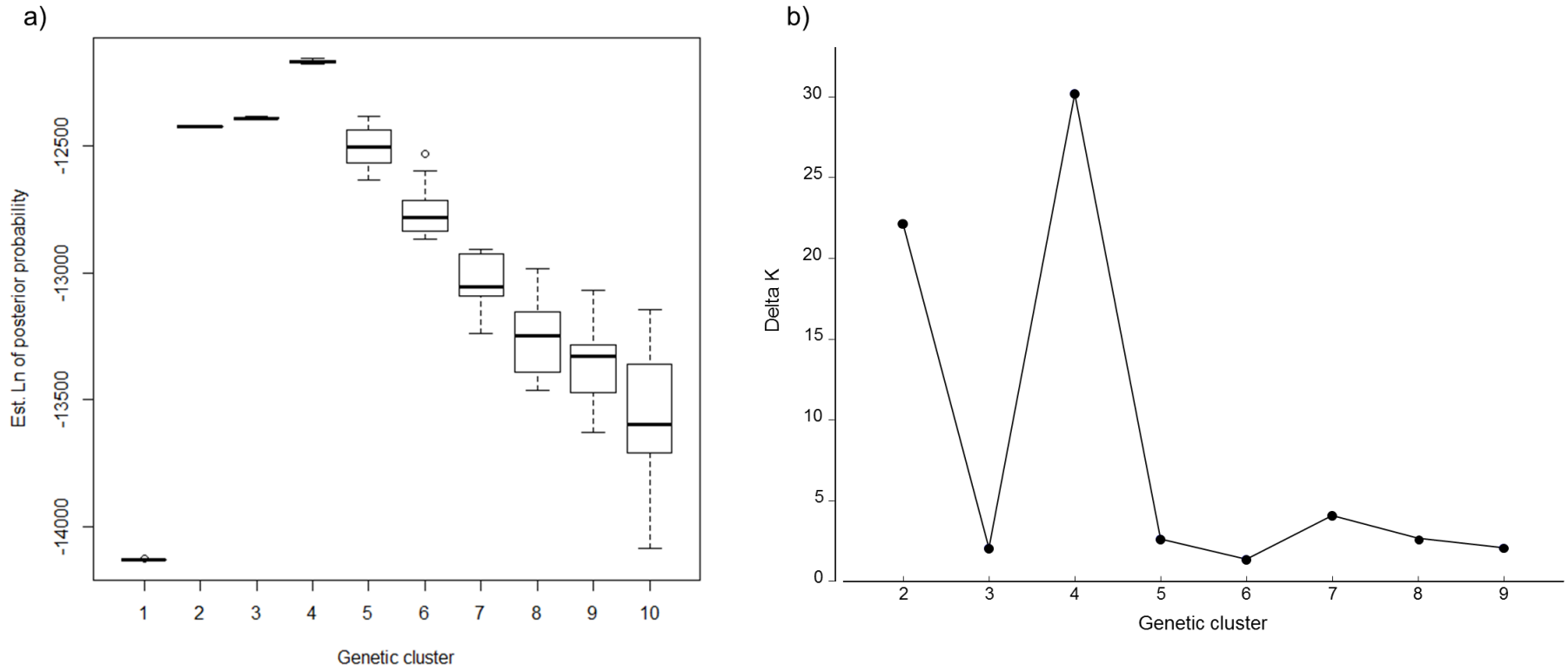


Figure 2 Predicted number of genetic clusters. A) log-likelihood, b) Delta K, indicated in STRUCTURE for all 215 *Nautilus pompilius* samples.

MICROSATELLITE ANALYSER (Dieringer & Schlötterer 2003) calculated pairwise F_{ST} (Weir & Cockerham 1984) between sampling locations, with Bonferroni corrections applied. To test an association between F_{ST} and geographic distance, Mantel's test for isolation by distance (IBD) was performed in SPAGEDI with 10,000 randomisations. A regression of the spatial distance against $F_{ST}/(1-F_{ST})$ was performed (Rousset 1997). Jost's differentiation index (D_{est}) values (Jost 2008) across loci were calculated using SMOGD (Crawford 2010).

4.3.4 *Approximate Bayesian Computation Methods*

To test different hypotheses which could explain the genetic similarity between PH and WA samples (see results), we conducted an approximate Bayesian computation (ABC) analysis (Beaumont *et al.* 2002). ABC aims to obtain the joint posterior distribution of complex models for which the likelihood function can be difficult or impossible to solve analytically, allowing a great flexibility in the scenarios being investigated (Marjoram & Tavaré 2006). Its rational is to bypass the need of an exact likelihood function by comparing summary statistics from observed data to summary statistics simulated under models of interest (Beaumont 2010; Csilléry *et al.* 2010).

We compared three evolutionary scenarios (fig. 3). $Model_{IWOM}$ assumes that an ancestral population of size N_A split at time t generations ago into two daughter populations PH and WA of effective sizes N_1 and N_2, respectively (fig. 3b). $Model_{IM}$ (Nielsen & Wakeley 2001) is equivalent to $Model_{IWOM}$ with the adjustment that populations PH and WA constantly exchange migrants since their split at rates m_{12} and m_{21}, respectively (fig. 3c). A null model was also tested as $Model_{PAN}$, this assumes that PH and WA are part of the same panmictic population of effective size N_A (fig. 3a). Note that to compute summary statistics comparable to the observed data (i.e. from two different populations), we set

$Model_{PAN}$ using $Model_{IWOM}$ parameters but fixed the divergence time to 1 generation - effectively modelling the whole history of the samples as a panmictic population.

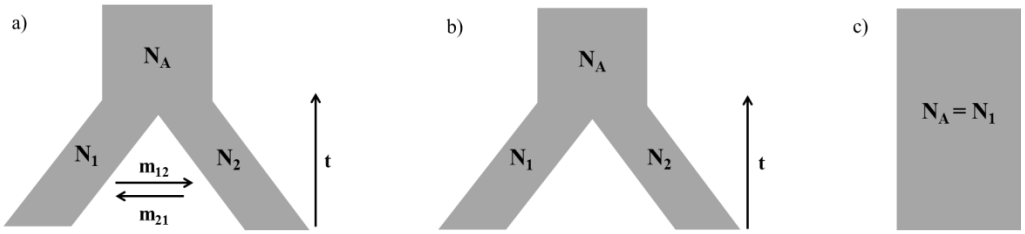


Figure 3 Schematic representation of the three demographic models compared using ABC under a coalescent framework. a) isolation with migration model, $model_{IM}$, (Nielsen & Wakeley 2001); N_A, effective size of the ancestral population; t, time since population split; N_1, N_2, effective size of current populations; m_{12}, m_{21}, migration occurring in both directions; b) isolation without migration, $model_{IWOM}$, where divergence resulted in current populations that have no gene exchange; c) A panmictic population, $model_{PAN}$, between both sampled sites, where $N_A=N_1$ with no divergence.

The prior distributions were uniform for all demographic parameters and the same range was used for common parameters between models (Table 2). For all demographic models, we assumed that microsatellites evolved under a stepwise-mutation model. Average mutation rates across loci were extracted from a normal prior distribution, and single locus mutation rates were drawn from a Gamma distribution as parameterized in ABC TOOLBOX (Wegmann *et al.* 2010), using uniform priors for the two parameters of the distribution. To avoid effects of substructure within the WA clade, and due to similar sample size, Ashmore Reef was chosen to represent the WA clade.

Table 2 Prior and posterior distributions of model parameters for $model_{iWOM}$ using ABC TOOLBOX.

Parameters	Prior range	Mean	Median	Mode	HPD95 low	HPD95 high
ARG_K	Uniform [0 - 6]	1.78785	1.62815	1.35678	0.105534	3.72541
MUT_U	Uniform [10^{-6} - 5×10^{-4}]	5.00E-05	4.11E-05	2.61E-05	-2.53E-07	0.000122236
NA	Uniform [0 - 5×10^{6}]	2.17E+06	2.04E+06	1.31E+06	62816.9	4.51E+06
N_ONE	Uniform [0 - 5×10^{6}]	3.08E+06	3.19E+06	3.79E+06	1.05E+06	4.99E+06
N_TWO	Uniform [0 - 5×10^{6}]	2.61E+06	2.56E+06	2.09E+06	665830	4.77E+06
TS	Uniform [0 - 10^{6}]	353845	296495	105529	-2511.56	839452

ARG_K, shape parameter for the gamma distribution; MUT_U, average loci mutation rate; N, effective population sizes (ONE=Philippines, TWO=Ashmore Reef, A=ancestral); TS, time of population split.

4.3.5 *SUMMARY STATISTICS AND SIMULATIONS*

ABC analyses were conducted using the package ABC TOOLBOX (Wegmann *et al.* 2010). One limitation of ABC is that models can look more or less likely dependent on the range of the parameter values and the weight assigned to them by the priors (Sousa *et al.* 2012). Exploratory simulations were therefore performed with varied sets of priors to allow an assessment of their effect on the posterior distribution and ensure that the whole posterior was contained within the final prior range.

We used FASTSIMCOAL (Excoffier & Foll 2011; Excoffier *et al.* 2013) to run one million coalescent simulations of our data set of 14 microsatellites under each model. ARLSUMSTAT (Excoffier & Lischer 2010) was used to calculate a set of 30 summary statistics (within and between populations; Table 3), chosen based on those shown to be informative in previous studies (Palero *et al.* 2009; Sousa *et al.* 2012; Butlin *et al.* 2013). To reduce the high dimensionality of the summary statistics, we used a partial least-squares (PLS) transformation (Wegmann *et al.* 2009) to extract their orthogonal components. PLS identifies components to explain variability of response variables (model parameters) by maximising the covariance matrix of predictor (raw summary statistics) and response variables (Wegmann *et al.* 2009).

Table 3 Summary statistics of the chosen model, $model_{iWOM}$, used in the ABC analyses.

Within populations			Between populations	
Statistic	Pop1 estimate	Pop2 estimate	Statistic	Estimate
K	14.357	14.00	sd_K	0.253
Ksd	7.078	6.75	Hsd	0.000
H	0.864	0.87	mean_GW	0.784
Hsd	0.091	0.08	sd_GW	0.017
GW	0.796	0.77	tot_GW	0.821
GWsd	0.145	0.13	sd_NGW	0.008
NGW	0.707	0.70	sd_R	0.000
NGWsd	0.156	0.14	FIS	0.024
R	18.429	18.43	FST	0.015
Rsd	11.161	11.61	FIT	0.038

Summary statistics using populations 1 (Philippines) and 2 (Ashmore Reef). K, mean number of alleles; sd, standard deviation; H, heterozygosity; GW, Garza-Williamson; tot_GW, mean Garza-Williamson statistic over poll of all pops (mean TOT_Ki/(TOT_RANGEi+1)); NGW, mean of the modified Garza-Williamson index; R, range

4.3.6 *MODEL CHOICE AND PARAMETER ESTIMATION*

For model comparison, producing marginal densities comparable between models was archived by using the PLS-transformed summary statistics for the rejection step, while all raw summary statistics were used to perform the post-sampling adjustment step using the ABC-GLM (General Linear Model) from ABC TOOLBOX (Wegmann *et al.* 2009). We retained the 5% of simulations closest to the observed data. We checked that our observed summary statistics (for both PLS components and raw summary statistics) fell within the distribution of summary statistics from the simulations retained. Bayes Factors and PP were derived from model choice procedure (Table 4). *P*-values were taken into account as an indication of each model's ability to explain the data.

Table 4 Posterior probabilities for $model_{IWOM}$, $model_{IM}$ and $model_{PAN}$. Bayes factor calculated for both pairwise comparisons.

Model	Marginal density (LOG10)	Posterior probability	Bayes factor 1 (LOG10)	Bayes factor 2 (LOG10)
$model_{IWOM}$	19.6539155	1	33.17072631	37.65430698
$model_{IM}$	-13.51681081	6.74953E-34	-33.17072631	4.483580665
$model_{PAN}$	-18.00039148	2.21663E-38	-4.483580665	-37.65430698

To validate our model choice procedure, we simulated 1000 pseudo-observed data sets for each model using the original priors. The original results files (of one million simulated data sets for each model) were used to perform our model choice procedure using each of the 1000 pseudo-observed data sets in turn. To test the robustness of discrimination between models using our model choice procedure, each pairwise comparison of simulated and observed data for the two models was performed four times. A model's original data were compared with the pseudo-data of the same model and that of the model being compared. This procedure was repeated for all pairwise model comparisons. Posterior probabilities were compared with a logistic regression. Confidence in model choice was calculated by estimating the FDR (Verhoeven *et al.* 2005): the frequency of the PP being equal to or larger than the real PP of the best model.

For parameter estimation, the distance step and post-sampling adjustment were both carried out using PLS components. It was performed independently for each model because different PLS components are extracted for each model. The GLM method implemented in ABC TOOLBOX (Leuenberger & Wegmann 2010) was used for post-sampling adjustment. Parameter estimation was verified by ensuring *p*-values were reasonably large (>0.05 as suggested in the ABC TOOLBOX manual) and checking that

posterior distributions were within the prior ranges (Fig. 4). The pseudo-observed data were also used to check for uniformity of the posterior quantiles; a departure from uniformity suggests a parameter is over or underestimated.

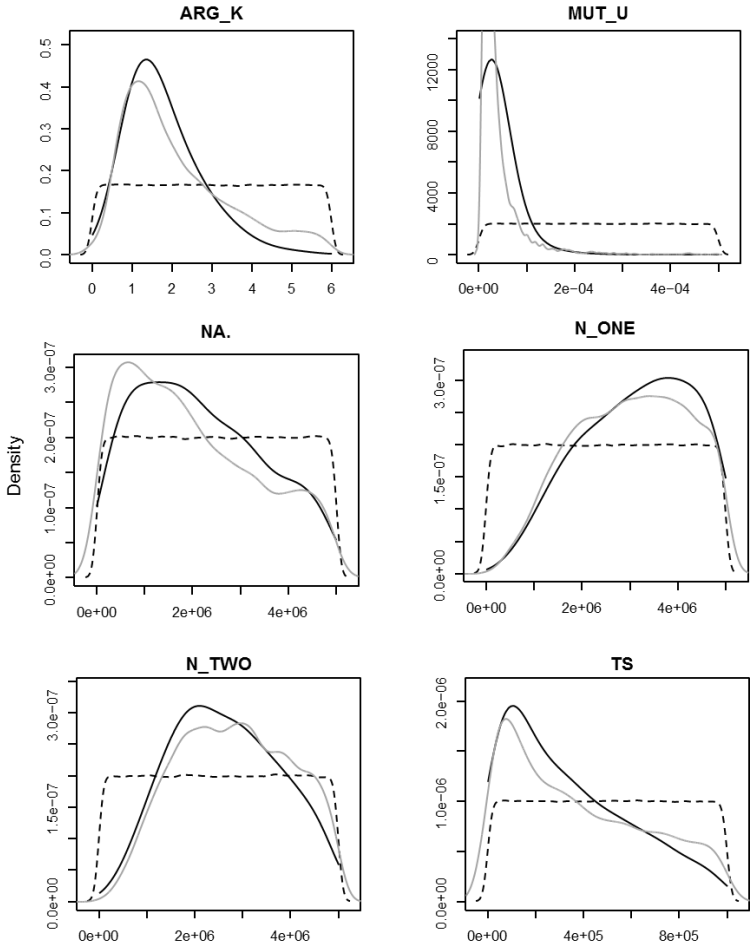


Figure 4 Posterior distributions (black) obtained under the chosen model ($model_{iWOM}$), marginal parameter distribution among the retained simulations (grey), prior distribution estimated from the first 50,000 simulations (dashed). *X*-axis shows the range of parameter values, *y*-axis the probability density.

4.4 *RESULTS*

4.4.1 *GENOTYPING VALIDATION*

A genotyping error rate of zero was determined between replicates. No evidence was found for large allelic drop out across all loci in all sampling locations. A shortage of heterozygous genotypes was found in locus *Npom08* in Osprey Reef and Scott Reef populations, possibly resulting from scoring errors due to stutter. This remained in analyses due to its quality in other reefs. Loci showed no evidence for null alleles (est. freq≤0.05). Departure from HWE was not detected consistently across all sampling locations for any loci. No groups of loci consistently showed LD in all populations suggesting no loci were physically linked. Allelic richness was calculated as lower in the east than that seen in the west (Fig. 5). F_{ST} values ranged from -0.04 to 0.35 (Table 5). IBD analysis revealed no overall association between F_{ST} and geographic distance and was not significant (r^2=0.139; *P*=0.095).

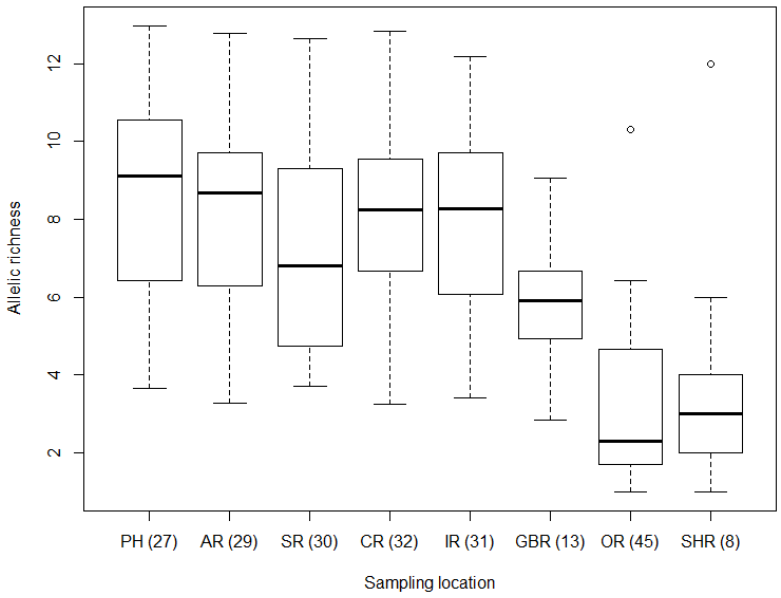


Figure 5 Allelic richness shown across 14 loci for each sampling location. Individual sample size (); PH, Philippines; AR, Ashmore Reef; SR, Scott Reef; CR, Clerke Reef; IR, Imperieuse Reef; GBR, Great Barrier Reef; OR, Osprey Reef; SHR, Shark Reef. Median shown by the line in the centre of the box; upper and lower quartiles (25% of data greater/less than this value) shown by the horizontal lines of each box; whiskers represent maximum and minimum values excluding outliers; outliers shown as single points

Table 5 Estimation of population differentiation among eight sites of *Nautilus pompilius.*

Population	Philippines	Ashmore Reef	Scott Reef	Clerke Reef	Imperieuse Reef	Great Barrier Reef	Osprey Reef	Shark Reef
Philippines ($N = 27$)	-	0.015*	0.044*	0.014*	0.024*	0.130*	0.330*	0.237*
Ashmore Reef ($N = 29$)	0.067	-	0.018*	-0.004	0.006	0.121*	0.319*	0.228*
Scott Reef ($N = 30$)	0.140	0.044	-	0.015*	0.015*	0.173*	0.354*	0.268*
Clerke Reef ($N = 32$)	0.045	-0.009	0.043	-	0.004	0.124*	0.322*	0.234*
Imperieuse Reef ($N = 31$)	0.058	0.012	0.051	0.008	-	0.144*	0.343*	0.255*
Great Barrier Reef ($N = 13$)	0.409	0.368	0.472	0.382	0.415	-	0.312*	0.229*
Osprey Reef ($N = 45$)	0.596	0.618	0.662	0.620	0.703	0.337	-	0.012
Shark Reef ($N = 8$)	0.520	0.609	0.662	0.591	0.688	0.317	0.000	-

Pairwise F_{ST} (Weir & Cockerham 1984) shown above the diagonal, values significant at $P < 0.01$ after Bonferroni correction indicated (*). D_{est} (Jost 2008) shown below the diagonal

4.4.2 *Population Structure*

Shark Reef was grouped with Osprey Reef for analyses based on their close geographic location and high relatedness results (Table 5), they will hereafter be referred to as Osprey Reef. Results incorporating spatial data in TESS (Fig. 6a) and GENELAND (Fig. 7) returned three and five genetic clusters respectively. TESS returned the first cluster including PH and all WA reefs, the second cluster of the Great Barrier Reef, and third cluster of Osprey Reef. GENELAND divided samples into five genetic clusters of: (i) PH, (ii) Ashmore, Imperieuse and Clerke Reefs, (iii) Scott Reef, (iv) Great Barrier Reef and (v) Osprey Reef.

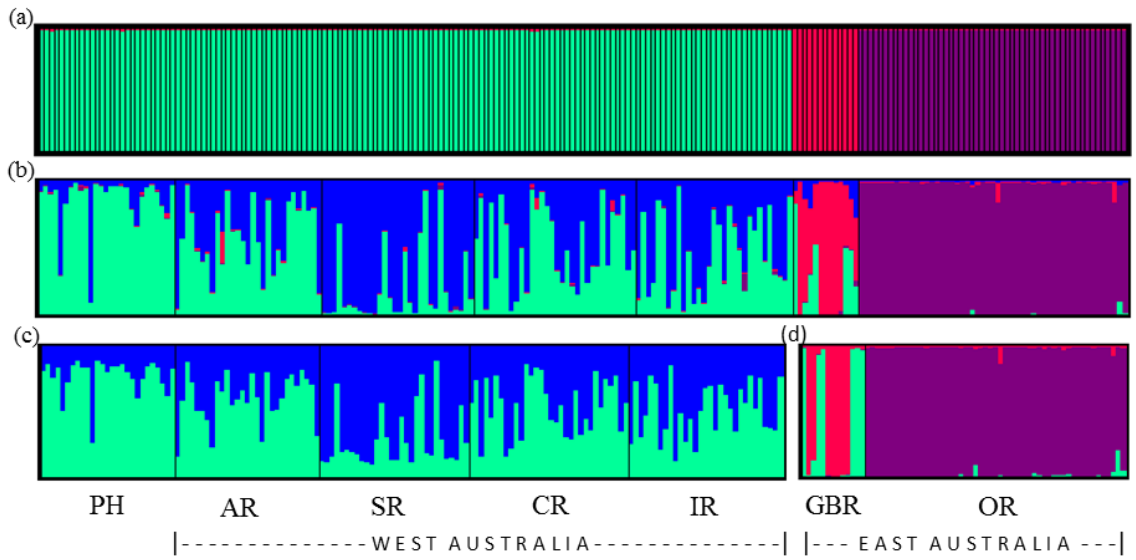


Figure 6 Bayesian assignment probabilities (*y-axis*) of individual *Nautilus pompilius* (*n*=215, *x-axis*). Inferred populations indicated; PH, Philippines; AR, Ashmore Reef; SR, Scott Reef; CR, Clerke Reef; IR, Imperieuse Reef; GBR, Great Barrier Reef; OR, Osprey Reef; SHR, Shark Reef. Colours represent the probability proportion to each cluster in (a) *K=3* clusters indicated in TESS, (b) *K=4* clusters indicated in STRUCTURE, (c) Philippines and west Australia in STRUCTURE (d) east Australia in STRUCTURE.

Plots of ΔK and LnP(K) generated from STRUCTURE results indicated four as the most likely number of genetic clusters present in the full data set (Fig. 6). The first two genetic clusters consisted of populations PH and WA, the third cluster consisted of EA Great Barrier Reef, and cluster four consisted of Coral Sea's Osprey Reef (Fig. 6b). Sub-setting these data to look for further division within clusters returned validating results (Fig. 6c and 6d).

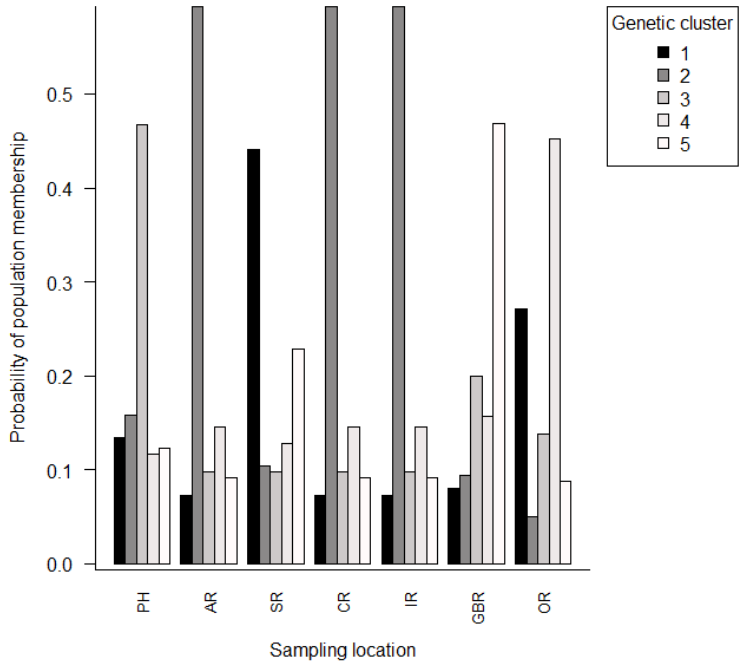


Figure 7 Probabilities of population membership generated in GENELAND. Sampling locations (*x*-axis) of *N. pompilius* were assigned to a total of 5 genetic clusters. A probability of zero was given for genetic clusters six to ten. PH, Philippines; AR, Ashmore Reef; ST, Scott Reef; CR, Clerke Reef; IR, Imperieuse Reef; GBR, Great Barrier Reef; OR, Osprey Reef.

4.4.3 *APPROXIMATE BAYESIAN COMPUTATION ANALYSIS*

The model comparison gave strong support to $model_{IWOM}$ (PP = 1.0, Table 4) and the FDR was low (0.2%); indicating with high confidence that these data are not the result of sustained migration between the Philippines and WA (Fig. 8).

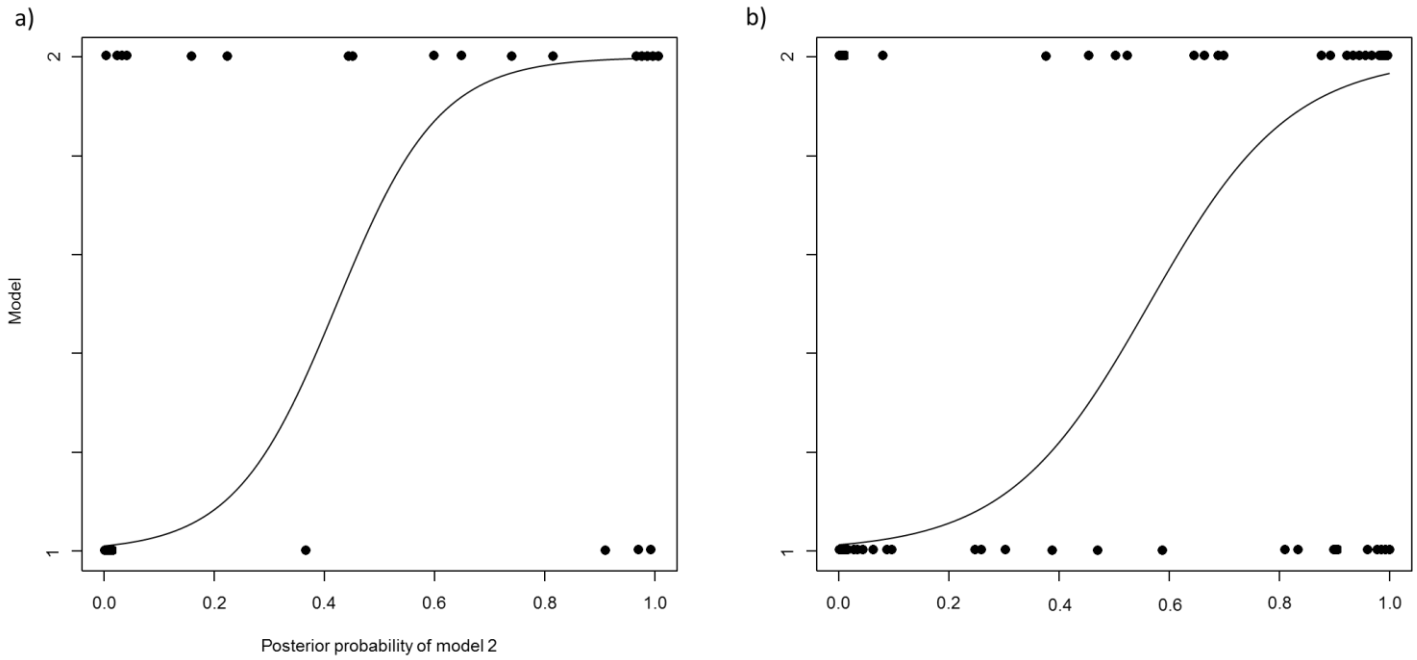


Figure 8 Logistic regressions for model validation. The model classifier (1 = model 1 divergence without migration, 2 = (a) model 2 divergence with migration, (b) panmictic) specifies the model under which the pseudo-observed data were generated. We simulated 1000 new data sets from the original priors for each model. This resulted in 2000 resulting vectors of summary statistics. We verified that each pseudo observed data set (generated summary statistic vector) could correctly identify the model from which it was generated. The approximate posterior probability was calculated by dividing the marginal density of a chosen model by the sum of the marginal densities of both the chosen model and the comparative one. These values are plotted against the categorical model classifier. The strength of the discrimination between models is represented by a logistic regression.

Moreover, this model fitted these data well (the observed summary statistics lay within the range of both the untransformed and PLS-transformed post-rejection simulated summary statistics) indicating that its best score was not a result of a bad fit by all models to these data. Parameter estimation under $model_{IWOM}$ calculated ancestral population size (median: 2035120; highest posterior density (HPD95): 62816.9, 4508320) as smaller than current population sizes of PH and WA (median: 3080000, 2610000 respectively). The distribution of posterior quantiles did not show strong departures from uniformity, indicative of a lack of bias in parameter estimation (Wegmann *et al.* 2009).

4.5 *DISCUSSION*

We found population structure between east Australian sampling sites, showing the genetic isolation of Osprey Reef and Shark Reef from the Great Barrier Reef. West Australian samples revealed low population structure but with significant pairwise F_{ST} for Scott Reef. The genetic similarity shown between the Philippines and west Australia was unexpected. Further investigation modelling different demographic scenarios revealed that this similarity was not the result of recent migration, but may be due to large ancestral population sizes until recently, and low genetic drift.

4.5.1 *MECHANISMS FOR POPULATION STRUCTURE*

Results from software STRUCTURE, TESS and GENELAND showed Osprey Reef and Shark Reef populations in the Coral Sea to be genetically distinct from Great Barrier Reef, west Australia, and Philippine populations. Ocean physiography (the physical geography of the ocean floor) appears influential in this differentiation. Ocean depths between the Coral Sea and the Great Barrier Reef exceed 1700 m (Dunstan *et al.* 2011b) and while movement through open water is feasible, it leaves individuals vulnerable to predation (Yomogida & Wani 2013). The response to teleost attacks on *N. pompilius* is to retreat

into their shell and show no defence or escape (Saunders & Landman 2010). The Great Barrier Reef was shown to be distinct not only from Osprey Reef but also from the western populations, which supports previous evolutionary work using partial COI sequences (Sinclair *et al.* 2007, 2011; Bonacum *et al.* 2011; Williams *et al.* 2012).

West Australian results were not consistent across software; STRUCTURE and TESS assigned the Philippines and west Australia to the same genetic structure, whereas GENELAND designates the Philippines and Scott Reef as separate genetic clusters. Geographically, Scott Reef is located between Ashmore and Clerke Reef, and the differentiation is seen between Scott Reef and surrounding west Australian reefs despite shallower surrounding sea depths. F_{ST} measures deviation from panmixia, D_{est} measures deviations from total differentiation (Whitlock 2011), and both F_{ST} and D_{est} values distinguish Scott Reef as a separate genetic cluster.

Due to their residing depth, surface current data are not capable of explaining *N. pompilius* dispersal patterns (Biuw *et al.* 2007). Currents have been shown to impact individual positions on a reef (O'Dor *et al.* 1993) with movements up to 6km recorded that may have been facilitated by currents (Dunstan *et al.* 2011b). However, *N. pompilius* has also demonstrated strong resistance to currents and an ability to utilise them to obtain food (O'Dor *et al.* 1990). The overall impact of currents on their population distribution is poorly documented.

No significant correlation was found between linear geographic distance and F_{ST} (Rousset 1997). IBD has been shown in cephalopods (Pérez-Losada *et al.* 2002; Kassahn *et al.* 2003; Cabranes *et al.* 2008) but like *Nautilus,* octopus (Doubleday *et al.* 2009; Moreira *et al.* 2011), cuttlefish (Zheng *et al.* 2009) and squid (Buresch *et al.* 2006) have all demonstrated genetic distances disproportionate to geographic distance. It has been

hypothesised that these are examples of natal philopatry (Kassahn *et al.* 2003; Buresch *et al.* 2006). Such behaviour has been speculated not to occur in nautiloids (Crook & Basil 2013), but gaps remain in our knowledge of *N. pompilius* ecology. Despite lacking the lensed eye and vertebrate-like brain of other cephalopods (including dedicated lobes for learning and memory), *N. pompilius* have been shown to be capable of both spatial learning and navigational strategy (Crook *et al.* 2009; Crook & Basil 2013). Migration on a small scale is not completely unfeasible, but seems unlikely at the scale investigated in our study.

4.5.2 *DIVERGENCE WITHOUT MIGRATION*

Our ABC model choice procedure does not support a scenario of sustained migration between the Philippines and west Australia as explanation for the genetic admixture shown in the structural analyses. Movement of individuals between the depths of the two sites was unknown, but depth limitations of *N. pompilius* are indicative of isolation over such geographic distance; it is possible that the genetic similarity observed has resulted from incomplete lineage sorting. Loci can appear misleading about relationships among populations due to retention and stochastic sorting of ancestral polymorphisms. If effective population size is large relative to lineage length (the time since population split) then this is especially likely (Maddison & Knowles 2006). Alleles then remain in both populations due to low genetic drift. $Model_{IWOM}$ indicated extremely large ancestral and current effective populations, resulting in a lower probability of loci being brought to fixation before divergence (Pamilo & Nei 1988). Current population size estimates for the Philippines (median=3190920) and Ashmore Reef (median=2562800) suggest genetic drift has yet to have significant impact. Such large current population estimates is potentially due in part to sub-structure within the sampled areas. Sampling from the Philippines was conducted in several locations, the connectivity between which is assumed but not confirmed. Sampling from Ashmore Reef

was from the most Northern of west Australian reefs, where gene flow had been previously established between surrounding reefs (Williams *et al.* 2012). This may have inflated the population estimate for Ashmore Reef.

Population estimates were calculated as 13.6 and 0.03 individuals per km^2 for Osprey Reef and Bohol Sea (Philippines) respectively (Barord *et al.* 2014). Generating such results from individual identification along transects, in comparison to our effective population size estimates, predictably produced results of lower abundance. Barord *et al.* (2014) documents evidence of sudden population size reduction, but our data showed allelic richness in the Philippines was no lower than other locations sampled (fig. 4). It is possible that the genetic consequences of population reduction have yet to take effect; low fecundity and long developmental time (Carlson *et al.* 1984; Landman & Cochran 2010) of nautiloids results in a long generation time compared to other cephalopods. Fishing for shells is relatively new, with no cultural or historical significance in studied areas such as Palawan (Dunstan *et al.* 2010) and so it is possible that we are seeing a delayed genetic response to exploitation.

The time split estimation (median=296495) by $model_{iWOM}$ indicates current populations have been evolving for a similar time as modern *N. pompilius* (O'Dor & Webber 1991). Additionally, the larger Philippine population size is in accordance with biological theory (Wray *et al.* 1995) of the progenitor population being located in the Philippines. The west Australian population is expectedly smaller, representing just one of the three subsequent evolutionary clades. Results from a second evolutionary clade, consisting of east Australian samples, also corroborates the proposed direction of colonisation; lower allelic richness is seen in the Great Barrier Reef with a further decline in the more genetically distinct Osprey and Shark Reefs of the Coral Sea (fig. 4). These data are in

accordance with the smaller population size estimates for Osprey Reef (Barord *et al.* 2014).

4.5.3 *MANAGEMENT IMPLICATIONS*

The absence of migration between the Philippines and west Australia highlights the need for mechanisms to protect these populations as discrete conservation units. As a unique population with a high possibility of sub-structure, adequate protection for the Philippines is imperative to the long term survival of this genetic cluster. The absence of migration into and out of the Philippines means that it is unlikely that the population would be repopulated if fished to extinction.

The variation seen in IBD within cephalopods demonstrates the need for species-specific range studies, especially when results are extrapolated for fisheries management. As fin fish stocks decline and the fishing industry targets novel resources, it is likely increased fishing pressure will be placed on cephalopod stocks (Dillane *et al.* 2005). The data supporting the case for *Nautilus* and *Allonautilus* protection is ever increasing (Dunstan *et al.* 2010; Bonacum *et al.* 2011; Williams *et al.* 2012; De Angelis 2012; Barord *et al.* 2014). Overexploitation is threatening marine species world-wide (Hutchings 2000; Worm *et al.* 2006; Doukakis *et al.* 2009; Neubauer *et al.* 2013; Watson *et al.* 2013) and our study highlights the need for multiple or finer-scale markers to determine connectivity patterns and establish adequate protection. For example, mitochondrial DNA data on the west Australian reefs (Williams *et al.* 2012) revealed a panmictic population, but the higher resolution data presented here show sub-structure within them. Our results show how management plans should incorporate discrete management units and should account for more than separation by geographic distance.

4.5.4 *CONCLUSIONS*

A range of molecular work has been conducted in coleoids (Allcock *et al.* 2015) including population structure analysis using minisatellites, microsatellites and mitochondrial DNA (Dillane *et al.* 2005; Zheng *et al.* 2009; Moreira *et al.* 2011), but this is the first study to use microsatellite markers on a nautiloid. We had hypothesised genetic division between east Australian and west Australian populations by reason of previous evolutionary work on these populations (Sinclair *et al.* 2007, 2011; Bonacum *et al.* 2011; Williams *et al.* 2012), but a greater level of genetic similarity was shown between samples from the Philippines and west Australia than had been previously considered. Concluding that this similarity is most likely not a result of migration emphasises the need to reduce overexploitation and prevent the local extinction of *N. pompilius* in the Philippines. Gaining CITES protection for *Nautilus* and *Allonautilus* would result in decreased incentive for continued exploitation. For relatively cryptic or inaccessible species, genetic data gives insight into migration and population dynamics. Such studies should be utilised to develop efficient species-specific management plans for declining populations. Enforcing these in collaboration with legislative protection is imperative for the conservation of marine populations (Neubauer *et al.* 2013).

CHAPTER FIVE:

PILOT STUDY: COMPARATIVE EFFECTS BETWEEN DRUGS ON TISSUE REACTIVITY IN *NAUTILUS POMPILIUS*

Nautilus pompilius, Osprey Reef, Coral Sea

Photo taken by Billy Sinclair

5.1 *ABSTRACT*

Receptors can be targeted *in-vitro* to indicate their presence or absence within a tissue. If we can understand the role that receptors play, we can establish their control over the physiological processes of organisms. Receptor sequences can be used to investigate taxonomic relationships using structures that are highly conserved across taxa. We tested for the presence of dopamine, noradrenaline, isoprenaline and 5-hydroxtryptamine receptors in cardiovascular, epithelial and respiratory tissues of *Nautilus pompilius*. Experiments were conducted with dissected tissue in organ baths. Cumulative concentration curves were then constructed in response to the agonist. Antagonists were also used in association with dopamine and noradrenaline. Results showed a significant reaction in all tissues to dopamine and noradrenaline. The reaction was delayed and reduced in response to the presence of an antagonist, corroborating that the reaction recorded had been in response to the agonist administered. Aortic tissue and respiratory blood vessels showed no response to isoprenaline or 5-hydroxtryptamine. Both drugs elicited a significant effect on the heart. Results indicate the presence of receptors in *N. pompilius* that have been previously identified in higher order animals. The experiments were conducted as a pilot study to firstly test the feasibility of looking at *N. pompilius* tissue in an organ bath system, and secondly to elicit tissue responses to test for the presence or absence of receptors while minimising the animals euthanized. This demonstration of sufficient activity confirms the scope for further research into the physiological role played by receptors in *N. pompilius.* Thus, we can phylogenetically determine taxonomic relationships at this fundamental level. Finding relatively high protein sequence homologies in cross species comparisons would be informative both on the evolution of nautiloids, and the functional role of receptors across taxa.

5.2 *INTRODUCTION*

Drugs react with specific binding sites termed receptors. This tenet in the field gives a fundamental starting point for more advanced pharmacological experiments (Gilchrist 2010). A receptor is a target molecule through which soluble physiological mediators, such as hormones and neurotransmitters, produce their effects. When applied to a system, drugs act on target proteins i.e. receptors, whose function is to recognise and respond to endogenous chemical signals (Rang *et al.* 2012). Understanding this basic principle enables insight into species physiology; for example simple molecules such as dopamine can facilitate a large range of physiological processes, mediated by adrenoceptors (Gilchrist 2010).

Catecholamines are compounds that contain a catechol moiety and an amine side chain. Pharmacologically, the most significant ones are: noradrenaline, a transmitter released by sympathetic nerve terminals; adrenaline, a hormone secreted by the adrenal medulla; dopamine, the metabolic precursor of noradrenaline and adrenaline which is also a transmitter/neuromodulator in the central nervous system; and isoprenaline, a synthetic derivative of noradrenaline (Rang *et al.* 2012). Dopamine, noradrenaline and adrenaline are three catecholamines that act as neurotransmitters (Messenger 1996; Yamamoto & Vernier 2011; Rang *et al.* 2012).

Much research has been conducted into human dopaminergic receptors due to their role in a number of disorders such as Parkinson's disease and schizophrenia. Invertebrates have a simpler nervous system and yet exhibit a huge variety of behaviours, making them interesting as model systems for investigating dopamine receptors and their role in behaviour and development (Rang *et al.* 2012).

The actions of dopamine are mediated via G protein-coupled receptors (GPCRs) and it has been estimated that 80% of all neurotransmitters and hormones are signalled through these receptors (Birnbaumer *et al.* 1990). In vertebrates, they have been divided into two groups based on sequence similarity, functional characteristics and pharmacological profiles (Mustard *et al.* 2005). The D_1 group of receptors consists of D_1 and D_5, while the D_2 group consists of D_2, D_3 and D_4 (Rang *et al.* 2012). The distinction between vertebrate and invertebrate receptors has been documented (see Mustard *et al.* 2005) but invertebrate characterisation is still very much under exploration comparative to that in vertebrates.

Identifying these receptor groups has had additional applications in animal behaviour; the isolation of dopamine receptors in the nematode *Caenorhabditis elegans* (Suo *et al.* 2002) led to understanding the role of biogenic amines in the regulation of key processes such as egg-laying and foraging (Komuniecki *et al.* 2004). The development of such research has allowed an assessment of using receptors to enable drug discovery (Smith *et al.* 2007).

The neurotransmitter more commonly known as serotonin is 5-hydroxytryptamine (5-HT). Found in the human central nervous system, it functions as both a neurotransmitter and as a local hormone. Its inclusion within the catecholamines is due to its metabolism which closely parallels that of noradrenaline, and its property of stimulating nociceptive (pain mediating) sensory nerve endings (Rang *et al.* 2012). In recent years, research efforts have focussed on the role that 5-HT has played in states of pain (Bardin 2011). While pain states in cephalopods have been investigated (Mather & Anderson 2007; Fiorito *et al.* 2014), this has yet to be extended to the nautiloids. Ethical considerations in cephalopods are greater than in any other invertebrate due to their recognised cognitive ability and measured responses to pain (Mather & Anderson 2007;

Moltschaniwskyj *et al.* 2007). Fascination regarding neurotransmitters in cephalopods and their role in behaviour such as colour change, has led to pharmacological research predominantly in 5-hydroxytryptamine (Messenger 1996).

The central nervous system of cephalopods has been shown as extremely complex, even in comparison to vertebrates, however research to characterise this excluded *Nautilus* (Wollesen *et al.* 2009). Their nervous system arrangement is similar to that of coleoids but difference in appearance is due to the wide separation of the nerve cords in *Nautilus.* This is most probably due to its connection with the oesophagus; the "brain" is comprised of a great concentration of neurons which is formed by cords around the oesophagus (Young 2010). The nervous system of *Nautilus* contains a much larger number of neurons than is found in any non-cephalopod mollusc and its organisation consists not of compact ganglia but of cords with cell bodies around the outside, and a neuropil at the centre (Saunders & Landman 2010).

A principal 'amine' in the central nervous system is noradrenaline; initial investigations failed to detect the presence of noradrenaline in molluscs (Euler 1961). The presence of both noradrenaline and dopamine has now been shown in the phylum and, further, in cephalopods (Dahl *et al.* 1966; Cottrell 1967). The ratio shows at least ten times more dopamine than noradrenaline in the central nervous system of invertebrates (Kerkut 1973). Both vertebrates and invertebrates release a biogenic amine in response to stress (Adamo 2012); noradrenaline is released by molluscs (Lacoste *et al.* 2001).

The first pure beta agonist to be developed was isoprenaline, a synthetic derivative of noradrenaline (Pearce & Hensley 1998; Rang *et al.* 2012). This is included in the catecholamines as one of a number of artificially created compounds. The drug has been shown to increase the rate and strength of contractions both in intact and isolated heart

muscle of humans (Greiner & Garb 1950) and so has been used to treat heart conditions. Isoprenaline can also elicit bronchial muscle relaxation and was therefore previously administered to treat asthma and patients with airway resistance (Zamel *et al.* 1966). Complications regarding its use in human health have been well documented; between 1960 and 1965 an increase in deaths relating to asthma were observed in England and Wales, particularly in young people (Speizer *et al.* 1968a). Over 170 deaths were investigated and in 84% of cases pressurised aerosol bronchodilators had been used as asthma treatment. In 72% of these cases, the drug used had been isoprenaline (Speizer *et al.* 1968b). The side effects were attributed to the high dose formulation of isoprenaline (known as isoprenaline-forte) because the epidemic was also experienced in New Zealand, Australia and Japan, but not in the United States where isoprenaline-forte was not licenced. Due to its ability to induce such drastic effects on humans, its effects on a system regarded as so different such as *Nautilus,* were unknown.

The present work was undertaken to initially assess whether *N. pompilius* tissue can be tested in an organ bath system. If possible, we then wanted to investigate the effects of dopamine, noradrenaline, isoprenaline and 5-hydroxtryptamine on cardiovascular, epithelial and respiratory tissues (respiratory blood vessels; RBV) of *Nautilus pompilius.* We investigated the presence or absence of receptors for these agents in the tissues tested. Previous research has predominantly been conducted on mammals, and any investigations on cephalopods have involved coleoids. These are the first experiments to test the reaction of *Nautilus* tissue in experiments of this nature. The work was conducted as a pilot study to inform further investigations. To do this successfully, it was first important to confirm the presence of the receptors and test the methodology while minimising the number of animals euthanized.

5.3 *METHODS*

5.3.1 *SAMPLE COLLECTION*

N. pompilius were collected from Osprey Reef in the Coral Sea on an Australian Fisheries Management Authority Scientific Permit (Permit number: 1002548). Traps were baited with 1kg uncooked chicken (*Gallus gallus*) and placed at dusk on the reefs at a depth of ~200 m. Traps were collected at dawn and individuals transferred into a tank containing aerated sea water and ice to maintain temperature at ~10^{o}C. Eleven animals were transported to research labs within the Faculty of Science and Engineering at CQUniversity, Rockhampton, Australia, where they were stabilised for 24 hours before laboratory work.

5.3.2 *DRUGS AND CHEMICALS*

All drugs used in this experiment (noradrenaline, dopamine, isoprenaline, 5-hydroxtryptamine, clozapine and prazosin) were purchased from the Sigma Chemical Company, St Louis MO, USA. The drugs were dissolved in distilled water to produce the serial dilutions of noradrenaline, dopamine, isoprenaline, 5-hydroxtryptamine, clozapine and prazosin. The latter two are associated antagonists for dopamine and noradrenaline receptors.

5.3.3 *GENERATING CUMULATIVE CONCENTRATION CURVES*

Animals were euthanized by immersion in an anaesthetising solution made up by dissolving 10 g of MS-222 into 1 ltr of seawater, and then 120 ml of this into 3 L of seawater. Animals were observed until all typical responses ceased, taking approximately 20 minutes, after which they were removed from the shell. The heart, thoracic aorta and one gill pair were isolated and placed in 0.17% glucose/seawater solution. Whilst in this solution, the aorta was cut free from the heart and dissected into 5mm long rings. Similarly the respiratory blood vessel was removed from the rest of the

gill and cut into 5mm long rings. Pieces of the heart were also dissected from the remainder of the organ. The aorta, vessels and heart were then suspended in 25ml organ baths that were maintained at 37 ^{o}C with carbogen (95% O_2 and 5% CO_2) on continuous supply through the solution. All tissue had a pre-set resting tension of 10 mN when suspended, this force was maintained throughout the experiment. The tissues had a 30 minute equilibration period, with buffer washings every 10 minutes.

A cumulative dose response curve was then generated for each drug/tissue combination (Fig. 1). The drugs acting on the receptors can be classified as agonists and antagonists; agonists induce changes and initiate effects whereas antagonists bind to a receptor without causing any change (Rang *et al.* 2012). Two parameters can determine agonist potency: affinity and efficacy. Affinity is the ability to bind to a receptor, and the efficacy is the ability to induce a change (Stephenson 1956); efficacy for antagonists is consequently zero. The agonists were administered to each tissue in the organ bath and results recorded. The tissue was then periodically washed with buffer five times before a 30 minute equilibrium period. The associated antagonist of dopamine and noradrenaline was then administered to the tissue before repeating the cumulative doses of the agonist. The tissues were repeatedly washed and this was performed with increasing concentration of antagonist until a visible response was no longer seen.

A tissue contraction was indicative of a response to the applied drug. Fluctuations to the pre-set tension after the addition of drugs were recorded using Chart 4 software in conjunction with a Mac G4 computer. Tissue contractions were indicative of the presence of corresponding receptors.

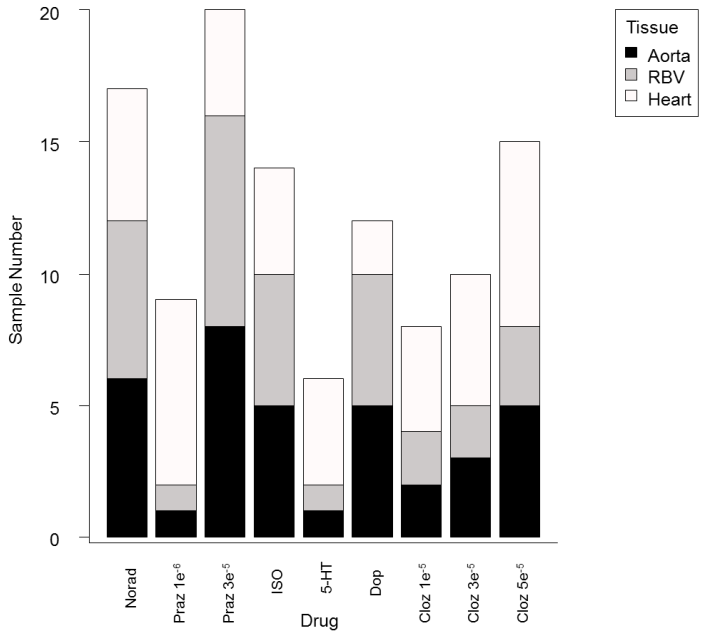


Figure 1. Sample numbers for each drug/tissue combination. Dugs (*x*-axis) were tested on three tissue types from *Nautilus pompilius:* aorta, respiratory blood vessel and heart. The number of individuals (*y*-axis) used for each drug/tissue combination are shown. NorAd, noradrenaline; Praz $1e^{-6}$, noradrenaline after prazosine $1e^{-6}$, Praz $3e^{-5}$, noradrenaline after prazosine $3e^{-5}$, ISO, isoprenaline; 5-HT, 5-hydroxtryptamine; Dop, dopamine; Cloz $1e^{-5}$, dopamine after clozapine $1e^{-5}$; Cloz $3e^{-5}$, dopamine after clozapine $3e^{-5}$; Cloz $5e^{-5}$, dopamine after clozapine $5e^{-5}$.

5.3.4 *STATISTICAL ANALYSIS*

Due to the repeated measures nature of the experiment and the potential for pseudoreplication, General Linear Mixed Models were used to analyse these data. We used R (R Core Team 2013) and lme4 (Bates *et al.* 2014) to perform linear mixed effects analysis of the relationship between drugs and tissues. We scaled dose to standardise the independent variable and allow for accurate interpretation. To meet the assumptions of the statistical tests, a log transformation was performed on the response variable of contraction. In the early stages of dose application, contraction response values are recorded as zero, and so 0.5 was added to every recorded contraction value. To ensure that this was the most appropriate transformation of these data, a box-cox transformation was first performed. This returned $\lambda=0$, equal to a log transformation.

For all models, visual inspection of residual plots did not reveal any obvious deviation from homoscedasticity or normality. Lme4 returns *t*-values, of which results were deemed significant at ± 1.96 (Bates 2006; Bates *et al.* 2014). *P*-values were obtained by likelihood ratio tests of the full model with the effect in question, against the null model without the effect in question. For *P*-values, $P < 0.05$ was regarded as significant. AIC values were also checked and considered for each model comparison.

A range of GLMM's were used to analyse these data. The effects of dopamine, noradrenaline, isoprenaline and 5-HT were tested on the individual tissues: aorta, RBV and heart. Drug and dose were entered into the model as fixed effects; random effects were defined as individual to account for multiple samples obtained from each animal.

The effect of tissue type in response to dopamine and noradrenaline was tested; dose and tissue type were entered into the model as fixed effects, with tissue type nested in individual and entered as random effects. To test whether all tissues significantly responded to tissue type, an interaction was tested between dose and tissue type. To assess whether isoprenaline and 5-HT had a significant effect on the heart in isolation, unpaired two tailed student's *t*-tests were performed for each drug.

The effects of all agonists: dopamine, noradrenaline, isoprenaline and 5-HT, were tested on the heart. Drug and dose were entered as fixed effects; random effects were listed as tissue type nested in individual. The effect of agonists on the RBV and aorta were tested using the same model including data for just dopamine and noradrenaline. The effect of dopamine and noradrenaline on all three tissues was tested using a model with dose and tissue entered as fixed effects, and tissue type nested in individual were entered as random effects.

5.4 *RESULTS*

N. pompilius tissue response, measured as tissue contraction, was recorded after exposure to cumulative doses of pharmacological drugs. We tested for the presence of receptors for the investigated drugs; a significant response was interpreted as indicative of receptor presence. This was further confirmed by the use of specific antagonists; when the tissue had been exposed to the antagonist, failure of the agonist to bind to the receptor indicated that designated receptors had in fact been activated. Results presented show the effect of agonists on the tissue, the effect of the antagonist on the agonists, and the effect of tissue in activation (Table 1).

Table 1 Average contractions across doses for each drug/tissue combination.

	Aorta	RBV	Heart
Dopamine	5.74±2.35	0.4±0.14	0.73±0.36
Dop (clozapine $1e^{-5}$)	4.08±1.42	8.41±1.14	0.87±0.40
Dop (clozapine $3e^{-5}$)	0.38±0.20	0.00±0.00	
Dop (clozapine $5e^{-5}$)	0.43±0.14	0±0.01	0.02±0.01
NA	3.92±1.95	3.46±0.74	0.25±0.10
NA (prazosin $1e^{-6}$)	1.93±1.23	0.00±0.00	
NA (prazosin $3e^{-5}$)	0.84±0.56	0.59±0.13	0.25±0.08
ISO	0.00±0.00	1.15±0.33	0.55±0.13
ISO (propranolol $3e^{-5}$)	0.03±0.01	0.00±0.00	
5-HT	0.00±0.00	0.00±0.00	0.24±0.06

Data are presented as mean ± S.E.M; Dop(cloz-), dopamine after the presence of clozapine at the concentration indicated; NA, noradrenaline; NA(praz-), noradrenaline after the presence of prazosin at the concentration indicated; ISO, isoprenaline; ISO(prop-), ISO after the presence of propranolol at the concentration indicated; 5-HT, 5- hydroxytryptamine

5.4.1 *THE EFFECT OF DOPAMINE*

Investigating the effect of dopamine on the aorta (Fig. 2a) demonstrated that the full model was significant when compared to the null model (likelihood ratio test: $\chi^2 = 36.88$, df = 3, $P < 0.001$). The reaction produced when dopamine was administered after the lowest dose of clozapine was not significantly different to the reaction recorded from solely adding dopamine (t = -1.68). As the concentrations of clozapine increased to clozapine $3e^{-5}$ and $5e^{-5}$, this competitively bound with the dopamine receptors and prevented dopamine from binding (t = -5.04, -6.03 respectively). The ability of dopamine's antagonist to competitively bind to the dopamine receptor indicates that dopamine receptors were successfully activated.

When testing the effect of dopamine on the RBV (Fig. 2b), we found that the full model was significant when compared to the null model (likelihood test: $\chi^2 = 41.61$, df = 3, $P < 0.001$). Results did not exhibit the same patterns shown by the dopamine/aorta experiments; clozapine $1e^{-5}$ produced a larger reaction than dopamine without clozapine (t=8.89). Clozapine $3e^{-5}$ and $5e^{-5}$ produced expected results of competitively binding (t=-2.68, -2.19 respectively).

The full model of dopamine on the heart (Fig. 2c) corroborated previous tissues and was significant when compared to the null model (likelihood test: $\chi^2 = 22.76$, df=2, $P < 0.001$). Clozapine $1e^{-5}$ did not cause a significantly different reaction (t = -1.75) unlike a higher dose of clozapine $5e^{-5}$ (t = -5.16).

Testing response of tissue type (aorta, heart and RBV) to dopamine (Fig. 2d) found that the full model was significant when compared to the null model (likelihood test: $\chi^2 = 9.38$, df = 2, $P < 0.05$), showing that tissue affects contraction. To investigate if all tissues significantly responded, the full model (dose + tissue type) was compared to the

model containing the interaction between dose and tissue type. This showed that the interaction was significant when compared to the full model (likelihood test: $\chi^2 = 15.46$, df $= 2$, $P < 0.005$) suggesting that the tissues respond differently to dopamine. When looking at these data, aorta showed a delayed reaction in comparison to the RBV, but statistically it was the strongest reaction, followed by heart and RBV respectively (Fig. 2d).

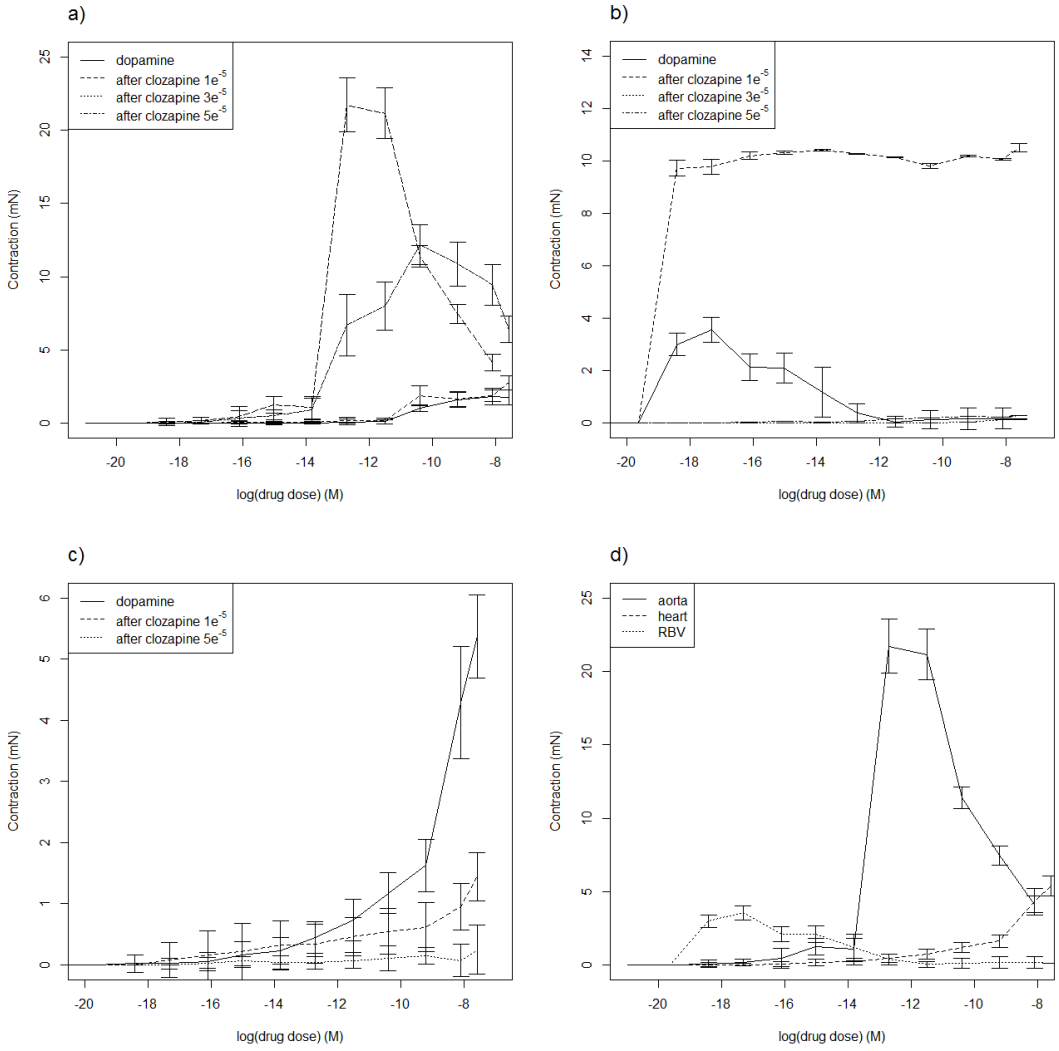


Figure 2 Effects of dopamine on *Nautilus* tissue. The effect of dopamine is shown as a single reaction, and after increasing concentrations of the antagonist clozapine in a) the aorta, b) the respiratory blood vessel c) the heart. A comparison of dopamine, without clozapine, is shown in d) on the aorta, respiratory blood vessel and heart.

5.4.2 *THE EFFECT OF NORADRENALINE*

Testing the effect of noradrenaline on the aorta (Fig. 3a) found that the full model was significant when compared to the null model (likelihood test: $\chi^2 = 15.5$, df $= 2$, $P < 0.001$). Testing noradrenaline after the presence of antagonist prazosine $1e^{-6}$, no difference was found in the strength of aortic contraction, however a higher dose of prazosin $3e^{-5}$ did inhibit reaction ($t = -3.95$).

The effect of noradrenaline on the RBV (Fig. 3b) or the heart (Fig. 3c) showed no significant difference upon comparison between the full and the null model. This is supported by testing the response of tissue type (aorta, heart and RBV) to noradrenaline (Fig. 3d), finding that the full model was significant when compared to the null model (likelihood test: $\chi^2 = 21.26$, df $= 2$, $P < 0.05$), showing that tissue affects contraction.

To investigate if all tissues significantly responded, the full model (dose + tissue type) was compared to the model containing the interaction between dose and tissue type. This showed that the interaction was non-significant when compared to the full model (likelihood test: $\chi^2 = 1.32$, df $= 2$, $P = 0.52$) suggesting that all tissues respond noradrenaline (Fig. 3d).

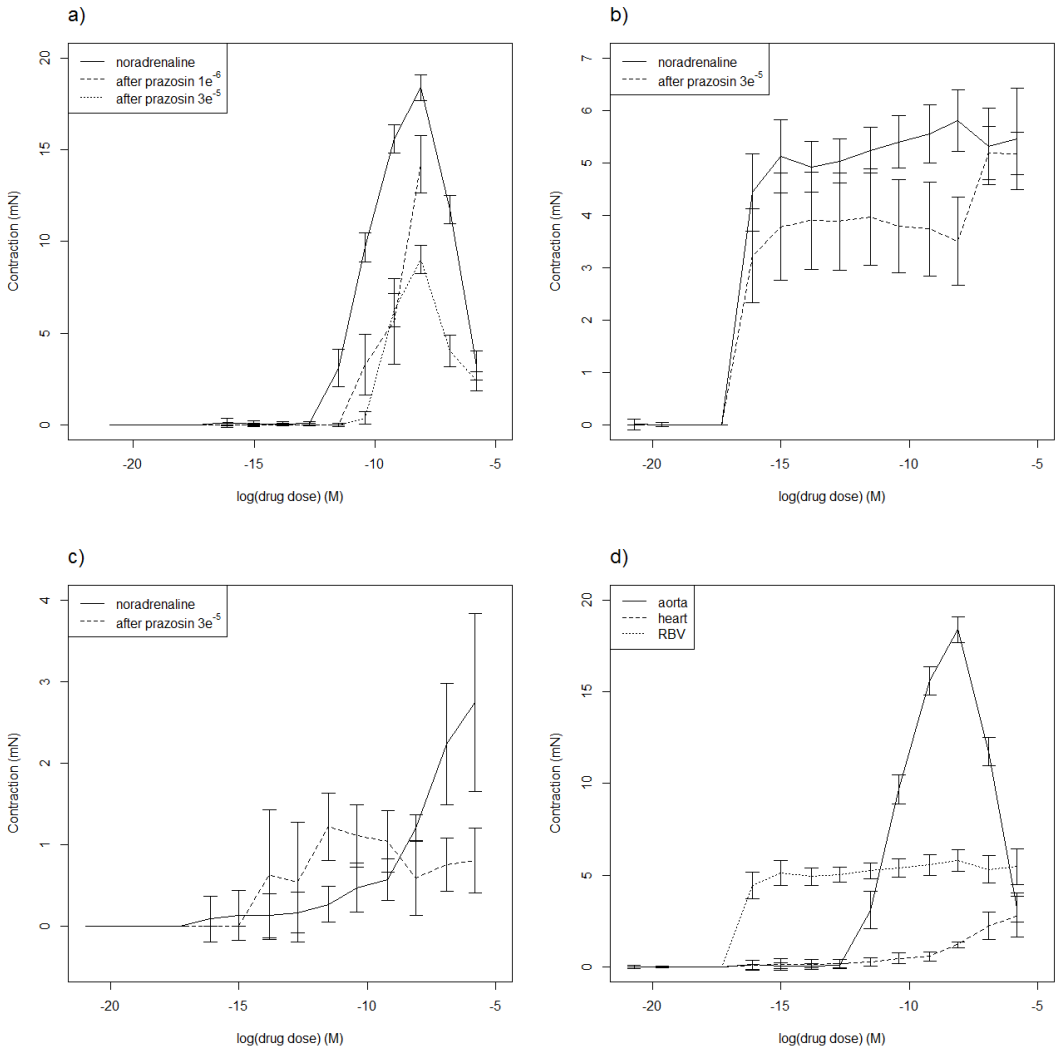


Figure 3 Effects of noradrenaline on *Nautilus* tissue. The effect of noradrenaline is shown as a single reaction, and after increasing concentrations of the antagonist prazosin in a) the aorta, b) the respiratory blood vessel, c) the heart. A comparison of noradrenaline, without prazosin, is shown in d) on the aorta, respiratory blood vessel and heart.

5.4.3 *THE EFFECT OF 5-HT AND ISOPRENALINE*

No response was shown by the aorta or RBV to 5-HT and isoprenaline. Both 5-HT and isoprenaline (Fig. 4a, 4b respectively) were shown to have a significant effect on the heart however (t=13.68, df=71, $P<0.001$; t=18.86, df=63, $P<0.001$ respectively).

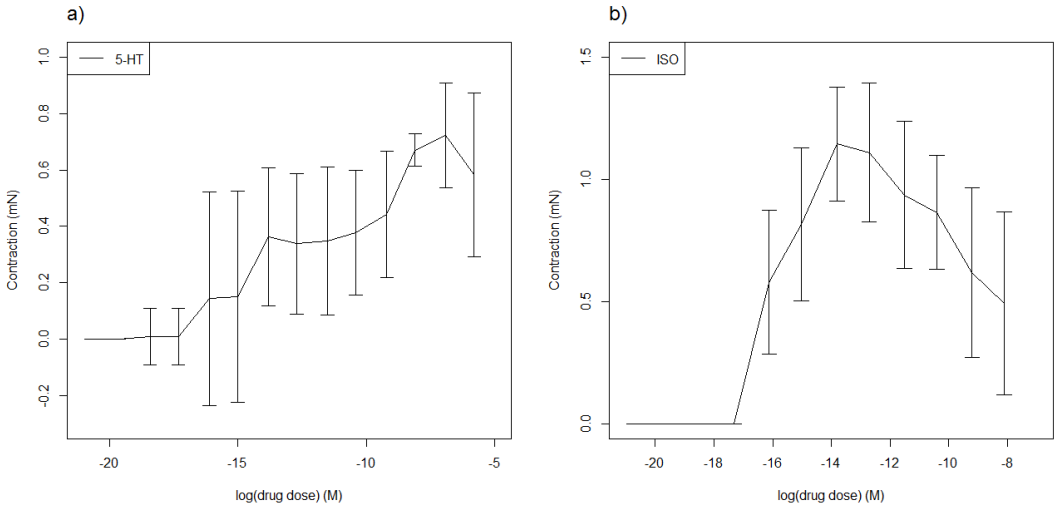


Figure 4 Effects of a) 5-hydroxtryptamine and b) isoprenaline on *Nautilus* heart.

5.4.4 *THE EFFECT OF AGONISTS ON ALL TISSUES*

The reactions of 5-HT and isoprenaline on the heart were corroborated by the significance of the GLMM when looking at the effect of all four agonists on the heart in comparison to the null model (likelihood test: $\chi^2 = 17.27$, df $= 3$, $P < 0.001$; Fig. 5a). Testing the effect of dopamine and noradrenaline on the aorta (Fig. 5b) showed a significant reaction when compared to the null model (likelihood test: $\chi^2 = 6.16$, df $= 1$, $P < 0.05$). Testing this effect on the RBV (Fig. 5c) also showed a significant reaction when compared to the null model (likelihood test: $\chi^2 = 15.18$, df $= 1$, $P < 0.001$).

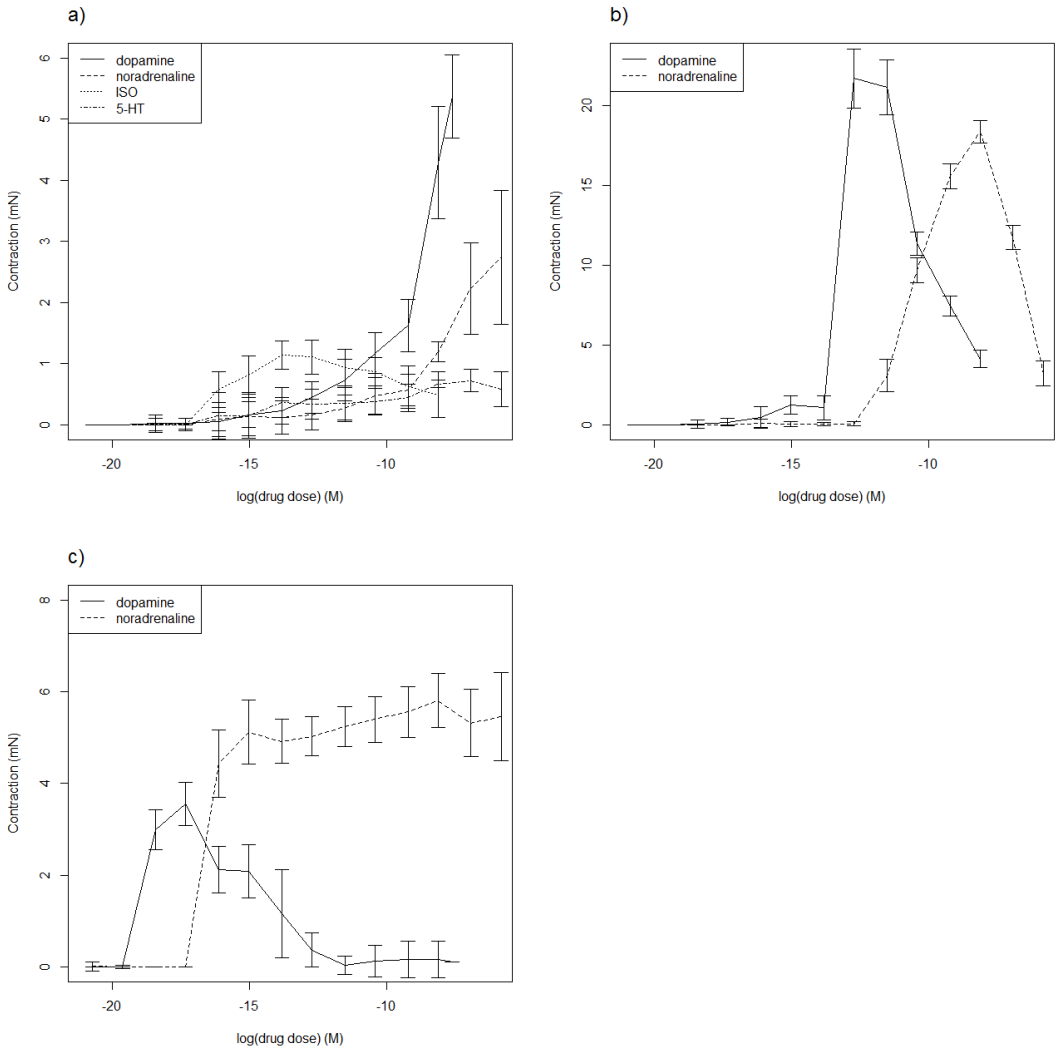


Figure 5 Effects of *Nautilus* tissue on pharmacological agonists. A) the effect of dopamine, noradrenaline, isoprenaline and 5-hydroxtryptamine on the heart. The effect of dopamine and noradrenaline in b) the aorta c) the respiratory blood vessel.

5.5 *DISCUSSION*

We aimed to analyse the functional pharmacology of cardiovascular, epithelial and respiratory tissues in *N. pompilius*. We tested for the presence of specific receptors to dopamine, noradrenaline, isoprenaline and 5-HT in these tissues. Responses to drugs have previously been tested in coleoids (e.g. Dahl *et al.* 1966; Juorio 1971; Kling & Schipp 1987). This was conducted by assessing colour reactions after paper chromatographic separation (Juorio 1971). Although techniques have progressed since the 1960s, their results are still highly informative and have provided a strong foundation on which to develop our experiments. We wanted to investigate whether *N. pompilius* tissue elicited responses that would allow us to confirm or deny the presence of these receptors within nautiloids. This work was conducted as a pilot study to allow us to assess whether the research validated further investigation.

We demonstrated reactions to both noradrenaline and dopamine, indicating the presence of receptors for both compounds in all three tissues. These results are consistent with those previously shown in other cephalopods (Juorio 1971). The tissue reaction to dopamine was delayed and reduced after an initial application of clozapine. As concentrations of clozapine increased, the responses recorded subsequently decreased. An anomaly in the results is seen in the test of dopamine on the RBV (Fig. 2b); the response to dopamine was smaller than the response to dopamine after clozapine $1e^{-5}$. We have found no evidence in the literature of this occurring in other experiments that could explain this response. Unfortunately, these data contributing to the experiment using clozapine $1e^{-5}$ was data taken from experiments on just two individuals. Retesting this drug/tissue combination would therefore be a priority when the experiment was repeated.

To build on these preliminary results, more specific agonists could be used to allow us to discriminate between the dopamine receptor classes (Sibley 1999; Mustard *et al.* 2005). It had been shown that differences in D_1 receptor sequences were present between taxa (see Callier *et al.* 2003). In the more primitive, jawless vertebrates, only one D_1-like sequence had been found in lampreys and hagfish (agnathans). As a result it had been hypothesised that only one type of D_1 receptor existed in species that descended from the earliest diverging vertebrates (Callier *et al.* 2003). More recently however, the D_2 receptor gene was identified in lampreys (*Petromyzon marinus*; Robertson *et al.* 2012). The gene structure was shown to resemble that of different vertebrate D_2 receptors, showing the fundamental function of the receptors is a conserved feature. Structural differences have been documented between vertebrates and invertebrates (Mustard *et al.* 2005) and *Nautilus* could contribute to this on-going research.

Reactions to dopamine were indicative of the potential for presence of noradrenaline; in humans, dopamine is converted to noradrenaline by dopamine β-hydroxylase. Noradrenaline is therefore unlikely to be produced in the absence of dopamine (Rang *et al.* 2012). We saw significant reactions to noradrenaline that were confirmed by the reduction in strength and speed of reaction by first exposing the tissue to prazosin. Prazosin $1e^{-6}$ was not available to the same dosage of that tested solely with noradrenaline or prazosin $3e^{-5}$. These data show the delay in the initial response to noradrenaline but unfortunately cannot be plotted to completion (Fig. 3a). We see another unexpected result when testing noradrenaline on the heart (Fig. 3c); similar to dopamine in the RBV, the test after the antagonist creates a faster reaction than that seen after noradrenaline alone. When plotting the average, the result is actually skewed by data from just one individual (Fig. 1). This is a priority for further investigation when repeated. Figure 5b shows a stronger reaction to dopamine than to noradrenaline, corroborating similar responses previously observed (Cottrell 1967; Kerkut 1973). In

the RBV, however, noradrenaline takes longer to react but then produces a stronger reaction and for a longer period of time (Fig. 5c).

No reaction was seen to 5-HT in the aorta or RBV, despite eliciting a reaction in the heart. This reaction is consistent with results on cuttlefish (*Sepia officinalis*) heart, from which strong reactions were elicited by the presence of 5-HT (Kling & Schipp 1987). While commonly detected in the intestine wall, blood and central nervous system in humans, the smooth muscle of many species (although to a lesser extent humans) is contracted by the presence of 5-HT (Rang *et al.* 2012). It has been shown that its effect on blood vessels is dependent on the size of the vessel and on the species (Rang *et al.* 2012). The reaction seen in the heart is both unusual and interesting. There are seven families of 5-HT receptors with further subtypes. While their locations and functions have been identified in humans, morphological differences mean that this information is not directly applicable to *Nautilus.* It has been previously highlighted that few pharmacological tools are available for accurate manipulation of 5-HT pathways in animals (Barbas *et al.* 2003). As a primitive start in this area of research, the evidence of a reaction by these receptors indicated in the heart can inform future work that can use a more specific range of agonists and antagonists to distinguish the actual receptor being activated.

No reaction was seen in the aorta and RBV as a result of exposure to isoprenaline, although a reaction was observed in heart tissue. The concentration response curve shows a small delay followed by a steep response and then subsequent relaxation. Human experience with the drug was indicative of the lack of contraction seen in the respiratory tissue. In human medicine, isoprenaline has been shown to elicit bronchial muscle relaxation (Zamel *et al.* 1966). With a "reaction" recorded as significant based on the strength of the contraction recorded, it was expected that tissue dilation would have

the opposite effect resulting in no contraction being recorded. Alternatively, in the human heart the drug was shown to increase the strength and rate of contraction (Greiner & Garb 1950). While this is testing the function of the heart and not the presence of receptors, it can still be interpreted as indicative of an effect in cardiac tissue.

5.5.1 *PROPOSED CHANGES TO FUTURE EXPERIMENTAL DESIGN*

There are unavoidable constraints that must be accounted for when working on deep water species, such as the limitations of sampling. A substantial aim for current research is to gather information on *N. pompilius* that can be used in a case for protection. The work presented here had not been investigated in nautiloids before, and so euthanizing animals without an initial small scale study seemed unnecessary. Due to the logistics of sampling deep water species, it was not possible to re-release animals if the work was not a success, and therefore sampling was conservative. While there are problems associated with studies of small sample size, this allowed us to test if there was scope for further investigation without wasting valuable animals. We propose the following alterations to the experimental design that will produce more informative and conclusive results when replicated in the future.

This limited sample size resulted in the same pieces of tissue being used multiple times with different drugs. The longer that tissue is *in-vitro*, the less responsive it will become. While the experiments were conducted efficiently, removing the need to use the same piece of tissue multiple times would prevent any doubt regarding the effect of tissue degradation on the reaction recorded.

As another consequence to a limited sample size, multiple samples were taken from the same individual and treated as separate samples. This runs a severe risk of

pseudoreplication (Hurlbert 1984). The experimental design aimed to test the reactions of tissues to drugs, while also allowing comparison between different drugs and different tissues. Each individual drug/tissue combination was treated as an individual data point. To prevent the experiments from actually testing the difference between individuals, the results from each animal could be averaged. This consequently drastically reduces the sample size. We were able to use mixed models to prevent pseudoreplication at the analysis stage (Bates *et al.* 2014). The statistical package implemented in R allows random factors to be entered into the model. Random effects account for a lack of fit due to outliers, extra variation or unexplained sources of variation. Specific to subjects, this allows within unit correlation at the level of the outcome. GLMMs have been proposed as the correct way to analyse dose response curves (Thorin *et al.* 2010), despite a strong dominance of two-way ANOVA in previous literature (eg. Mallem *et al.* 2005). The literature does also not consistently provide confirmation of meeting the statistical assumptions.

With a larger sample size, multiple pieces of tissue could still have been taken from each individual, but only using one piece per tissue type in each drug/tissue combination. For example, aortic pieces *"a-e"* from individual *I* could have been used more efficiently. Each tissue could have been used as a replicate for drug experiments; tissue *"a"* would test the agonist, *"b"* would test the agonist after the lowest dose of antagonist, *"c"* after the second lowest dose of antagonist etc. This would have eliminated the repeated measures nature of the study and the potential for the tissue to die or become saturated for experiments conducted later, because each tissue piece would only be tested with one drug. Individual *I* would then participate in each drug/tissue combination experiment, but data would not be lost due to averaging all data points from one individual. These data would still be analysed using mixed models to account for individual variation.

Additional tissue should also be used to conduct replications with the presence and absence of the endothelium. The endothelium was not removed from the aorta in the presented experiments, as tested in previous experiments following similar protocols (Mallem *et al.* 2005). Its presence can be confirmed by the observation of at least 60% relaxation to acetylcholine (1 μM; Mallem *et al.* 2005). The role of the endothelium does not always have a significant impact on the effect (Raimundo *et al.* 2008), but to standardise the procedure a control should be introduced in future work to account for the possibility of an influence.

The method with which the antagonist was applied to the experiment could also be improved when retested. The antagonist was added first, followed by cumulative doses of the agonist. While this was effective in demonstrating that we had activated (or failed to activate) the receptors we were targeting, it would have been beneficial to also reverse the process. The agonist could have been applied, followed by the antagonist at the optimal dose of the agonist (identified in previous experiments). This would have assessed the affinity of the antagonist and if it could competitively bind to the receptor by displacing the agonist at the peak of the response. Antagonists not specific to the agonist (for example use clozapine with noradrenaline) could also be used to demonstrate that this failed to block a reaction. This work did nothing to demonstrate that it was not merely the presence of another drug that affected the tissue reaction.

The stronger reactions to dopamine are informative on *Nautilus* evolution at a very basic level. The evidence presented reveals that receptors present in higher order animals are also present in nautiloids. We could add further strength to the study by simultaneously generating concentration curves in multiple species. A comparative approach with model species such as rats or mice would be informative for a direct comparison. This

could be extended to test the effects within cephalopods. Work conducted within the class has documented the effect of the inclusion of nautiloids in the analysis (Wood & O'Dor 2000; Wollesen *et al.* 2009). Generating our own comparison would allow us to distinguish if differences with modern species were specific to cephalopods or detectable solely in *Nautilus*.

On several occasions during the experiments, the application of the drug to maintain the increase in concentration was terminated too early to see the saturation of the receptor, thereby failing to see the plateau in the contraction curve. This was unfortunately a result of drug supply. The experiments should only be repeated in the future if the drug supply exceeds that used in the current work. The plateau in the contraction is informative for calculating the EC_{50} and IC_{50} values; the EC_{50} is the concentration of agonist that provokes a response halfway between the baseline and the maximum response. It is commonly used as a measure of an agonist's potency, or effectiveness, but it is not a measure of drug affinity. The IC_{50} is used for inhibitors (I for inhibition rather than E for effectiveness). With more inhibitor the response decreases and so the dose-response curve decreases (GraphPad 2014) .

5.5.2 *FUTURE RESEARCH AIMS*

If the proposed changes were implemented and the improvements allowed specific receptors to be identified with a greater variability of drugs, we could identify whether the receptors found in *N. pompilius* are those found in higher order animals. We could then attempt to quantify these similarities or differences with comparative sequence analysis using methods similar to those of Kubikova *et al.* (2010). When their presence had been indicated using the organ baths, we would then clone cDNAs of the receptors using primers for family receptors (such as D_1 and D_2). Genbank would be used to collect

already published sequences, and then highly conserved areas would be used to design primers.

RT-PCR would be performed on total RNA from the specific *N. pompilius* tissue with the appropriate primer pairs. Preferably, primers would amplify regions with sequences specific to a given receptor. PCR products would then be examined on agarose gels, and if predicted size bands were present they would be cut and cloned into the pGEMTeasy plasmid. The plasmids could then be transformed into XL-1 blue *Escherichia coli* supercompetent cells. DNA mini preps could then be performed, and successful cloning confirmed with BLAST.

The partial receptor cDNA sequences could potentially identify and predict the full-length coding sequences from genomes of taxonomically similar species. This remains difficult in a species like *N. pompilius,* but highly conserved regions may be successful in molluscs with sequenced genomes such as the California sea hare (*Aplysia California*) or the Giant owl limpet (*Lottia gigantean*). This would allow us to phylogenetically determine taxonomic relationships at this fundamental level, between *Nautilus* and other taxa on which this research has been conducted (Tierney 2001; Crom *et al.* 2003). Finding relatively high protein sequence homologies in cross species comparisons would be informative of both the evolution of *Nautilus* and the evolution of receptors. If the research could follow the trajectory of the nematode research (Komuniecki *et al.* 2004; Smith *et al.* 2007), there is huge potential for behavioural understanding and human application.

CHAPTER SIX:

FINAL DISCUSSION

6.1 *SUMMARY AND FUTURE DIRECTION*

6.1.1 *MAIN FINDINGS OF THE THESIS*

Within this thesis we aim to provide new knowledge on the evolutionary biology of *Nautilus pompilius.* We generated data on the evolutionary divergence and genetic structure between populations. Responding to the research questions and project objectives of Chapter One, the main findings presented in this thesis are:

1. **Populations separate genetically into three evolutionary clades.** Sample division was seen based on partial sequencing of the cytochrome c oxidase sub unit I (COI) gene region. The clades consist of (i) Philippine and west Australian samples, (ii) east Australian samples, and (iii) west Pacific samples. Additionally, divergence is occurring within two of these clades; findings corroborated results from previous analyses of east Australian (Sinclair *et al.* 2007, 2011) and west Pacific samples (Bonacum *et al.* 2011) to show division within both clades. The east Australian samples revealed a genetic divide between the Great Barrier Reef and those from the Coral Sea. Sequences generated from the west Pacific clade (Bonacum *et al.* 2011) indicated divergence between Fiji, Vanuatu and American Samoa. The west Australian clade showed no intra-clade divergence.

These data allowed a novel assessment of population similarity across their range using a highly conserved DNA barcoding marker. This analysis showed full support for hypotheses on *Nautilus* evolutionary history: the division of *N. pompilius* populations into three genetic clades (Wray *et al.* 1995). The results can be used to support the use of DNA barcoding to assign samples to genetic clades, but it cannot be used to identify

specific geographic locations within the clade. Results showed 49 discrete haplotypes for 128 individuals, indicating that sequences are conserved within clades. Despite some apparent divergence at the molecular level, intra-clade diversity was consistently lower than inter-clade diversity. This suggests that management plans need to be specific to geographic regions, promoting the treatment of them as operational taxonomic units.

2. **Microsatellites were successfully isolated from *N. pompilius*.** The isolation and amplification of microsatellites was successful from DNA extracted from tentacles. This led to characterisation of novel loci that did not display linkage disequilibrium, and that were in Hardy-Weinberg equilibrium. Microsatellites have never before been isolated in *Nautilus* and proved challenging. Scoring the alleles for analysis was notably more complicated than is commonly experienced with other groups, such as Aves. This may be attributed to the unexplained genetic difficulties associated with molluscs, which have been experienced by other researchers in the field (Mallet *et al.* 1985; Borsa *et al.* 1991; Chacón *et al.* 2013).

Due to *Nautilus* microsatellite complications, a strict systematic technique had to be developed when scoring genotypes to advocate their reliability in such rigorous analyses. The isolation and development of microsatellite primers in *Nautilus* contributes to the overall microsatellite research programme on cephalopods (such as Girard & Angers 2006; Cabranes *et al.* 2008; Toussaint *et al.* 2011; Miller *et al.* 2011; Tomano *et al.* 2013) and represents the first nautiloid contribution to this area of work. The primers developed were of quality suitable to be used in structural analyses, the results of which will be informative in developing and subsequently implementing management strategies for their conservation.

3. Microsatellites reveal low genetic structure between the Philippines and west Australia, analyses show that this is not a result of gene flow. Analysis using 14 novel microsatellite loci showed the individuals sampled from Great Barrier Reef to be genetically isolated from individuals sampled from Osprey Reef and Shark Reef in the Coral Sea (F_{ST}=0.312, 0.229 respectively). Unexpectedly low genetic structure was demonstrated between west Australian and Philippine samples (F_{ST}=0.015), contradictory to anticipated isolation. Approximate Bayesian computation (ABC) analysis indicated that these populations had diverged from a progenitor population, and that the observed genetic similarity was not a result of current gene flow. We suggest this may be explained by large population sizes at both locations, indicating a minor role for genetic drift. Their evolution from a progenitor population in the Philippines had been hypothesised (Wray *et al.* 1995) but ABC analysis (or equivalent) had not previously been used to test this. Simulating movement between sampling locations helps to understand the impact of fishing in these areas; we can conclude from results that Philippine populations would not be naturally repopulated if fished to local extinction.

While the thesis, and specifically this chapter, does not directly address speciation within *N. pompilius*, the data do indicate that Coral Sea samples (Osprey Reef and Shark Reef) show enough genetic differentiation to be classified as a separate sub-species, or potentially a separate species. Combined with morphological work, this could also be said for Great Barrier Reef samples. Caution has been taken, however, to address speciation based solely on structural data as this would depend not only on the level of divergence caused by drift, but most importantly on the amount of divergent selection experienced by populations. This amount of selection would not be possible to predict from a demographic study based on neutral markers like microsatellites. These data do

offer a strong contribution though, and should definitely be considered should a case for speciation be addressed in the future.

4. **Receptors shown to be present in humans are also present in *N. pompilius.*** Our experimental work demonstrated that it was possible to conduct investigations on *Nautilus* tissue in a conventional organ bath system, similar to that used in more model systems such as rats and mice. Both dopamine and noradrenaline were shown to elicit contractile tissue responses in the heart, the aorta and the respiratory blood vessels. Isoprenaline displayed similar patterns to those shown in humans (Greiner & Garb 1950; Zamel *et al.* 1966), causing contraction in the heart but no reaction in the respiratory blood vessels. Results from this pilot study demonstrate scope for further investigation into *Nautilus* receptors and their functional diversity at this fundamental level.

6.1.2 *UNANSWERED QUESTIONS WITHIN THE THESIS*

The next logical step in this area of research is to fully explore the ultimate aims of the pharmacological work presented in Chapter Five. The work was conducted as a pilot study and, as discussed, results warrant progression of the investigations. This would involve initially re-sampling. Implementing the revised experimental design discussed in Chapter Five discussion would then allow a multi species comparison. There is also the possibility to determine if dopamine receptors have any involvement in *N. pompilius* behaviour. Behavioural observations of *Nautilus* are fairly limited (Westermann & Beuerlein 2005), and therefore individual interaction or social structure is unknown. As a result this may remain an outstanding question in the field for some time, but is a consideration for the future.

The rapid development of technology means that genetic techniques have already improved and become more accessible since the presented research began. The use of microsatellites in Chapter Four built on the findings using mitochondrial DNA in Chapter Two, by using a finer scale marker and larger number of samples. This research can now be explored with greater depth by employing whole genome work. Genome-wide association scans, among many things, can look at genetic similarities between individuals without problems associated with specific markers (Estoup *et al.* 2002). With an initial outlay in cost, the scope for variability in analyses exceeds that of using single markers.

6.1.3 *STRENGTHS AND WEAKNESSES OF THE RESEARCH*

The strengths of the research lie in the exploration of an understudied species. An abundant array of molecular work has been conducted on cephalopods (see Allcock *et al.* 2015), but this research is dominated by coleoid studies. Using DNA barcoding allows direct comparisons to be made between species within Cephalopoda, therefore producing work that can be incorporated into this catalogue of research on the class. More specifically to *N. pompilius,* the microsatellite analyses have answered questions regarding their current movement as a species, and consequently questions on their evolutionary patterns. These data feed directly into informing their protection, and can subsequently be used as an example to show the necessity of taking individual movement into consideration when designing management plans.

The thesis uses novel analyses such as ABC (Wegmann *et al.* 2010; Csilléry *et al.* 2010) in microsatellite research of Chapter Four, which has been shown as hugely influential in understanding species evolution and current gene flow between populations (Palero *et al.* 2009; Guillemaud *et al.* 2010; Butlin *et al.* 2013; Fountain *et al.* 2014). Molecular data used for analyses were generated using standardised techniques such as DNA extraction

with Qiagen DNeasy tissue kits (QIAGEN Ltd, Manchester, UK). Error was accounted for at each stage of the laboratory work by using controls and by running sample replicates. We are therefore confident that final results are standardised and reliable.

The pharmacological research conducted thus far in Chapter Five can answer questions in isolation, such as presence and absence of receptors, but also warrants additional work. This chapter involved provisional experiments, data of which contribute to the ultimate aims within this area of *N. pompilius* research. Its completion of receptor sequencing, and consequent analyses, was not possible during this investigation. This may, however, be advantageous to the overall project; time and therefore innovation could be largely beneficial to this aspect of the project still to be conducted.

Weaknesses within the project fall under the common criticisms of research: the need to increase the quantity and quality of data generated:

- In Chapter Two, using additional markers would add strength to the findings. Using a barcoding marker allowed us to combine our generated sequences to those from other research (Bonacum *et al.* 2011). An additional marker such as Cytochrome-b could have provided further depth and variation to the results.

- In Chapter Four, a larger number of loci would have contributed to the research; a total of 55 primer sets were tested and only those with enough strength to be reliably used in statistical analyses were carried forward, leaving a final total of 14. The level of complication when working with *N. pompilius* on molecular work of this nature was unknown. To compensate for this, a large number of primers were developed using both Sanger and MiSeq sequencing techniques. The final number is sufficient for the analyses, but larger numbers are preferred.

- In Chapter Five, as discussed in detail, sample size was restricted during experiments. This is common for a pilot study, and using General Linear Mixed Models to avoid pseudoreplication meant that a small amount of data were not detrimental to the conclusions drawn.

Due to the nature of the sampling environment, individuals were not sexed during sample collection; in the absence of a researcher, tentacles were collected by dive guides on commercial dive boats. As such, date and location were recorded, but information such as sex was not retained about the animal. Markers displaying an excess of homozygotes or deviating from Hardy-Weinberg equilibrium, were removed from the population structure analyses. The lack of reliable data regarding gender prevented us from identifying the location of the removed markers. If we assume that this species has two sex chromosomes, all individuals of the homogametic sex would appear homozygous (hemizygous) whereas individuals of the heterogametic sex may appear heterozygous or homozygous. The availability of known sex individuals would have allowed the identification of sex-linked markers and the confirmation of other markers as autosomal. ABC analysis concluded that genetic similarity over large distances between the Philippines and west Australia was not as a result of current gene flow. Due to their unknown gender, sex-biased dispersal could not be investigated (Lukoschek *et al.* 2008). Any information on sex-based movement would have contributed towards the unanswered questions regarding the disproportionate sex ratios recorded in *Nautilus* (Haven 1977; Saunders & Spinosa 1978; Saunders *et al.* 1991; Dunstan *et al.* 2011c).

Due to the nature of molecular biology, molecular techniques become quickly dated. This does not detract from the accomplishments of the presented data, but does provoke evaluation in regards to how three years have influenced the nature in which we would now address the research questions.

6.1.4 *WIDER IMPLICATIONS OF THIS RESEARCH*

The levels of extractive exploitation currently being experienced by *Nautilus* across much of its range may have long-term genetic implications. Establishing patterns of gene flow between populations both with and without pressure can help us to predict long term impacts. The genetic data presented here demonstrate limited movement of individuals between populations, and consequently the genetic isolation of populations. These data do not indicate current consequences to population declines in the Philippines (Chapter Four, fig. 5), however *Nautilus* have only recently been targeted by the Philippine fisheries (Dunstan *et al.* 2010) and as such, a delayed response is expected in the populations of a *k*-selected species. Although their ability to recover from a potential bottleneck event is unknown (De Angelis 2012), the low fecundity and a long developmental time of *Nautilus* spescies (Carlson *et al.* 1984; Landman & Cochran 2010) would indicate a low threshold for coping with the long term impacts of overexploitation. Populations in areas of high fishing pressure have shown severe declines (Barord *et al.* 2014) and here we provide evidence that, as a result of little or no migration into the Philippines, local extinction events are a distinct possibility. This thesis documents the genetically unique nature of populations, highlighting the requirement for specific individual management plans.

It is estimated that up to 36,000 species may be becoming extinct each year, with the largest contributions of data being for mammals, amphibians and birds (Monastersky 2014). It is important that any data gathered on the more understudied species be used towards long term management. Evidence presented in this thesis supports the necessity to halt the declines of *N. pompilius* populations, and these data would thus be beneficial to a case for protection for *N. pompilius.*

Commercially targeted marine species are undergoing significant declines (Hutchings 2000; Worm *et al.* 2006; Neubauer *et al.* 2013; Watson *et al.* 2013). Trade agreements such as CITES are in place, which use biological data to help control the continuing impact on declining species. Data presented in this thesis documents the necessity for species specific assessments. Dispersal differences within Cephalopoda have been demonstrated (Buresch *et al.* 2006), and they substantiate why catch limits cannot be applied generically across species. Broadcast spawning species with a juvenile stage which facilitates large scale dispersal, or species that utilise oceanic features such as currents, will respond differently to fishing pressure than species with little or no juvenile dispersal (Mirams *et al.* 2011).

Our findings could also feed into understanding the ocean physiography along west Australia. The significant F_{ST} seen between Scott Reef and other Australian Reefs is unexplained by ocean depths in the area. There may be more unique features to this reef that are currently unidentified; significant F_{ST} has also been seen between Scott Reef and surrounding reefs in the olive sea snake (*Aipysurus laevis*; Lukoschek *et al.* 2008) and coral reef fish (Underwood *et al.* 2012). With no exploitation pressure, there appears no imminent risk to species here. Multiple examples of animals sampled at Scott reef show genetic differences in comparison to those sampled at relatively close geographic locations, therefore further investigation could reveal interesting characteristics to the biogeography of the area.

The pharmacological characterisation of the *Nautilus* functional genome is of more direct application to human research than to marine conservation. Providing information on the range and activity of chemical receptors found in humans feeds back into their evolution and time line of appearance (Crom *et al.* 2003; Yamamoto & Vernier 2011). There is an on-going search for compounds to use in agricultural, pharmaceutical

and cosmeceutical products. Amongst many other examples, saponins have been discovered in the viscera of the Australian sea cucumber *(Holothuria lesson)* that have biotechnical applications (Bahrami *et al.* 2014), compounds of the Thai bael fruit *(Aegle marmelos)* have been identified as functional food ingredients (Suvimol & Pranee 2008), and marine sponge derived metabolites (such as *Tectitethya crypta*) continue to show promise as a source of new drug leads for cancer treatments (Essack *et al.* 2011). Copper based blood is extracted from the horseshoe crab (Limulidae) for its role in medical applications and the detection of endotoxins (Iwanaga *et al.* 1978), and the presence of copper based blood in *Nautilus* (Saunders & Landman 2010) provide a potential avenue for novel pharmacological research.

Identifying natural products for human application can be beneficial for their conservation; locating paclitaxel (Stierle *et al.* 1993), a natural-source drug for cancer, from the bark of the Pacific yew tree (*Taxus brevifolia*) stimulated the protection and propagation of the trees until the substance could be artificially synthesised (Bland *et al.* 1991). Investigations into the function and application of natural products are on-going and we aim to discover if the pharmaceutical properties of *Nautilus* can be used in this way.

6.2 *CONSERVATION OF NAUTILUS*

6.2.1 *COMMUNITY ENGAGEMENT*

For the conservation of *N. pompilius,* biological data are necessary for the legal case to protect against overfishing (De Angelis 2012). Another approach which can be used to aid in their protection is community outreach. Philippine communities use *Nautilus* fishing as their source of income (Dunstan *et al.* 2010) and so preventing this activity entirely could have severe repercussions. Working with local people to develop community based marine protected areas (as discussed in Chapter One) may aid the

conservation of the species and the economy of the local communities. This approach to conservation has been shown to be successful (Brooks *et al.* 2013) but it requires community involvement in the design, which would require participation from each fishery. Locals must see the advantages in order to continue this long term (Halpern 2003). It is most likely that we will unfortunately see a delayed response in *N. pompilius* recovery due to their *k*-selected traits, and so it would be important to communicate this expected delay with fishing communities.

6.2.2 *Combining knowledge on Nautilus evolution with Nautilus conservation*

The international trade of *Nautilus* products (De Angelis 2012) would suggest that research should be aimed towards conservation efforts, but as such an iconic species their evolution remains a source of general fascination. Combining research for their conservation with research on their evolution, for the benefit of their protection, may be a possibility. *Nautilus* shells have a striking and well recognised pattern of semi-irregular stripes, the colour of which varies between individuals from the most commonly seen brown, to purple and deep red colour morphs. Anomalies to this have been reported; a shell collected in the Philippines was noted as being absent of any shell pattern (Mapes & Landman 2012). This was not the first encounter of such an "albino", as concluded by the authors; a patternless *N. pompilius* individual was collected during a 2007 sampling trip to Osprey Reef in the Coral Sea (Fig. 1). While being held during sampling and recording prior to release, this individual repeatedly demonstrated aggressive behaviour towards the other individuals in the tank (pers. comm. Dr Billy Sinclair). This is an unusual behaviour pattern for *Nautilus* in such circumstances and it is unknown if this was related to its phenotypic difference or not. The individual appeared mature, and so the fitness consequences of this variation in shell patterning are unknown. A sample taken from this individual was part of the data set analysed in

Chapter Two and Four (sample number OR2400), and this showed no unexpected genetic separation compared to other samples from the same location.

a) b)

Figure 1 *Nautilus pompilius* stripe morphology. a) *N. pompilius* lacking typical shell stripes; the individual was photographed and sampled in 2007 at Osprey Reef in the Coral Sea. The shell lacks the colour stripes typically seen on all *Nautilus* species (photo by Jürgen Freund). b) an individual displaying standard shell markings, also sampled at Osprey Reef in 2012.

It is unknown whether the shell patterns hold any significance to individual *Nautilus* in addition to camouflage. Variation in shell stripe patterns has not been characterised to any extent across their range. Photos were taken of each individual encountered on all Coral Sea and Great Barrier Reef sampling trips. Pattern analysis could be used to see if any difference exists in shell patterns, and if so, whether this is correlated to the differences in genetic diversity. If CITES protection comes into place but illegal trade continues, understanding the shell pattern distinction across their range has the potential to identify the origin of a fished shell. This work could therefore be informative on a signature feature of the *N. pompilius* phenotype, and be utilised for their protection.

6.3 *CONCLUSION*

Throughout this thesis I have presented my research on the evolutionary biology of *N. pompilius.* I hope that this research will be of value to the scientific community, and

more specifically, to the conservation of marine species. The decline in animal populations has been accounted to habitat degradation (31%), climate change (7%) and invasive species (5%), among others. The most dominant factor (37%), is exploitation (Monastersky 2014). It is important to gather biological data on exploited species before their disappearance contributes to these statistics. As a species that is regarded as data deficient, it is imperative that we gather the information on *N. pompilius* that can be used to facilitate their protection before their local extinction in the Philippines becomes a reality.

"Yet, even as we gain more insight into the complex and heterogeneous world of the seas, the signs of destructive human impact are visible everywhere. What we once thought limitless, isn't; what we once thought as resilient seems less so"

— M Tundi Agardy (1994)

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APPENDIX I

GenBank accession numbers and sampling information for all samples used

Sample Reference	GenBank Accession #	Sampling Location
FNGBR1c	JQ862293	Far North Great Barrier Reef, east Australia
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	
OR1361	JQ862308	Osprey Reef, east Australia
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	
SR090801	JQ890081	Scott Reef, east Australia
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	
AR01	JN227639	Ashmore Reef, west Australia
AR02	JN227640	
AR03	JN227641	
AR04	JN227642	
AR05	JN227643	
AR06	JN227644	
AR07	JN227645	
AR08	JN227646	
AR09	JN227647	
AR11	JN227648	
AR12	JQ429507	
AR13	JQ429508	
AR14	JQ429509	
AR15	JQ429510	
AR16	JQ429511	
CR01	JN227649	Clerke Reef, west Australia
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	
IR01	JN227659	Imperieuse Reef, west Australia
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	
S1736	JQ862326	Shark Reef, west Australia
S1737	JQ862327	
S1738	JQ862328	
S1739	JQ862329	
S1740	JQ862330	
S1741	JQ862331	
S1742	JQ862332	
S1744	JQ862333	
S1745	JQ862334	
GQ280240	GQ280240	Vanuatu
GQ280241	GQ280241	
GQ280242	GQ280242	
GQ280243	GQ280243	
GQ280244	GQ280244	
GQ280245	GQ280245	
GQ280246	GQ280246	
GQ280247	GQ280247	
GQ280248	GQ280248	
GQ280249	GQ280249	
GQ280190	GQ280190	Ambon Strait, Indonesia
GQ280191	GQ280191	
GQ280192	GQ280192	Pangalao Island, Philippines
GQ280193	GQ280193	Balayan Bay, Philippines
GQ280194	GQ280194	
GQ280201	GQ280201	Port Moresby, Papua New Guinea
GQ280202	GQ280202	
GQ280206	GQ280206	Little Ndrova Island, Papua New Guinea
GQ280207	GQ280207	
GQ280208	GQ280208	
GQ280209	GQ280209	
GQ280210	GQ280210	
GQ280211	GQ280211	
GQ280212	GQ280212	
GQ280213	GQ280213	
GQ280204	GQ280204	Lorengau, Manus Island, Papua New Guinea
GQ280205	GQ280205	Komuli Island, Papua New Guinea
GQ280214	GQ280214	Pago Pago, American Samoa
GQ280215	GQ280215	Suva, Fiji
GQ280216	GQ280216	

APPENDIX II

Williams *et al.* (2012): DNA Barcoding in *Nautilus pompilius* (Mollusca, Cephalopoda): evolutionary divergence of an ancient species in modern times. Chapter Two published in INVERTEBRATE SYSTEMATICS

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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.

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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

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:

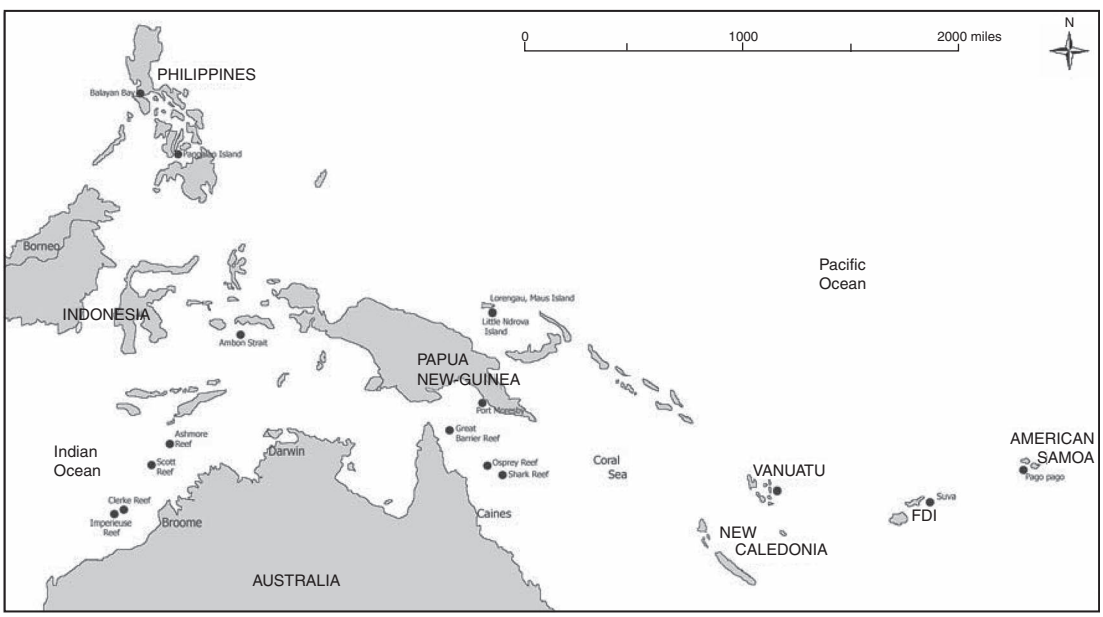

Fig. 1. Geographical locations of the sampling sites for *Nautilus pompilius*. From west Australia: Imperieuse, Clerke, Scott and Ashmore Reefs; east Australia: Shark, Osprey and Great Barrier Reefs; and samples sourced from GenBank: Philippines (Balayan Bay and Pangalao Island), Indonesia (Ambon Strait), 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

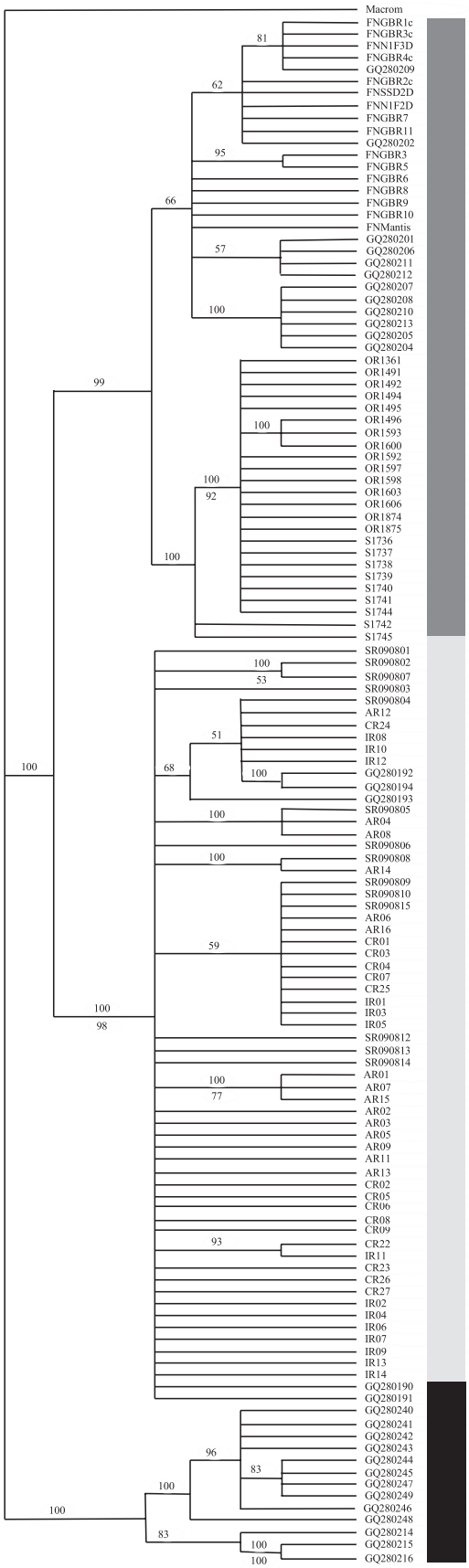

Fig. 2. Phylogenetic analyses of *N. pompilius* COI DNA sequences. Maximum-likelihood analysis generated a consensus tree constructed from the alignment of partial COI sequences of 412 bp from 128 *N. pompilius* sequences, and rooted against *Nautilus macromphalus*. Bootstrap values (1000 resampling 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

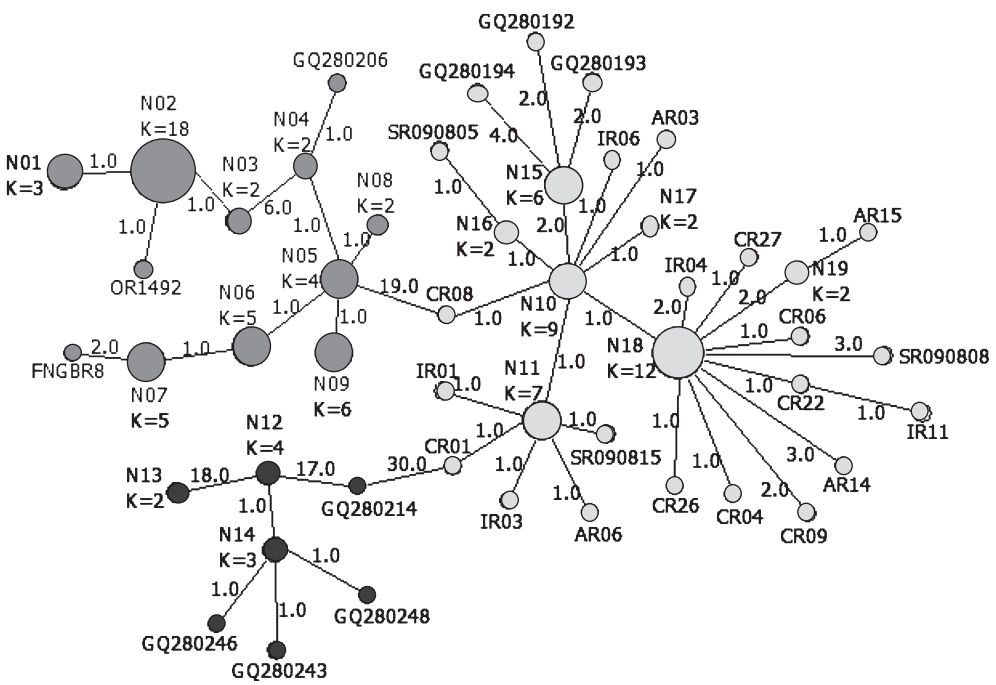

Fig. 3. Minimum Spanning Tree produced using Arlequin v.3.5.1.2. Nodes represent haplotypes, k values signify the frequency of individuals represented in a particular node, values between the nodes demonstrate base pair differences between haplotypes. Node colour corresponds to the three geographical 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.

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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

Appendix III

Williams *et al.* (2015a): Isolation and characterisation of microsatellite loci for the ancient cephalopod, *Nautilus pompilius*. Chapter Three published in CONSERVATION GENETICS RESOURCES

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MICROSATELLITE LETTERS

Isolation and characterisation of microsatellite loci for the ancient cephalopod, *Nautilus pompilius*

Rachel C. Williams · Deborah A. Dawson · Gavin J. Horsburgh · William Sinclair

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Abstract A microsatellite-enriched genomic library was created from a single *Nautilus pompilius* individual and clones/fragments sequenced using Sanger and Illumina MiSeq sequencing. Forty-four markers were tested in four individuals and 30 markers selected for further testing in a population of unrelated individuals from Imperieuse Reef, west Australia. Observed levels of heterozygosity in the IR population ranged from 0.17–1.00 with a mean of 17.2 alleles per locus. No groups of loci displayed evidence of significant linkage disequilibrium. Twenty-one markers were in Hardy–Weinberg equilibrium and will be used to study population structure in this species.

Keywords Cephalopod · Conservation · Microsatellite · Mollusc · *Nautilus* · Shell trade

We present the first set of microsatellite markers isolated from *Nautilus pompilius* (Cepalopoda). Published data supports qualification for *Nautilus* as "endangered" on the IUCN red list (Dunstan et al. 2010), yet a paucity of information currently inhibits the true classification. Previous research has shown genetic isolation occurring between three evolutionary clades across the *N. pompilius* range (Williams et al. 2012). *Nautilus* are unsustainably overfished for the ornamental shell trade and numbers are declining dramatically (Dunstan et al. 2010). Investigating diversity between and within these isolated populations will highlight any populations with an immediate requirement for protection.

N. pompilius were sampled from Imperieuse Reef (IR), west Australia (17°35′S, 118°55′E). Genomic DNA was extracted from tentacle tissue using a Qiagen DNeasy tissue kit (Qiagen Pty. Ltd.). Sample collection is non-lethal and was collected under AFMA Scientific Permit. DNA concentration was quantified using a fluorimeter (Fluostar Optima) and its quality assessed by electrophoresis. The library was created from one individual (IR38) by digesting its DNA with *Mbo*I and enriching the *Mbo*I-fragments for the dinucleotide microsatellite motifs $(CA)_n$, $(GA)_n$, $(TC)_n$, $(TG)_n$ (following Armour et al. 1994). Transformant colonies (n = 192) were Sanger sequenced using BigDye Terminators v3.1 (Applied Biosystems) in both directions, to create a consensus sequence. An Illumina paired-end library was also created using 1 µg of the IR38 dinucleotide-enriched genomic DNA. The SureSelect Library Prep Kit, ILM (Agilent Technologies Inc.) protocol was followed and DNA sequencing was conducted using a MiSeq Benchtop Sequencer (Illumina). Primer sets were designed from 44 microsatellite sequences (39 Sanger and five MiSeqs; EMBL accession numbers HG918068-HG918111) using PRIMER3 v4.0.0 (Rozen and Skaletsky 1999). Primer sets were tested in four individuals from four different populations.

Products were amplified in 2 µl multiplex PCR reactions, including 1.0 µl DNA (air dried), 1 µl primer mix (fluoro-labelled forward and reverse primer(s) at 0.2 mM) and 1 µl Qiagen Multiplex Master mix. Multiplexes were amplified under the following profile: 95 °C for 15 min, followed by 44 cycles of 94 °C for 30 s, 56 °C for 90 s, 72 °C for 90 s and finally 72 °C for 10 min. PCR products were genotyped on an ABI 3730 48-well capillary DNA

R. C. Williams (✉) · W. Sinclair
Centre for Wildlife Conservation, University of Cumbria, Ambleside LA22 9BJ, UK
e-mail: Williams.RachelC@gmail.com

R. C. Williams · D. A. Dawson · G. J. Horsburgh
NERC Biomolecular Analysis Facility, Department of Animal and Plant Sciences, University of Sheffield, Western Bank, Sheffield, South Yorkshire S10 2TN, UK

Table 1 Characterisation of 30 *Nautilus pompilius* microsatellite loci in the Imperieuse Reef population, west Australia

Locus	Clone name and EMBL accession number	Primer sequences and fluoro label (5′- 3′)	Multiplex set	Repeat motif	Expected and observed allele size of library individual (bp)	n	# Alleles obs.	Allele size range (bp)	Hobs	Hexp	HWE p value < 0.05	Est. f(null)
Npom01	Naut106_B01	F: [6FAM]ACCAAATCAACATAATCTGGTGTG	–	(GA)28	221	31	31	184–298	0.92	0.98	0.00*	0.02
	HG918068	R: CCTTAAATTTGCTTACCATGTGC			221, 221							
Npom02	Naut106_B09	F: [VIC]GCCCAAATGGATAAATACAAGC	6	(CA)14	191	31	19	180–226	0.74	0.85	0.17	0.06
	HG918069	R: TCAAATCTCGCCATCACATC			188, 209							
Npom03	Naut106_C10	F: [PET]ACGGAGCCGAAACCACTAC	1	(GA)17	177	31	18	159–184	0.90	0.91	0.34	0.00
	HG918070	R: ACACGTGGAATTTCCTTTGC			167, 179							
Npom04	Naut106_D01	F: [PET]GTGGTTTATTGGTGGGAAGG	2	(GT)15	162	22	20	150–212	0.86	0.94	0.16	0.03
	HG918071	R: CCGCTCTTCTGTCAACAGTG			152, 167							
Npom05	Naut106_D12	F: [6FAM]TTTACTTTGGAGAACTTTCGCTAATC	3	(GA)39	209	31	25	166–267	1.00	0.97	0.11	−0.03
	HG918072	R: GTGGTGACCTTCAACGGTTC			205, 205							
Npom06	Naut106_E07	F: [NED]GATCACGCAGCAAAGTCG	5	(CA)11	191	31	10	179–202	0.79	0.72	0.95	0.05
	HG918073	R: GAGCGAGCGGTACGATTC			187, 191							
Npom07	Naut106_E11	F: [PET]TTCATCCGTTAATTCGTATTGC	3	(CA)20	147	34	24	124–196	0.97	0.94	0.01*	−0.03
	HG918074	R: CTCCCAAATTCTTATACGTCAGC			153, 157							
Npom08	Naut106_E12	F: [PET]TTGGCTAAATCGACGAAACATAG	5	(GA)24	127	29	12	115–160	0.17	0.91	0.00*	0.68
	HG918075	R: GCCGATTCACAATAACAGTGG			137, 137							
Npom09	Naut106_F05	F: [VIC]TTCCAACAAATGCCTTCAAAC	5	(CA)17	188	31	29	176–239	0.96	0.97	0.63	0.00
	HG918076	R: GCTTGGAATAGCTGCACAGAG			185, 206							
Npom10	Naut106_G01	F: [6FAM]GGTCGACGCTGGAACAAT	4	(GT)17	85	31	16	67–102	0.94	0.92	0.39	−0.01
	HG918077	R: TGTCACGACTACGGAATAGGTG			81, 85							
Npom11	Naut106_G08	F: [NED]CCTATTTCAGTGAGTGGAGCTG	1	(CT)20	124	31	8	116–128	0.79	0.78	0.76	0.00
	HG918078	R: TTCATGTGGCTGATGTAAGAAAG			122, 124							
Npom12	Naut106_G10	F: [NED]AGGACAATTAGACTGACGTCTGAAG	4	(GT)17	148	31	31	124–205	0.97	0.96	0.54	−0.01
	HG918079	R: GATGGCGTGCTTATTGGTG			156, 171							
Npom13	Naut116_A10	F: [6FAM]ATTGCCGTCTCGATGTGC	5	(CT)14	297	31	31	272–365	1.00	0.98	1.00	−0.02
	HG918080	R: GCCTCCAGACAGTCGAACC			295, 299							
Npom14	Naut116_B04	F: [6FAM]CAATGGACGTACACCAGAGG	–	(CT)21	142	31	16	118–176	0.25	0.93	0.00*	0.57
	HG918081	R: AAGACAAACTTCCCTCCGAAT			144, 144							
Npom15	Naut116_B05	F: [6FAM]AAGCACGTACAGGTGTGCAG	2	(GT)10	99	25	7	87–102	0.79	0.82	0.99	0.01
	HG918082	R: TGTTATTTCGCTCTAGCAACAGTC			94, 98							
Npom16	Naut116_C04	F: [VIC]GACTATTTGCGTAGAAACACAAGG	1	(CA)17	154	31	29	137–209	0.94	0.96	0.84	0.00
	HG918083	R: CGTTAATCCTAATCCAACTATCTCG			156,202							

Table 1 continued

Locus	Clone name and EMBL accession number	Primer sequences and fluoro label (5′- 3′)	Multiplex set	Repeat motif	Expected and observed allele size of library individual (bp)	n	# Alleles obs.	Allele size range (bp)	Hobs	Hexp	HWE p value < 0.05	Est. f(null)
Npom17	Naut116_E02	F: [VIC]TCCCACCAGTTTACATACAATAGC	3	(CA)15	179	31	24	134–209	0.84	0.95	0.18	0.05
	HG918084	R: GAAACCTGGAATTCTGATTATGC			164,178							
Npom18	Naut116_E08	F: [6FAM]TAGCATGCTCGCTGGTTATG	6	(GT)11	182	31	8	177–191	0.92	0.82	0.91	−0.06
	HG918085	R: CTTGAGTACCAACCAAGACCAAG			177,183							
Npom19	Naut116_F01	F: [6FAM]TATAGGGATGCGTCGTCACC	–	(GA)8	132	31	8	123–139	0.17	0.83	0.00*	0.66
	HG918086	R: CCCTAAGGCTTTATGAAGTCAGC			129,129							
Npom20	Naut116_G04	F: [NED]CGGACAGACCTAATGCAATG	3	(CT)33	208	31	26	175–232	1.00	0.96	0.96	−0.03
	HG918087	R: CAAATGAAACTCGGCAGAAAC			189,223							
Npom21	Naut116_G05	F: [VIC]TGATGGACCGGACTAGGAA	4	(GA)16	123	31	11	99–143	0.88	0.86	0.04*	−0.03
	HG918088	R: CGCCAATGACCAGTCAGA			113,122							
Npom22	Naut116_H02	F: [HEX]CTGGTTACTATCATTATGGTTTCTCG	2	(CA)36	186	31	20	134–185	1.00	0.96	0.00*	−0.03
	HG918089	R: CGACATCGTCCTGCATTTAG			127,127							
Npom23	Naut116_H06	F: [6FAM]CTGTCCTGGCTGCTAACCTAC	–	(CA)10	178	31	4	168–178	0.26	0.52	0.01*	0.32
	HG918090	R: CTTCGATTCATCAGAACCTAATACC			174,176							
Npom24	Naut116_H09	F: [6FAM]CAAAGAATTTGAAGCTCGAACAG	5	(CA)25	146	31	26	114–189	0.88	0.95	0.11	0.03
	HG918091	R: TTTCCACAAATCGTGTCTTGAG			132,144							
Npom25	Naut116_H12	F: [PET]GTGCTTTACTGACAGTTACATATCGTG	6	(GT)9	183	31	5	176–184	0.75	0.63	0.72	−0.12
	HG918092	R: CCTAGACCATCGATGCAAGC			185,187							
Npom26	Naut2904	F: [6FAM]AACCCGGTACAGGAGCAAC	1	(CT)11	162	31	9	138–162	0.92	0.83	0.99	−0.62
	HG918093	R: ACGAGCGAAGAACCACTTTG			148,160							
Npom27	Naut5395	F: [PET]AAAGTTTCCCGGCTTCTTTG	4	(CA)10	147	31	15	144–188	0.92	0.84	0.71	−0.07
	HG918094	R: CGGAGGATTAATATTGATTATTTGTTG			147,175							
Npom28	Naut5442	F: [NED]AAACAGTTCGGTGCATCCTC	6	(GT)10	167	25	13	169–194	0.76	0.89	0.65	0.07
	HG918095	R: CATCCAGCAAATCAGTCGTG			175,190							
Npom29	Naut5466	F: [VIC]CTGCAGCAAAGTAGGCTGTG	2	(CT)12	152	31	16	136–184	0.92	0.94	0.95	0.00
	HG918096	R: AAGTGGCCATGGGTATTTTG			146,154							
Npom30	Naut7474	F: [HEX]ATCAAACGCTCGGATGTAAAC	–	(GT)10	100	31	6	89–113	0.56	0.74	0.01*	0.13
	HG918097	R: ACGCATTCGTCTCTATTCTGC			94,98							

Loci sequenced using Sanger (*Npom01–Npom25),* Miseq (*Npom26–Npom30*)

n, total individuals genotyped; # Alleles obs, total alleles observed; *bp* base pairs; library individual, the individual genotype from which the library was developed; *Hobs* observed heterozygosity; *Hexp* expected heterozygosity; *HWE* Hardy–Weinberg equilibrium

* significant deviation from HWE ($p < 0.05$); Est. f(null), estimated null allele frequency; -, excluded from multiplex. Primer melting temperatures 59–61 °C

Table 2 Details of 14 *Nautilus pompilius* microsatellite loci rejected after initial amplification due to difficulty in scoring following testing in the Imperieuse Reef population

Locus	Clone name and EMBL accession number	Primer sequences and fluoro label (5′- 3′)	Repeat motif	Expected allele size (bp)
Npom31	Naut106_B03	F: [HEX]CGACTCAAGGGACTACATTCG	(CT)32	250
	HG918098	R: AAACAGTAACGCCTAAACACCAC		
Npom32	Naut106_B12	F: [6FAM]CGACTGCTGAACCTACAAAGC	(GA)30	170
	HG918099	R: GCGGGAGAACAAATCAAGAC		
Npom33	Naut106_C07	F: [6FAM]CGGAATTTAGCACGGTGAC	(CA)12	148
	HG918100	R: AGCGGTGTTGGGAAGAATAC		
Npom34	Naut106_C11	F: [6FAM]TTCCTTAAATTTGCCACCGTA	(GA)16	259
	HG918101	R: ATCACTTAAACCATCAGTTACGACA		
Npom35	Naut106_D05	F: [6FAM]AAATTTCCTTGGTCGATATACGG	(GA)33	169
	HG918102	R: TTCTGCCAGTCTATCCTGACG		
Npom36	Naut106_E08	F: [HEX]CCGAAGCTGAAAGAATTTGC	(CT)37	183
	HG918103	R: TGGCTATGCCCATATTAGCC		
Npom37	Naut106_E10	F: [6FAM]TCTAATGCCACCAAATGAAGTC	(GA)29	203
	HG918104	R: TGTTTGTTCTGCCATCATCC		
Npom38	Naut106_F07	F: [HEX]AGAAAGACCCGATACGCAAC	(GA)32	282
	HG918105	R: TCTGCTATTTCGCAGCGTAG		
Npom39	Naut116_A09	F: [HEX]CGTTCACTGTTGGCCATACTT	(GT)7	92
	HG918106	R: GCCGACAGGCCTCTACTCT		
Npom40	Naut116_B02	F: [HEX]GCGCAAAGTGAAAGCTGAC	(GT)8	141
	HG918107	R: CCCTGACCCTTTCACATACTTC		
Npom41	Naut116_C06	F: [HEX]AGCCCTTCAGCCTACATTCTG	(CA)9	227
	HG918108	R: ATAATCGGTCGTCGCATTTG		
Npom42	Naut116_E12	F: [HEX]CGACTAAATTGCTTCAGCAGAC	(GA)10	146
	HG918109	R: CTCCAGGTAGGCGCTCAG		
Npom43	Naut116_G08	F: [HEX]CTTGTAGGGCAAAGTTTGTGG	(GT)9	323
	HG918110	R: GATCATAGGCAACTCAACAGACAC		
Npom44	Naut116_H07	F: [HEX]GGATAATTGAAATTCTGGAGTTGG	(GT)10	190
	HG918111	R: AACCGTAAGTTATCCCGCAAC		

F forward; *R* reverse; *bp* base pairs. Primer melting temperatures 59–61 °C

Analyser using LIZ GS500 size standard (Applied Biosystems Inc.). Alleles were scored using GENEMAPPER v3.7 software. Thirty markers that could be scored reliably were tested in 24 individuals from the IR population (Table 1). Markers that were indistinct or did not amplify consistently were dropped at this stage (Table 2).

Approximately 60 % of individuals were re-extracted and re-genotyped across the selected loci. Alleles not matching between reruns were identified using Microsatellite Toolkit and scoring inaccuracies rectified. All individuals genotyped were confirmed as unrelated using SPAGEDI (Hardy and Vekemans 2002). Observed and expected heterozygosities were calculated using CERVUS v3.0.3. Tests for deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were calculated in GENEPOP v4.2 software. To correct *p* values in multiple tests, the False Discovery Rate (FDR) was applied to LD *p* values.

Twenty-one markers were in HWE. There was no evidence of large allele drop out (MicroChecker) and no loci were deficient in heterozygotes. Observed levels of heterozygosity ranged from 0.17 to 1.00 with a mean of 17.2 alleles per locus. No groups of loci displayed LD, suggesting that there is no evidence for physical linkage between loci based on the few individuals genotyped.

These loci were developed as part of project to investigate the vulnerability of the isolated populations of this overexploited ancient species.

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Appendix IIII

Williams *et al.* (2015b): The genetic structure of *Nautilus pompilius* populations surrounding Australia and the Philippines. Chapter Four published in MOLECULAR ECOLOGY

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The genetic structure of *Nautilus pompilius* populations surrounding Australia and the Philippines

RACHEL C. WILLIAMS,*† BENJAMIN C. JACKSON,‡[1] LUDOVIC DUVAUX,‡[1] DEBORAH A. DAWSON,† TERRY BURKE† and WILLIAM SINCLAIR*

Centre for Wildlife Conservation, University of Cumbria, Ambleside LA22 9BJ, UK, †Department of Animal and Plant Sciences, NERC Biomolecular Analysis Facility, University of Sheffield, Sheffield S10 2TN, UK, ‡Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, UK

Abstract

Understanding the distribution of genetic diversity in exploited species is fundamental to successful conservation. Genetic structure and the degree of gene flow among populations must be assessed to design appropriate strategies to prevent the loss of distinct populations. The cephalopod *Nautilus pompilius* is fished unsustainably in the Philippines for the ornamental shell trade and has limited legislative protection, despite the species' recent dramatic decline in the region. Here, we use 14 microsatellite markers to evaluate the population structure of *N. pompilius* around Australia and the Philippines. Despite their relative geographical proximity, Great Barrier Reef individuals are genetically isolated from Osprey Reef and Shark Reef in the Coral Sea (F_{ST} = 0.312, 0.229, respectively). Conversely, despite the larger geographical distances between the Philippines and west Australian reefs, samples display a small degree of genetic structure (F_{ST} = 0.015). Demographic scenarios modelled using approximate Bayesian computation analysis indicate that this limited divergence is not due to contemporary gene flow between the Philippines and west Australia. Instead, present-day genetic similarity can be explained by very limited genetic drift that has occurred due to large average effective population sizes that persisted at both locations following their separation. The lack of connectivity among populations suggests that immigrants from west Australia would not facilitate natural recolonization if Philippine populations were fished to extinction. These data help to rectify the paucity of information on the species' biology currently inhibiting their conservation classification. Understanding population structure can allow us to facilitate sustainable harvesting, thereby preserving the diversity of genetically distinct stocks.

Keywords: approximate Bayesian computation, cephalopod, marine conservation, microsatellite, population structure

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Introduction

Throughout the world, many commercial marine species are experiencing significant population declines (Hutchings 2000; Worm *et al.* 2006; Neubauer *et al.* 2013; Watson *et al.* 2013). The harvesting of marine animals began 42 000 years ago (O'Connor *et al.* 2011), up to 50 000 years after the anthropogenically triggered extinctions of large mammals seen on land (Koch & Barnosky 2006). Human access to the marine environment is no longer technologically inhibited, and defaunation of the oceans is now occurring (McCauley *et al.* 2015). Present extinction rates are probably a thousand times higher than extinction rates in the absence of human actions (Pimm *et al.* 2014). Identifying species with a high risk of extinction (Davidson *et al.* 2012) can facilitate policy changes and prevent declines. An international agreement between governments that was

Correspondence: Rachel C. Williams,
E-mail: Williams.RachelC@gmail.com
[1]These authors contributed equally.

developed to regulate declines is the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). CITES aims to ensure that the survival of species is not threatened by international trade, and the convention is reliant on biological data to construct a specific framework for participating countries to implement (CITES 2015). Unfortunately, the absence of biological information and global industrial data can prevent qualification for CITES protection (De Angelis 2012).

Population connectivity data can be used by CITES to design effective conservation strategies for marine resources. Contemporary movement and migration among marine populations have been monitored using tracking devices, but success has been variable (see Semmens *et al.* 2007). Movement among populations can be estimated using population genetic analyses (Pearse & Crandall 2004). Although specific individuals cannot always be tracked, molecular techniques do allow the detection of gene flow among populations through patterns of shared genetic variation (Levin 2006; Cowen & Sponaugle 2009). For vulnerable species, knowledge of the genetic structure of populations can be used to inform sustainable harvesting practices, prevent local extinctions and preserve the diversity of genetically distinct stocks (Carvalho & Hauser 1994). Oceans are considered to present few physical barriers to gene flow so that widely separated areas can remain connected, making absolute vicariance rare in a marine environment (Palumbi 1994; Mirams *et al.* 2011). Population structure and speciation have, however, been attributed to oceanic features, such as salinity (Lessios *et al.* 2003; Rocha 2003), depth and temperature (Zardi *et al.* 2007). Ocean fronts (Galarza *et al.* 2009) and currents (White *et al.* 2010) have also been shown to represent major barriers to gene flow, suggesting that marine environments may contain more barriers to dispersal than is generally appreciated.

Oceanic features with the potential to act as a barrier to movement can result in genetic structure that ranges from panmixia (Lessios *et al.* 2003) to complete separation (Baums *et al.* 2012). The inference of demographic history over long timescales can answer questions regarding long-term gene flow between, and distribution of genetic diversity among, populations (Semmens *et al.* 2007). This ability to infer a species' movements can be used in various areas of conservation, for example, to evaluate whether marine protected areas are appropriate (i.e. to assess that species movement does not extend beyond protected boundaries; Grüss *et al.* 2011), to trace the origins of a catch (Hobson 1999), or to ensure that illegal catches are not being missold to the consumer (Griffiths *et al.* 2013).

Despite the technology and conservation strategies available, population declines continue in most species (Neubauer *et al.* 2013). The poor success of attempts to aid the recovery of commercial marine species (Hutchings 2000) suggests that anthropogenic pressure is too high and that there are gaps in scientific knowledge that must be resolved to enable effective conservation (Sale *et al.* 2005). A key example of a species currently lacking biological and population connectivity data is the shelled cephalopod, *Nautilus pompilius.* Nautiloids (*Nautilus* spp. Linnaeus and *Allonautilus* spp. Ward and Saunders) are heavily overfished for the ornamental shell trade. The long-term effects of fishing on populations, or their ability to recover, are unknown (De Angelis 2012). An 80% decline in catch per unit effort for *N. pompilius* during 1980–2010 has been reported from Philippine fisheries (Dunstan *et al.* 2010). The current deficiency of data on the species' biology has inhibited the development of appropriate legislative mechanisms to prevent the species' overexploitation and decline. Understanding migration and its effect on population dynamics is important when deciding on appropriate protection measures for populations through *in situ* means, such as marine protected areas (Grüss *et al.* 2011).

Cephalopod migration, however, is poorly understood, and this is exacerbated by the difficulties associated with tracking them (Stark *et al.* 2005; Semmens *et al.* 2007). In coleoids (octopus, cuttlefish, squid), juveniles are often too small to be tagged, tag placement in adults can be difficult, and capture rates fluctuate and are often reliant on fishermen reporting catches (Sauer *et al.* 2000). Improvements in technology, with concomitant decreases in device size and cost, will aid progress in this area.

Historical movement of *N. pompilius* populations has been investigated using molecular approaches; hypothesized differentiation of *N. pompilius* populations was confirmed by comparing variation at *cytochrome c oxidase subunit I* (*COI*). Populations separated into three geographically distinct monophyletic clades, which comprise a west Australian/Indonesian clade, an east Australian/Papua New Guinean clade and a west Pacific clade (Wray *et al.* 1995; Sinclair *et al.* 2007, 2011; Bonacum *et al.* 2011; Williams *et al.* 2012). The observed population structure was proposed to be the result of dispersal from an ancestral population in the Philippines (Wray *et al.* 1995). Historical expansions of nautiloid distribution will have been restricted by at least three biogeographical barriers (Crick 1993): water depth, distance between adjacent shelf seas and sea temperature. These constraints are also relevant to modern *Nautilus* and *Allonautilus,* whose movements are limited to some extent by their morphology. Nautiloids

are typically found between the depths of 130 and 700 m (Dunstan *et al.* 2011), remaining close to the reef for protection from predators. The internal arrangement of the *Nautilus* shell means that their deepest position in the water column is limited by their risk of shell implosion. As a consequence, the maximum depth at which an individual would be encountered is considered to be approximately 800 m (Saunders & Wehman 1977; Kanie *et al.* 1980). These limitations create a dispersive barrier and, despite small geographical distances, genetic differentiation has occurred (Sinclair *et al.* 2007), furthered by the absence of a juvenile larval stage that might aid dispersal (Saunders & Landman 2010). Understanding gene flow between current populations can help to inform sustainable fishing and aid the design of specific genetic management, and yet nothing has been known about the current connectivity of *N. pompilius* populations. Understanding population connectivity will help to establish the impact that fishing is having across their distribution following *N. pompilius* population declines in the Philippines (Barord *et al.* 2014).

Here, we use molecular techniques and statistical analyses to assess the connectivity of *N. pompilius* populations surrounding Australia and the Philippines. These data will contribute towards rectifying the information deficiency that currently inhibits the legal classification of *N. pompilius* as an endangered species, and our findings can be used to assess the species' qualification for CITES listing. Approximate Bayesian computation (ABC) analysis has enabled a powerful assessment of the species' genetic diversity and made it apparent that, despite the persistence of high levels of variability that are usually associated with large populations, there is limited gene flow into shrinking contemporary populations. This implies that overfishing currently threatens some populations with extinction.

Materials and methods

Sample collection

Australian samples were collected from seven reefs in the Indo-Pacific Ocean (Fig. 1) under an Australian Fisheries Management Authority Scientific Permit (number 1002548). West Australian (WA) samples were taken from four reefs: Clerke Reef, Imperieuse Reef, Ashmore Reef and Scott Reef. East Australian (EA) samples consisted of Osprey Reef and Shark Reef in the Coral Sea, and the far north Great Barrier Reef. Samples from the Philippines (PH) were collected from three locations: Tinitian, Roxas and Palawan. Collections were made under a Gratuitous Permit from the Department of Agriculture in the Republic of the Philippines.

N. pompilius was caught using traps positioned on the reefs at a depth of ~200 m (Sinclair *et al.* 2011) baited with ~1 kg of uncooked chicken (*Gallus gallus*), set at dusk and collected at dawn. Tissue collection was non-lethal: a 1- to 2-cm-long labial tentacle sample was collected before each individual was released. Tentacles were immediately placed into a 20% DMSO (dimethylsulphoxide), 100 mM EDTA pH 8, saturated $NaCl_2$ solution and stored at 4 °C in the field. Samples were later washed in TE buffer (1 M Tris-HCl pH 7.5; 0.5 M EDTA pH 8.0; Sambrook *et al.* 1989) and placed into 1 mL absolute ethanol in the laboratory for storage at room temperature (Sinclair *et al.* 2011).

Microsatellite genotyping and validity

Genomic DNA was extracted using Qiagen DNeasy tissue kits (QIAGEN Ltd, Manchester, UK). DNA concentration was quantified using a fluorometer (Fluostar Optima) and its quality assessed with electrophoresis on a 1% agarose gel. Fourteen polymorphic *N. pompilius* microsatellite loci (Williams *et al.* 2015) were selected based on the satisfactory results from quality checks and used to genotype all 215 individuals sampled. PCR amplification was performed in 2 μL PCRs, including 10 ng air-dried DNA, 0.2 μM reverse primer, 0.2 μM forward fluorescent primer (6FAM, HEX, VIC or PET labelled) and 1 μL Qiagen Multiplex Master mix. Multiplexes were amplified under the following profile: 95 °C for 15 min, followed by 44 cycles of 94 °C for 30 s, 56 °C for 90 s, 72 °C for 90 s and finally 72 °C for 10 min. PCR products were analysed on an ABI 3730 48-well capillary DNA analyser (Applied Biosystems Inc.) using LIZ GS500 size standard (Applied Biosystems Inc.). Allele sizes were assigned using the GENEMAPPER v3.7 software (Applied Biosystems Inc.).

Relatedness between individuals was estimated with SPAGEDI (Hardy & Vekemans 2002) using Queller & Goodnight's (1989) measure of relatedness. Relatives were removed, and departure from Hardy–Weinberg equilibrium (HWE; $P < 0.05$) and linkage disequilibrium (LD) was calculated using GENEPOP (Raymond & Rousset 1995; Rousset 2008). LD was assessed using 1000 iterations per population and *P*-values corrected using the false discovery rate adjustment (FDR; Verhoeven *et al.* 2005). Corrections were made on a population-by-population basis to avoid overinflating the number of tests in which the correction was required. Each microsatellite locus was assessed to estimate the frequency of null alleles and identify scoring errors due to stutter using MICRO-CHECKER (Van Oosterhout *et al.* 2004). Null allele frequency per locus was estimated using CERVUS v3.0 (Kalinowski 2005). To assess genotyping error rate, 60% of samples were re-extracted and regenotyped across all

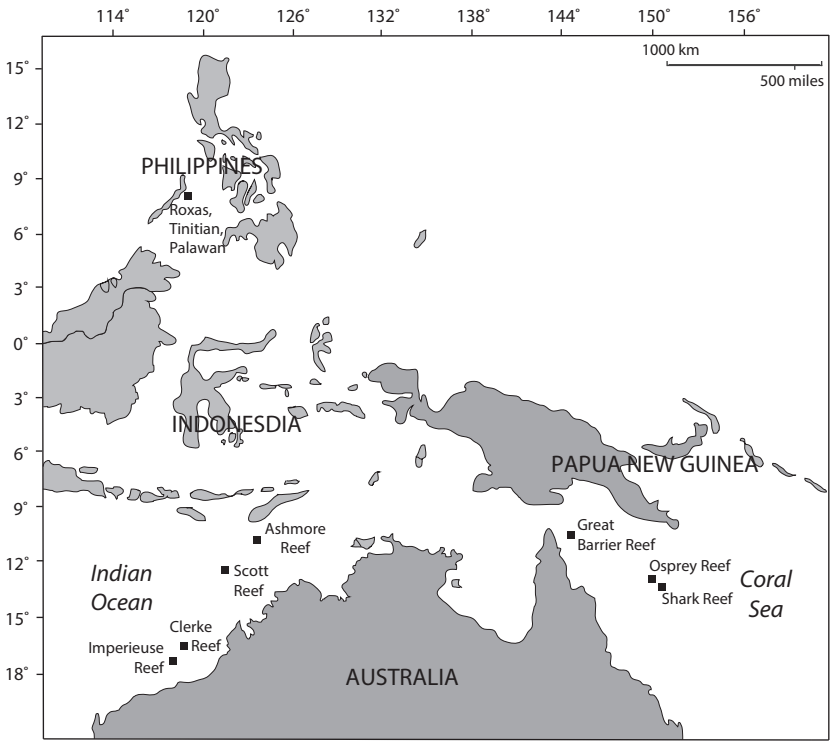

Fig. 1 *Nautilus pompilius* distribution; locations sampled are indicated. A total of 215 *N. pompilius* were sampled from eight collection sites (range 8–45 individuals per location).

loci. Error rates per reaction were calculated according to Hoffman & Amos (2005).

Population structure

Three Bayesian clustering methods were used to determine the most likely number of genetic clusters within the data set: STRUCTURE (Pritchard *et al.* 2000), TESS (Durand *et al.* 2009) and GENELAND (Guillot *et al.* 2005). The software STRUCTURE was run with an admixture model and no prior information on the sampling locations (Table S1, Supporting information). To avoid the influence of kinship on inferred structure, all individuals within a population with a relatedness of 0.5 were removed from the data set before analysis (Queller & Goodnight 1989). Plotting the natural logarithm of the posterior probability (P_P) of K given the data over multiple runs determined the predicted number of clusters (Fig. S2, Supporting information), and this was compared with ΔK (Evanno *et al.* 2005) as determined in STRUCTURE HARVESTER v.0.6.93 (Earl & VonHoldt 2011). Independent runs for all data sets were averaged in CLUMPP v.1.1.2 (Jakobsson & Rosenberg 2007) using the *Greedy* algorithm with 10 000 repeats to develop a consensus value for K. Graphical representation was produced in DISTRUCT v.1.1 (Rosenberg 2003). Bayesian clustering of TESS was run without admixture, and K was inferred from the modal value of the replicate with the highest likelihood. A correlated allele frequency model was used in GENELAND and the burn-in length was based on the appearance of the posterior density log, as suggested by the software manual. The number of proposed clusters was selected from the highest mean log P_P (Guillot *et al.* 2009).

MICROSATELLITE ANALYSER (Dieringer & Schlötterer 2003) was used to calculate pairwise F_{ST} values (Weir & Cockerham 1984) between sampling locations, with Bonferroni corrections applied. To test for an association between F_{ST} and geographical distance, Mantel's test for isolation by distance (IBD) was performed in SPAGEDI with 10 000 randomizations. A regression of the spatial distance against $F_{ST}/(1\text{-}F_{ST})$ was performed (Rousset 1997). Jost's differentiation index (D_{est}) values (Jost 2008) across loci were calculated using DEMETICS (Gerlach *et al.* 2010).

Approximate Bayesian computation methods

To test alternative hypotheses that could explain the genetic similarity between PH and WA samples (see Results), we conducted an ABC analysis (Beaumont *et al.* 2002). ABC aims to obtain the joint posterior distribution of complex models for which the likelihood function can be difficult or impossible to solve analytically, allowing a great flexibility in the scenarios being investigated (Marjoram & Tavaré 2006). Its rationale is to bypass the need of an exact likelihood function by comparing summary statistics from observed data to

the summary statistics obtained by simulating the models of interest (Beaumont 2010; Csilléry *et al.* 2010).

We compared three evolutionary scenarios (Fig. 2). Model IWOM assumes that an ancestral population of size N_A split t generations ago into two daughter populations, PH and WA, of effective sizes N_1 and N_2, respectively (Fig. 2b). Model IM (Nielsen & Wakeley 2001) is equivalent to model IWOM with the adjustment that populations PH and WA have constantly exchanged migrants since their split at rates m_{12} and m_{21}, respectively (Fig. 2a). A null model was also tested as model_{PAN}; this assumes that PH and WA are part of the same panmictic population of effective size N_P (Fig. 2c). Note that to compute summary statistics comparable to the observed data (i.e. from two different populations), we set model_{PAN} using model_{IWOM} parameters but fixed the divergence time to 1 generation—effectively modelling the samples as a panmictic population throughout their whole history.

The prior distributions were uniform for all demographic parameters, and the same range was used for common parameters between models (Table 1). For all demographic models, we assumed that microsatellites evolved under a stepwise-mutation model. Mean mutation rates across loci were extracted from a normal prior distribution, and single-locus mutation rates were drawn from a gamma distribution as parameterized in ABC TOOLBOX (Wegmann *et al.* 2010), using uniform priors for the two parameters of the distribution. To avoid effects of substructure within the WA clade, and due to similar sample size, Ashmore Reef was chosen to represent the WA clade.

Summary statistics and simulations

ABC analyses were conducted using the package ABC TOOLBOX (Wegmann *et al.* 2010). One limitation of ABC is that models can appear more or less likely dependent on the range of the parameter values and the weight assigned to them by the priors (Sousa *et al.* 2012). Exploratory simulations were therefore performed with varied sets of priors to allow an assessment of their effect on the posterior distribution and ensure that the whole posterior was contained within the final prior range.

We used FASTSIMCOAL (Excoffier & Foll 2011; Excoffier *et al.* 2013) to run one million coalescent simulations of our data set of 14 microsatellites under each model. ARLSUMSTAT (Excoffier & Lischer 2010) was used to calculate a set of 30 summary statistics (within and between populations; Table S2, Supporting information), chosen based on those shown to be informative in previous studies (Palero *et al.* 2009; Sousa *et al.* 2012; Butlin *et al.* 2013). To reduce the high dimensionality of the summary statistics, we used a partial least-squares (PLS) transformation (Wegmann *et al.* 2009) to extract their orthogonal components. PLS identifies components to explain variability of response variables (model parameters) by maximizing the covariance matrix of predictor (raw summary statistics) and response variables (Wegmann *et al.* 2009).

Model choice and parameter estimation

For model comparison, marginal densities comparable between models were produced using the PLS-transformed summary statistics for the rejection step, while all raw summary statistics were used to perform the postsampling adjustment step using the ABC-GLM (general linear model) in ABC TOOLBOX (Wegmann *et al.* 2009). We retained the 5% of simulations closest to the observed data. We checked that our observed summary statistics (for both PLS components and raw summary statistics) fell within the distribution of summary statistics from the simulations retained. Bayes factors and P_P were derived from the model choice procedure (Table S3, Supporting information). *P*-values were taken as an indication of each model's ability to explain the data.

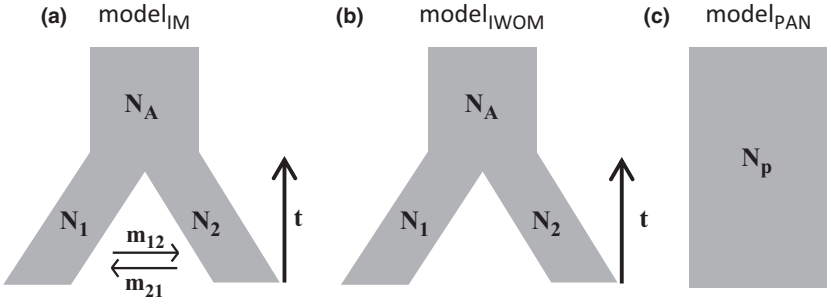

Fig. 2 Schematic representation of the three demographic models compared using ABC under a coalescent framework. (a) Isolation with migration model, model_{IM}, (Nielsen & Wakeley 2001); N_A, effective size of the ancestral population; N_1, N_2, effective size of current populations (*1* = Philippines, *2* = Ashmore Reef); t, time since the population split; m_{12}, m_{21}, migration occurring in both directions; (b) isolation without migration, model_{IWOM}, where divergence resulted in current populations that have no gene exchange; (c) A panmictic population, model_{PAN}, between both sampled sites; N_P, current panmictic population.

Table 1 Prior and posterior distributions of model parameters for $model_{IWOM}$ using ABC TOOLBOX

Parameters	Prior range	Mean	Median	Mode	HPD95 low	HPD95 high
ARG_K	Uniform [0–6]	1.79	1.63	1.36	0.11	3.73
MUT_U	Uniform [10^{-6}–5×10^{-4}]	5×10^{-5}	4.11×10^{-5}	2.61×10^{-5}	-2.53×10^{-7}	1.22×10^{-4}
N_A	Uniform [0–5×10^6]	2.17×10^6	2.04×10^6	1.31×10^6	6.28×10^4	4.51×10^6
N_1	Uniform [0–5×10^6]	3.08×10^6	3.19×10^6	3.79×10^6	1.05×10^6	4.99×10^6
N_2	Uniform [0–5×10^6]	2.61×10^6	2.56×10^6	2.09×10^6	6.05×10^5	4.77×10^6
t	Uniform [0–10^6]	3.53×10^5	296×10^5	1.05×10^5	-2.51×10^3	8.39×10^5

ARG_K, shape parameter for the gamma distribution; MUT_U, mean mutation rate; N, effective population sizes (1 = Philippines, 2 = Ashmore Reef, A = ancestral); t, time since the population split in generations.

To validate our model choice procedure, we simulated 1000 pseudo-observed data sets for each model using the original priors. The original results files (of one million simulated data sets for each model) were used to perform our model choice procedure using each of the 1000 pseudo-observed data sets in turn. To test the robustness of discrimination between models using our model choice procedure, each pairwise comparison of simulated and observed data for the two models was performed. A model's original data were compared with the pseudo-data of the same model and those of the model being compared. Posterior probabilities were compared with a logistic regression. Confidence in model choice was calculated by estimating the FDR (Verhoeven *et al.* 2005): the frequency of the P_P being equal to or larger than the real P_P of the best model.

For parameter estimation, the distance step and postsampling adjustment were both carried out using PLS components. This was performed independently for each model because different PLS components are extracted for each model. The GLM method implemented in ABC TOOLBOX (Leuenberger & Wegmann 2010) was used for postsampling adjustment. Parameter estimation was verified by ensuring that *P*-values were reasonably large (>0.05 as suggested in the ABC TOOLBOX manual) and checking that posterior distributions were within the prior ranges (Fig. S3, Supporting information). The pseudo-observed data were also used to check for uniformity of the posterior quantiles; a departure from uniformity suggests a parameter is over- or underestimated.

Results

Genotyping validation

A genotyping error rate of zero was determined between replicates. No evidence was found for frequent allelic dropout across any loci in any sampling locations. A shortage of heterozygous genotypes was found for locus *Npom08* in the Osprey Reef and Scott Reef populations, possibly resulting from scoring errors due to stutter. This locus was retained in analyses due to its quality in other reef populations. The estimated frequency of null alleles was low for all loci (≤0.05). Departure from HWE was not detected consistently across all sampling locations at any loci. No pairs of loci consistently showed LD in all populations, suggesting that no loci were physically linked. F_{ST} values ranged from −0.04 to 0.35 and D_{est} ranged from −0.04 to 0.75 (Table 2). IBD analysis revealed no overall association between F_{ST} and geographical distance and was not significant ($r^2 = 0.139$; $P = 0.095$).

Population structure

Shark Reef was grouped with Osprey Reef for analyses based on their close geographical location and high degree of relatedness (Table 2); they will hereafter be referred to as Osprey Reef. Results incorporating spatial data in TESS (Fig. 3a) and GENELAND (Fig. S1, Supporting information) returned three and five genetic clusters, respectively. TESS returned the first cluster, including PH and all WA reefs, the second cluster of the Great Barrier Reef and the third cluster of Osprey Reef. GENELAND divided the samples into five genetic clusters of (i) PH, (ii) Ashmore, Imperieuse and Clerke Reefs, (iii) Scott Reef, (iv) Great Barrier Reef and (v) Osprey Reef.

Plots of ΔK and LnP(K) generated from STRUCTURE results indicated four as the most likely number of genetic clusters present in the full data set (Fig. 3). The first two genetic clusters consisted of populations PH and WA, the third cluster consisted of EA Great Barrier Reef and cluster four consisted of Coral Sea's Osprey Reef (Fig. 3b). Subsetting the data to look for further division within clusters returned validating results (Fig. 3c,d).

Table 2 Estimation of population differentiation among eight sites for *Nautilus pompilius*

Population	Philippines	Ashmore Reef	Scott Reef	Clerke Reef	Imp Reef	GB Reef	Osprey Reef	Shark Reef
Philippines (N = 27)	—	0.015*	0.044*	0.014*	0.024*	0.130*	0.330*	0.237*
Ashmore Reef (N = 29)	0.136*	—	0.018*	−0.004	0.006	0.121*	0.319*	0.228*
Scott Reef (N = 30)	0.263*	0.097*	—	0.015*	0.015*	0.173*	0.354*	0.268*
Clerke Reef (N = 32)	0.114*	−0.028	0.084*	—	0.004	0.124*	0.322*	0.234*
Imp Reef (N = 31)	0.158*	0.044	0.111*	−0.042	—	0.144*	0.343*	0.255*
GB Reef (N = 13)	0.527*	0.485*	0.586*	0.494*	0.528*	—	0.312*	0.229*
Osprey Reef (N = 45)	0.693*	0.696*	0.740*	0.703*	0.745*	0.5*	—	0.012
Shark Reef (N = 8)	0.651*	0.612*	0.736*	0.678*	0.731*	0.483*	−0.014	—

Pairwise F_{ST} (Weir & Cockerham 1984) shown above the diagonal, D_{est} (Jost 2008) shown below the diagonal. Imp Reef, Imperieuse Reef; GB Reef, Great Barrier Reef. Values significant at $P < 0.05$ after Bonferroni correction indicated (*).

Approximate Bayesian computation analysis

The model comparison gave strong support to $model_{IWOM}$ (P_p = 1.0, Table S3, Supporting information) and the FDR was low (0.2%), indicating with high confidence that the data were not the result of sustained migration between the Philippines and WA (see also Fig. S4, Supporting information). Moreover, this model fitted the data well (the observed summary statistics lay within the range of both the untransformed and PLS-transformed postrejection simulated summary statistics), indicating that its best score was not a result of a bad fit by all models to the data. Parameter estimation under $model_{IWOM}$ enabled the estimation of the ancestral effective population size (median: 2 035 120; highest posterior density (HPD95 low, high): 62 816.9, 4 508 320), which was smaller than the estimated current population sizes of PH (median: 3 080 000) and WA (median: 2 610 000). The distribution of posterior quantiles did not show strong departures from uniformity, which is indicative of a lack of bias in parameter estimation (Wegmann *et al.* 2009).

Discussion

We detected population structure among east Australian sampling sites, indicating genetic isolation of Osprey Reef and Shark Reef from the Great Barrier Reef. West

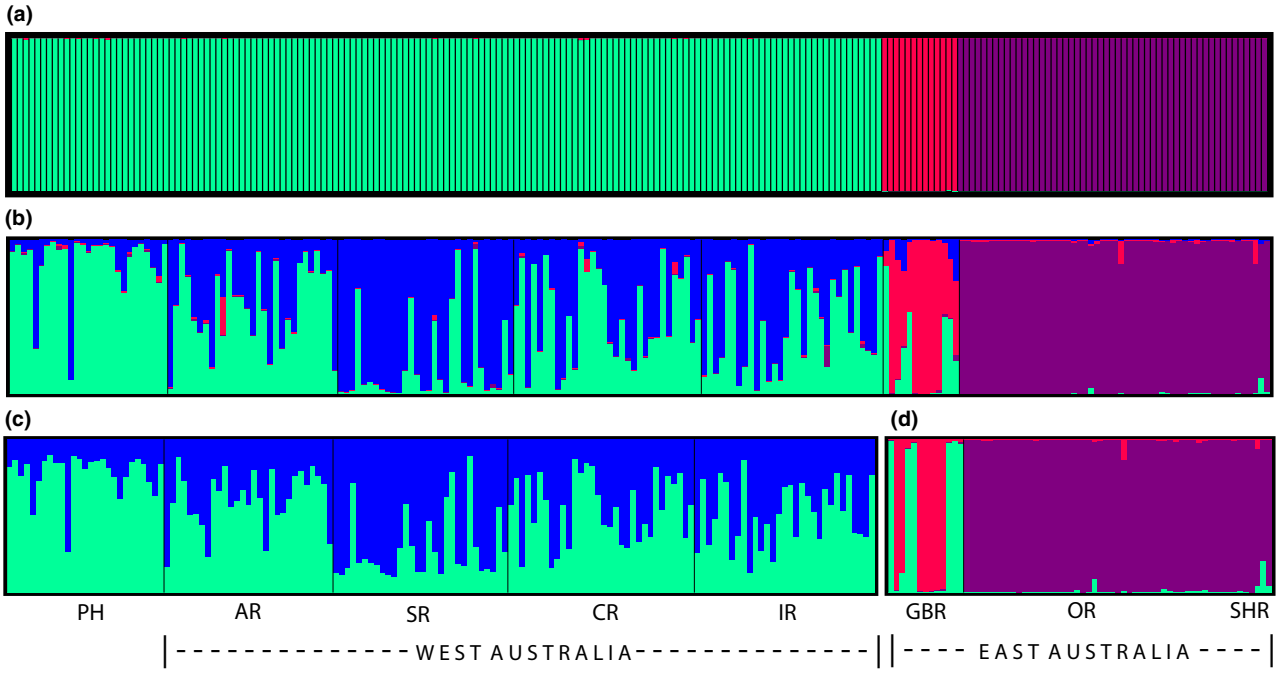

Fig. 3 Bayesian assignment probabilities (*y-axis*) of individual *Nautilus pompilius* (n = 215, *x-axis*). Inferred populations indicated: PH, Philippines; AR, Ashmore Reef; SR, Scott Reef; CR, Clerke Reef; IR, Imperieuse Reef; GBR, Great Barrier Reef; OR, Osprey Reef; and SHR, Shark Reef. Colours represent the probability proportion in each cluster in (a) K = 3 clusters indicated in TESS, (b) K = 4 clusters indicated in STRUCTURE, (c) Philippines and west Australia in STRUCTURE and (d) east Australia in STRUCTURE.

Australian samples revealed limited population structure but with significant pairwise F_{ST} and D_{est} between Scott Reef and the other west Australian reefs. The genetic similarity found between the Philippines and west Australia was unexpected. Further investigation modelling different demographic scenarios revealed that this similarity was not the result of migration, but may be attributable to ancestral population sizes that were until recently large (population declines have been shown in areas under fishing pressure; Barord *et al.* 2014), and consequently exhibiting limited genetic drift.

Mechanisms for population structure

Results from software STRUCTURE, TESS and GENELAND showed Osprey Reef and Shark Reef populations in the Coral Sea to be genetically distinct from the Great Barrier Reef, west Australia and Philippine populations. Ocean physiography (the physical geography of the ocean floor) appears to have been influential in this differentiation. Ocean depths in the Coral Sea between Osprey Reef and the Great Barrier Reef exceed 1700 m (Dunstan *et al.* 2011), and while movement through open water is feasible, it leaves individuals vulnerable to predation (Yomogida & Wani 2013). The response of *N. pompilius* individuals to attacks by teleosts showed that they retreated into their shells and demonstrated no defence or escape response (Saunders & Landman 2010). The Great Barrier Reef was shown to be distinct, not only from Osprey Reef but also from the western populations, which supports the conclusions of previous evolutionary studies using partial *COI* sequences (Sinclair *et al.* 2007, 2011; Bonacum *et al.* 2011; Williams *et al.* 2012).

West Australian results were not consistent across software; STRUCTURE and TESS assign the Philippines and west Australia to the same genetic population, whereas GENELAND designates the Philippines and Scott Reef as separate genetic clusters. Geographically, Scott Reef is located between Ashmore and Clerke Reefs, and the differentiation is seen between Scott Reef and the surrounding west Australian reefs, despite shallower surrounding sea depths. F_{ST} measures deviation from panmixia, D_{est} measures deviations from total differentiation (Whitlock 2011), and both F_{ST} and D_{est} values distinguish Scott Reef as a separate genetic cluster.

Due to their residing depth, data on currents at the surface cannot explain *N. pompilius* dispersal patterns (Biuw *et al.* 2007). Currents have been shown to impact individual positions on a reef (O'Dor *et al.* 1993), with recorded movements of up to 6 km that may have been facilitated by currents (Dunstan *et al.* 2011). However, *N. pompilius* has also demonstrated strong resistance to currents and an ability to utilize them to obtain food (O'Dor *et al.* 1990). The overall impact of currents on the species' population distribution is poorly documented.

No significant correlation was found between F_{ST} and linear geographical distance (Rousset 1997). IBD has been shown in other cephalopods (Pérez-Losada *et al.* 2002; Kassahn *et al.* 2003; Cabranes *et al.* 2008) but, like *Nautilus,* the octopus *Octopus vulgaris* (Moreira *et al.* 2011), the cuttlefish *Sepia esculenta* (Zheng *et al.* 2009) and the squid *Loligo pealeii* (Buresch *et al.* 2006) have all demonstrated genetic distances disproportionate to geographical distances. It has been hypothesized that this was in each case due to natal philopatry (Kassahn *et al.* 2003; Buresch *et al.* 2006). It has been speculated that this behaviour does not occur in nautiloids (Crook & Basil 2013), but gaps remain in our knowledge of *N. pompilius* ecology. Despite lacking the lensed eye and vertebrate-like brain of other cephalopods (including dedicated lobes for learning and memory), *N. pompilius* has been shown to be capable of both spatial learning and navigational strategy (Crook *et al.* 2009; Crook & Basil 2013). Migration on a small scale is not completely unfeasible, but seems unlikely at the scale investigated in our study.

Divergence without migration

Our ABC model does not support the possibility of sustained migration between the Philippines and west Australia as an explanation for the genetic admixture shown in the structural analyses. Movement of individuals between the two sites was unknown, but depth limitations of *N. pompilius* are indicative of isolation over such a geographical distance; it is possible that the genetic similarity observed has resulted from incomplete lineage sorting. Marker data can be misleading about relationships among populations due to the retention and stochastic sorting of ancestral polymorphisms. This is especially likely if the effective population size is large relative to lineage length (the time since the populations split; Maddison & Knowles 2006). Alleles can then persist in both populations due to limited genetic drift. Model$_{IWOM}$ indicated extremely large ancestral and current effective population sizes, resulting in a lower probability of alleles becoming fixed before divergence (Pamilo & Nei 1988). Current population size estimates for the Philippines (median = 3 190 920) and Ashmore Reef (median = 2 562 800) suggest that genetic drift has yet to have a significant impact. Such large current population estimates are potentially due, in part, to substructure within the sampled areas. Sampling from the Philippines was conducted in several locations, the connectivity between which was assumed but not confirmed. West Australian samples were from the most northern of these reefs, Ashmore Reef; gene flow was previously

established between the sampled west Australian reefs (Williams *et al.* 2012). This local gene flow may have inflated the population estimate for Ashmore Reef, reflecting the population size of the NW shelf populations.

Population density estimates made using baited remote underwater video systems were calculated as 13.6 and 0.03 individuals per km^2 for Osprey Reef and Bohol Sea (Philippines), respectively (Barord *et al.* 2014). Using different methods to generate these results produced predictably dissimilar abundance data. Additionally, Barord *et al.* (2014) document evidence of a sudden population size reduction, but our data showed that allelic richness in the Philippines was no lower than at other locations sampled (Fig. 4). It is possible that the genetic consequences of population reduction have yet to take effect; low fecundity and long developmental time (Carlson *et al.* 1984; Landman & Cochran 2010) of nautiloids results in a long generation time compared to other cephalopods. Fishing for shells is relatively new, with no cultural or historical significance in studied areas such as Palawan (Dunstan *et al.* 2010), and so it is possible that we will see a genetic response to exploitation that is not yet detectable with our current set of markers.

The allelic richness in Philippine samples also supports the proposed direction of colonization from the progenitor population located in the Philippines (Wray *et al.* 1995). There is lower allelic richness in the Great Barrier Reef, with a further decline in the more genetically distinct Osprey and Shark Reefs of the Coral Sea (Fig. 4). The time split estimation [median = 296 495 generations/1 660 366 years based on a generation time of 5.6 years (Saunders & Landman 2010)] by $model_{IWOM}$ indicates that current populations have been evolving independently for a similar time as the accepted species of modern *N. pompilius* (Kröger *et al.* 2011).

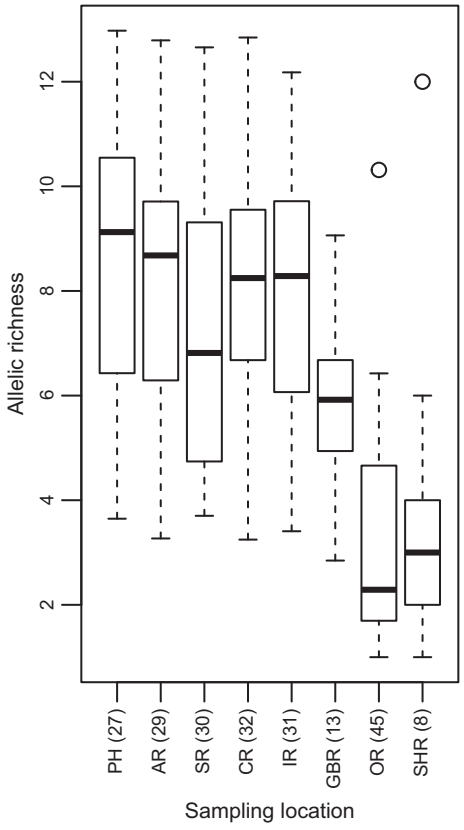

Fig. 4 Allelic richness across 14 loci for each sampling location. PH, Philippines; AR, Ashmore Reef; SR, Scott Reef; CR, Clerke Reef; IR, Imperieuse Reef; GBR, Great Barrier Reef; OR, Osprey Reef; and SHR, Shark Reef. Sample size indicated.

Management implications

The absence of migration between the Philippines and west Australia highlights the need for mechanisms to protect these populations as discrete conservation units (Moritz 1994). Environmental differences between sites have not been measured and so it is unknown whether resulting divergent selection has occurred, but as a unique population with the possibility of local adaptation, adequate protection for the Philippines is imperative to the long-term survival of this genetic cluster. The absence of contemporary migration indicates that it is unlikely that the Philippines would be repopulated, should fishing in this area continue to extinction. Informative results must now reach policymakers to enable legislative protection.

The variation seen in IBD within cephalopods demonstrates the need for species-specific range studies, especially when results are extrapolated for fisheries management. As fin fish stocks decline and the fishing industry targets novel resources, it is likely that cephalopod stocks will experience increased fishing pressure (Dillane *et al.* 2005). The data supporting the need for *Nautilus* and *Allonautilus* protection are ever increasing (Dunstan *et al.* 2010; Bonacum *et al.* 2011; De Angelis 2012; Williams *et al.* 2012; Barord *et al.* 2014). Overexploitation is threatening marine species worldwide (Hutchings 2000; Worm *et al.* 2006; Doukakis *et al.* 2009; Neubauer *et al.* 2013; Watson *et al.* 2013), and our study highlights the need for multiple or finer-scale markers to determine the connectivity patterns and establish adequate protection. For example, mitochondrial DNA data on the west Australian reefs (Williams *et al.* 2012) suggested that the population was panmictic, but the higher-resolution data presented here reveal substructure. Our results show how management plans should incorporate discrete management units and should account for more than separation by geographical distance.

Conclusions

A range of molecular studies has been conducted on coleoids (Allcock *et al.* 2015), including population structure analysis using minisatellites, microsatellites and mitochondrial DNA (Dillane *et al.* 2005; Zheng *et al.* 2009; Moreira *et al.* 2011), but this is the first study to use microsatellite markers in a nautiloid. We had hypothesized genetic division between east and west Australia based on previous evolutionary studies on these populations (Sinclair *et al.* 2007, 2011; Bonacum *et al.* 2011; Williams *et al.* 2012), but we found a greater degree of genetic similarity between samples from the Philippines and west Australia than had been previously considered. Our conclusion that this similarity was not the result of migration emphasizes the need to reduce overexploitation and prevent the local extinction of *N. pompilius* in the Philippines. Protection for *Nautilus* and *Allonautilus* under CITES would decrease the incentive for continued exploitation. In relatively inaccessible species, genetic data can provide an insight into migration and population dynamics. Such genetic studies should be utilized to develop efficient species-specific management plans for declining populations. Enforcing these, in collaboration with legislative protection, is imperative for the conservation of marine populations (Neubauer *et al.* 2013).

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R.C.W. and W.S. obtained samples. R.C.W. performed the genotyping. R.C.W. and D.A.D. assessed the marker quality. R.C.W., B.C.J. and L.D. analysed and interpreted the data. W.S., D.A.D., T.B. oversaw the project. R.C.W. drafted the manuscript, and all authors contributed edits and comments.

Data accessibility

Microsatellite primer sequences for *N. pompilius* are available through NCBI: Accession no. HG918068-HG918111. Microsatellite genotyping data and the geographical information for the Mantel's test are available in Dryad, doi:10.5061/dryad.j251f.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Settings for structural analyses run in STRUCTURE, TESS and GENELAND

Table S2 Summary statistics of the chosen model, $model_{IWOM}$, used in the ABC analyses

Table S3 Posterior probabilities for $model_{IWOM}$, $model_{IM}$ and $model_{PAN}$. Bayes factor calculated for both pairwise comparisons

Fig. S1 Probabilities of population membership generated in GENELAND.

Fig. S2 Predicted number of genetic clusters. (a) log-likelihood, (b) delta K, as indicated in STRUCTURE for all 215 *Nautilus pompilius* samples.

Fig. S3 Posterior distributions (red) obtained under the chosen model ($model_{IWOM}$), marginal parameter distribution among the retained simulations (blue), prior distribution estimated from the first 50 000 simulations (black).

Fig. S4 Logistic regressions for model validation