Molecular phylogenetics and the evolutionary history of reproductive strategies in benthic shallow-water octopuses (Cephalopoda: Octopodinae)

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ABSTRACT

The adaptive nature of egg size and juvenile types is of fundamental interest to the life history theory of benthic marine invertebrates. One tenet of life history theory for these organisms predicts that the evolution and maintenance of dichotomous reproductive strategies is a fecundity-survival trade-off and environmental factors strongly influence the evolutionary history of these strategies. In this thesis I aimed to examine the evolutionary relationships among the benthic shallow-water octopuses (subfamily Octopodinae) using a molecular phylogenetic approach. The best phylogenetic hypothesis was then used in a comparative phylogenetic analysis to examine the evolutionary history of reproductive strategies. I was interested in examining whether evolutionary transitions in egg size have been influenced by macro-environmental variation during their evolutionary history.

A molecular phylogenetic analysis was used to reconstruct a broad-scale phylogeny of the benthic shallow-water octopuses from the amino acid sequences of two mitochondrial DNA genes: Cytochrome oxidase subunit III and Cytochrome *b* apoenzyme and, the nuclear DNA gene, Elongation Factor-1α. Maximum Likelihood and Bayesian approaches were implemented to estimate the phylogeny and non-parametric bootstrap was used to verify confidence intervals for Bayesian topologies. Overall the genes used in this study were better suited to the examination of recent phylogenetic relationships, which has helped to resolve the relationships among closely related taxa, rather than deeper divergences among genera and species groups. The phylogenies revealed strong evidence that the genus *Octopus* is not a monophyletic group. Interestingly, a number of monophyletic sub-groups comprising closely related terminal taxa exist within the genus. Based on these findings it is clear that the systematics of the subfamily Octopodinae requires major revision. Deep relationships within this group remain only partially resolved and to improve resolution among distantly related species sequence data from conserved genes should be examined.

The dichotomous reproductive strategies that exist among species of the benthic shallow-water octopuses are an exceptional life history feature as they are only one of two groups within the Cephalopoda that maintain such a dichotomy. The reconstructed pattern of evolution in inferred juvenile types showed that the planktonic juvenile type

was ancestral among 22 species and three independent evolutionary transitions to the benthic juvenile type were observed with no subsequent reversals among taxa. The comparative phylogenetic analysis revealed that egg size covaries with variation in latitudinal gradient and more weakly with body size. These findings suggest that, evolutionarily, egg size is an adaptive trait that responds to a number of selection pressures including those associated with macro-environmental variation. Based on these results it is suggested that the dichotomy in egg sizes may be maintained by a fecundity-survival trade-off that responds to natural selection associated with the environmental conditions that a species inhabits.

Under the assumption that egg size and juvenile type are tightly correlated traits I propose a number of hypotheses regarding the evolution of reproductive strategies in octopuses. Small eggs and planktonic juvenile types are likely to be the ancestral states for shallow-water octopuses in general. Based on the covariation of egg size with latitudinal variation, inter-specific evolution in both egg size and juvenile type is likely to reflect adaptations to natural selection resulting from large-scale ecological factors; a finding that is consistent with benthic marine invertebrate life history theory. Large eggs and benthic juveniles may be an adaptation to high-risk conditions such as deep-sea and/or cold environments as supported by the tendency for transitions in reproductive strategy to occur most frequently in the direction of small egg size - planktonic juvenile type to large egg - benthic juvenile type. Evidence that egg sizes are constrained by phylogeny was observed, which may also indicate a constraint on reproductive strategies such that transitions in strategy are rare.

The dichotomous reproductive strategies that exist among species of the benthic shallow-water octopuses are an exceptional life history feature that is only observed in one other cephalopod family, the Idiosepiidae. Many other benthic marine invertebrates also maintain dual reproductive strategies between species and a large body of theory exists regarding how these traits have evolved and been maintained throughout evolutionary history. Using a comparative phylogenetic approach it was possible to investigate hypotheses generated by optimality models and experimental observations in an historical context and to examine the patterns of evolution in traits.

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DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

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CHAPTER 1 General Introduction: Using phylogenetic methods to examine evolution of reproductive life history strategies in the benthic shallow-water octopuses.

This chapter is a general introduction to the Octopodinae. I introduce their reproductive life history strategies and the major concepts on the evolution and maintenance of these traits among benthic shallow-water octopus species. Further outlined is research into benthic marine invertebrates in general where non-historical methods have largely been used to examine evolution of reproductive strategies and the maintenance of high variation in these traits among species. In this context, I introduce the advantages of using a comparative phylogenetic method to examine reproductive strategy evolution in the shallow-water octopuses. In Chapter 2, a broad-scale molecular analysis of phylogenetic relationships among shallow-water octopus species is presented. Prior to this study evolutionary relationships among species were poorly known. The phylogeny presented here reveals that distinct species groups exist within the genus *Octopus* and that this genus is not a monophyletic group. This phylogeny was employed in Chapter 3 to examine the evolution of reproductive strategies in octopuses using a comparative phylogenetic analysis. In this framework, it was observed that the evolution and maintenance of a dichotomy in egg size and associated traits has been influenced by latitude, and factors associated with this macro-environmental gradient are likely to be selective forces involved in maintenance of these traits. Interestingly, adult body size has also had an influence on the evolution of these traits, but this relationship was observed to be separate to the covariation of egg size and latitude.

1.1.1 Introduction to benthic shallow-water octopuses

The unique and cosmopolitan benthic shallow-water octopuses (subfamily Octopodinae) belong to the largest and only benthic family (Octopodidae) within the suborder Incirrata and species are distributed broadly across most coastal regions of the world (Norman 2000). For thousands of years the shallow-water octopuses have captured the imagination of humans because of their keen intellect, complex behaviours, vibrant colour patterns and their highly cryptic natures. Octopuses are also valued as a lucrative fisheries resource and are harvested globally. Despite their broad appeal, the present taxonomy of octopuses is poorly understood, which is mainly due to

extensive allocation of species to the genus *Octopus* (Norman and Sweeney 1997; Nesis 1998). The evolutionary relationships among species are also not well understood and are likely to be much more complex than their present day taxonomy suggests. Understanding these relationships is vital to future management and conservation of species.

Members of the Octopodinae share a basic structural plan (e.g. eight arms, biserial sucker rows and an ink sac) but their skin patterning and colouration, behaviours and life history strategies are extremely diverse. This high level of heterogeneity in traits is thought to have first arisen during a period of rapid divergence in the coleoid cephalopods during the Devonian (Aronson 1991) and additionally during the separation of the two octopod suborders (Cirrata and Incirrata) prior to the Late Cretaceous (Young et al. 1998). At this time many facets of octopus life-style were dramatically altered, in particular their reproductive systems, due to the occupation of diverse shallow-water habitats by species (Packard 1972; Young et al. 1998). An increase in the vulnerability of exposed eggs to predation is thought to have resulted in the evolution of embryo brood care (Young et al. 1998). It is also possible that around this time other complex life history strategies evolved and a dichotomy in reproductive strategies was subsequently maintained throughout octopus history.

Most of the Octopodinae are inhabitants of complex shallow-water environments. Their primarily benthic life cycles are semelparous (Boyle 1987; Calow 1987), which means they commonly live for about one year (a single reproductive season), although there are a number of exceptional species that reproduce over multiple seasons (Mangold 1983, 1987; Mangold et al. 1993). As short-lived organisms, individual fitness is maximised by devoting all resources into the next generation (Sibly and Calow 1985; Calow 1987; Stearns 1992). This is exemplified by the high cost of embryo brood care by all Octopodids (Calow 1987). Interestingly though, the Octopodinae belong to one of only two cephalopod groups where dichotomous inter-specific variation in life history traits is observed. The Octopodidae and the Idiosepiidae are the only families in the Cephalopoda that have both the planktonic and benthic juvenile lifestyles among species (Boletzky 1977). For most other cephalopods the juvenile life style is the same as that of the adults (i.e. pelagic cephalopods have planktonic juveniles and benthic cephalopods have benthic juveniles).

The two reproductive strategies in the Octopodinae, i.e. “small egg - planktonic juvenile type” and “large egg - benthic juvenile type” (Boletzky 1977, 1987b), are markedly different means to achieving a benthic only adult life style (see Plate 1.1 for examples of both strategies). Benthic progeny arise from large eggs at hatching and are very well developed, particularly in their neural capacity and the strength and dexterity of their arms (Boletzky 1987a) (see Plate 1.1a-c). Alternatively, species that are planktonic at hatching are born from small eggs and are extremely underdeveloped (see Plate 1.1a and 1.1d-e). Individuals endure a period of growth, development and planktotrophic feeding in the plankton prior to settlement on the benthos (Rees 1950; Nixon and Mangold 1996). Although “true larvae” no longer exist in cephalopods, the planktonic stage is regarded as a “paralarva” because of its distinctive form at hatching compared to benthic juveniles and adults (Young and Harman 1988). The distinguishing features of this juvenile mode are both the pelagic ecology and transformation at settlement of individuals, which is comparable to a transition in form via metamorphosis (Young and Harman 1988).

Insights into the evolutionary history and the potential present-day selective factors that maintain the dual reproductive strategies among the Octopodinae are worthy of study for a number of reasons. Firstly, these reproductive traits affect species dispersal and distribution and are potentially important to biogeography and speciation. Secondly, life history traits such as egg size and juvenile life style directly affect fitness (Futuyma 1986) and survival of species. Thirdly, despite the potential importance of these traits to the evolution of species, very little is known of their evolution and the factors that influence them. Finally, new knowledge gained regarding the evolution of these traits in the Octopodinae may apply more broadly to benthic marine invertebrates generally.

The primary aims of this research were two-fold. The first aim was to explore phylogenetic relationships among the benthic shallow-water octopuses. Secondly, this new knowledge of phylogenetic relationships allowed investigation of the evolution of reproductive life history traits in the shallow-water octopuses through a comparative phylogenetic approach. Octopuses are a vital component of the shallow-water marine fauna particularly as predators and their life history strategies reveal strong similarities with other benthic marine invertebrates. For this reason, it is appropriate to review life

history theories established for other benthic marine invertebrates to understand processes that are likely to have maintained inter-specific life history dichotomies in octopuses.

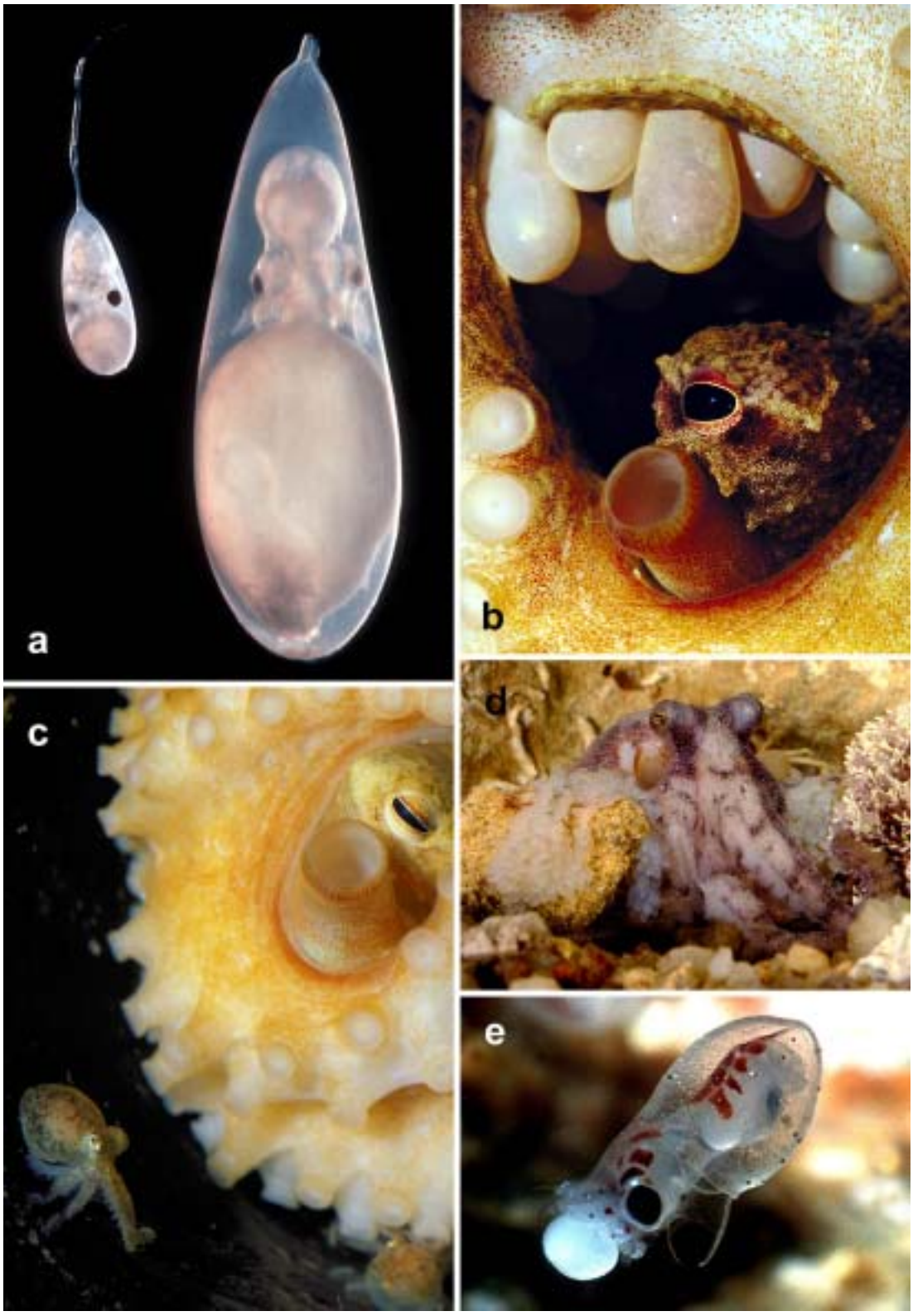

Plate 1.1: Photographs of a. relative egg sizes of *Octopus bimaculatus* (left) and *O. bimaculoides* (right) (photo by John Forsythe); b. *O. berrima* female with eggs; c. *O. berrima* female with a hatchling; d. *O. warringa* female with eggs; e. an *O. warringa* hatchling (photos b - d by David Paul).

1.1.2 Life history strategies of benthic marine invertebrates

Theories of life history evolution have largely been developed through general observation of present day patterns and trends in traits. Overall, the intention of life history theory has been to understand the adaptive significance and selective pressures that have influenced trait evolution among and within species. There are thought to be two significant trends that influence the inter-specific variation observed in egg size and juvenile type in benthic invertebrates. Firstly, egg size and juvenile type are strongly correlated traits, a relationship that is consistently demonstrated experimentally (Sinervo 1990; Hart 1995). Secondly, both traits vary geographically along latitudinal gradients, both within species in organisms such as cephalopods (Moltschaniwskyj and Martinez 1998; Forsythe et al. 2001; Pecl 2001; Jackson and Moltschaniwskyj 2002), freshwater shrimp (Hancock 1998), frogs (Cardillo 2002) and arthropods (Fox and Czesak 2000) and between species of benthic marine invertebrates (Thorson 1950; Jablonski and Lutz 1983; Lessios 1990) for example. It is predicted therefore, that egg size and juvenile type are adaptive traits that respond to variation in environmental conditions (Thorson 1950; Jablonski and Lutz 1983).

1.1.3 Methods for examining adaptation in life history traits

To investigate the adaptive significance of egg sizes and juvenile types prior studies have employed a number of specific approaches. In particular, optimality modelling, experimental approaches, testing for correlation between traits and to a lesser extent, the comparative phylogenetic method. The latter approach is widely merited as an important tool for examining historical processes but has not been used as commonly as the other methods listed above.

Optimality models are mathematical representations that estimate the optimal strategy under specified conditions. Based on an assumption that "subject to constraints natural selection should maximise Darwinian fitness" (Harvey and Pagel 1991:23), optimality modelling generates testable predictions and generalisations about life history evolution by inferring the best strategy given the conditions (Harvey and Pagel 1991). Models that describe the evolution of reproductive strategies in marine invertebrates (e.g. Vance 1973; Christiansen and Fenchel 1979; Havenhand 1995; McEdward 1997; Levitan

2000) have focused on the relationship between reproductive strategy and fitness (McEdward and Miner 2003). A favoured model predicts that these traits reflect a fecundity-survival trade-off because of the influence of size on juvenile survivorship (e.g. Vance 1973; Smith and Fretwell 1974; Christiansen and Fenchel 1979). Under this hypothesis, dichotomous reproductive strategies are maintained because they are individually suited to different environmental conditions. Models such as these are important for investigating trade-offs and potential explanations of trait adaptation but their predictions often require evidence from empirical data to be conclusive.

Experimental studies are a powerful tool for investigating the adaptive potential of present day traits and are commonly used to test predictions made by optimality models. In this way egg size has been shown to be an adaptive trait within species particularly under adverse conditions in many different types of invertebrates (Parker and Begon 1986; Sibly and Calow 1986; Sinervo and McEdward 1988; Azevedo et al. 1996; Fox 2000) and in this way the direct relationship between egg size, juvenile size and level of development at hatching have been demonstrated to influence juvenile survivorship (Sinervo 1990; Hart 1995). Such findings support the hypothesised trade-off between fecundity and mortality risk where high rates of juvenile mortality intensify selection on early life stages (Vance 1973; Sibly and Calow 1985; Fox 2000). The benefit of using empirical studies to examine natural selection is that they provide direct evidence of adaptive potential in present day traits. This is because they can identify cause and effect by controlling conditions where individual variables differ singly between treatments each time. The primary difficulty with such methods though, is that information about historical processes and evolution of traits is inherently absent from these studies.

Tests of correlation (not adjusted for phylogeny) that test for causal relationships between traits among taxa are commonly used to explain trait diversity in ecological studies (Miles and Dunham 1993). On its own, this approach is widely recognised as a weak method for inferring historical adaptation in traits (e.g. Dobson 1985; Gould 1986; Donoghue 1989; Lauder 1990) because relationships between traits among extant taxa may reflect common descent rather than adaptation. In this way it is impossible to distinguish between common selective forces and inheritance from a common ancestor.

However, these methods can provide an indicator of possible associations between traits and may be used in exploratory analyses.

Studies that use the above methods can only speculate on historical patterns and events, as they do not incorporate an historical component. To examine historical processes in marine invertebrate life history traits explicitly, it is necessary to adopt a comparative phylogenetic approach (McHugh and Rouse 1998).

1.1.4 The comparative phylogenetic method

Variation in life history traits is often phylogenetically constrained by associations within lineages to physiological or environmental factors (Harvey and Pagel 1991; Stearns 1992). The comparative method allows the adaptive significance of traits among extant taxa to be tested. The inclusion of phylogeny provides an historical context for hypothesis testing and permits identification of unique independent changes, adaptive changes and evolution maintained through common descent in traits among taxa (e.g. Clutton-Brock and Harvey 1984; Felsenstein 1985a; Harvey and Pagel 1991; Harvey and Purvis 1991). It is also possible to discriminate between three important factors, phylogenetic inertia, adaptation by natural selection and independent evolution of traits (Clutton-Brock and Harvey 1984; Felsenstein 1985a). Of particular interest is the method of tracing patterns of evolution in a trait over phylogeny. This allows estimation of the frequency of evolutionary state transformations. Similarly, inference of ancestral states can indicate the sequence, direction and magnitude of change (Miles and Dunham 1993). Examination of adaptive significance of traits with tests for evolutionary covariation between traits is valuable because parallel and convergent evolutionary transitions can indicate independent responses to similar selective pressures and can infer correlated responses (Harvey and Keymer 1991). In this way new insight into hypothesised trade-offs in life history evolution may be gained.

A number of studies have used a comparative approach to shed new light on life history evolution in many types of organisms, for example birds (Ward 2000), mammals (Purvis and Harvey 1995; Fa and Purvis 1997; Fisher et al. 2001; Fisher et al. 2002), fish (Crespi and Teo 2002; Reynolds et al. 2002) and arthropods (García-Barros 2000). Such studies demonstrate that questions of trait diversity can be better explored in a phylogenetic context and that non-historical approaches can be unreliable when

examining character state change because shared phenotypic traits in extant species may reflect phyletic heritage rather than adaptation. Without including phylogenetic relationships it is difficult to discriminate between common selective factors and inheritance from a common ancestor.

Studies that have used the comparative phylogenetic approach for examining evolution in egg size and juvenile type have made a number of important observations. Firstly, that evolutionary shifts in development time are correlated with inversely proportional shifts in egg size (Levitan 2000), supporting predictions of a theoretical optimality model proposed by the same author and suggesting egg size has directly influenced the evolution of juvenile strategy. Variation in larval types in asterinid sea stars appear unconstrained by phylogeny (Hart et al. 1997), while non-planktonic lineages are rarely thought to revert back to the ancestral larval mode after a transition to the apomorphic state (Lieberman et al. 1993). Of particular interest here is the observation that egg size appears to be constrained by phylogeny but does respond adaptively to variation in productivity levels (Lessios 1990), a result which may also apply to reproductive strategies in shallow-water octopuses of the Octopodinae.

CHAPTER 2 Molecular phylogeny of the benthic shallow-water octopuses (Cephalopoda: Octopodinae).

2.1 INTRODUCTION

The genus *Octopus* is one of the largest and most cosmopolitan genera within the order Octopoda, with over 150 named species (Sweeney and Roper 1998). Defined by a biserial sucker row on each arm and an ink sac (Cuvier 1797), representatives are found in most shallow-water regions of the world. Extreme species diversity exists in the Indo-West Pacific (IWP) including Australia (Stranks 1988b, 1998; Stranks and Norman 1992; Norman 1992a, b, c; Norman and Sweeney 1997; Toll and Voss 1998) and four other shallow-water genera are also recognised in this region.

The genus *Octopus* is widely regarded as a "catch all" genus (Nesis 1998) and its monophyly is dubious (Voight 1993; Norman and Sweeney 1997; Carlini et al. 2001). Identification of evolutionary relationships among shallow-water octopuses using systematics has proven difficult due to strong similarity in structural morphology among species and genera (Robson 1929; Roper and Hochberg 1988; Voight 1994). In an attempt to partition taxa within the genus, Robson (1929) identified nine species groups based on systematic relationships. Five of these groups encompass taxa from the IWP and Australia: *Octopus macropus*, *O. aegina*, *O. vulgaris*, *O. pallidus* and *O. australis* groups and later Norman (1993) raised the *O. horridus* species group (Plate 2.1a-e). More recently it has been suggested these groups be recognised as independent genera (Stranks and Norman 1992; Norman 1993; Norman and Sweeney 1997; Norman and Finn 2001). Based on this principle Norman and Finn (2001) assigned the subgeneric name *Abdopus* to define the *Octopus horridus* group.

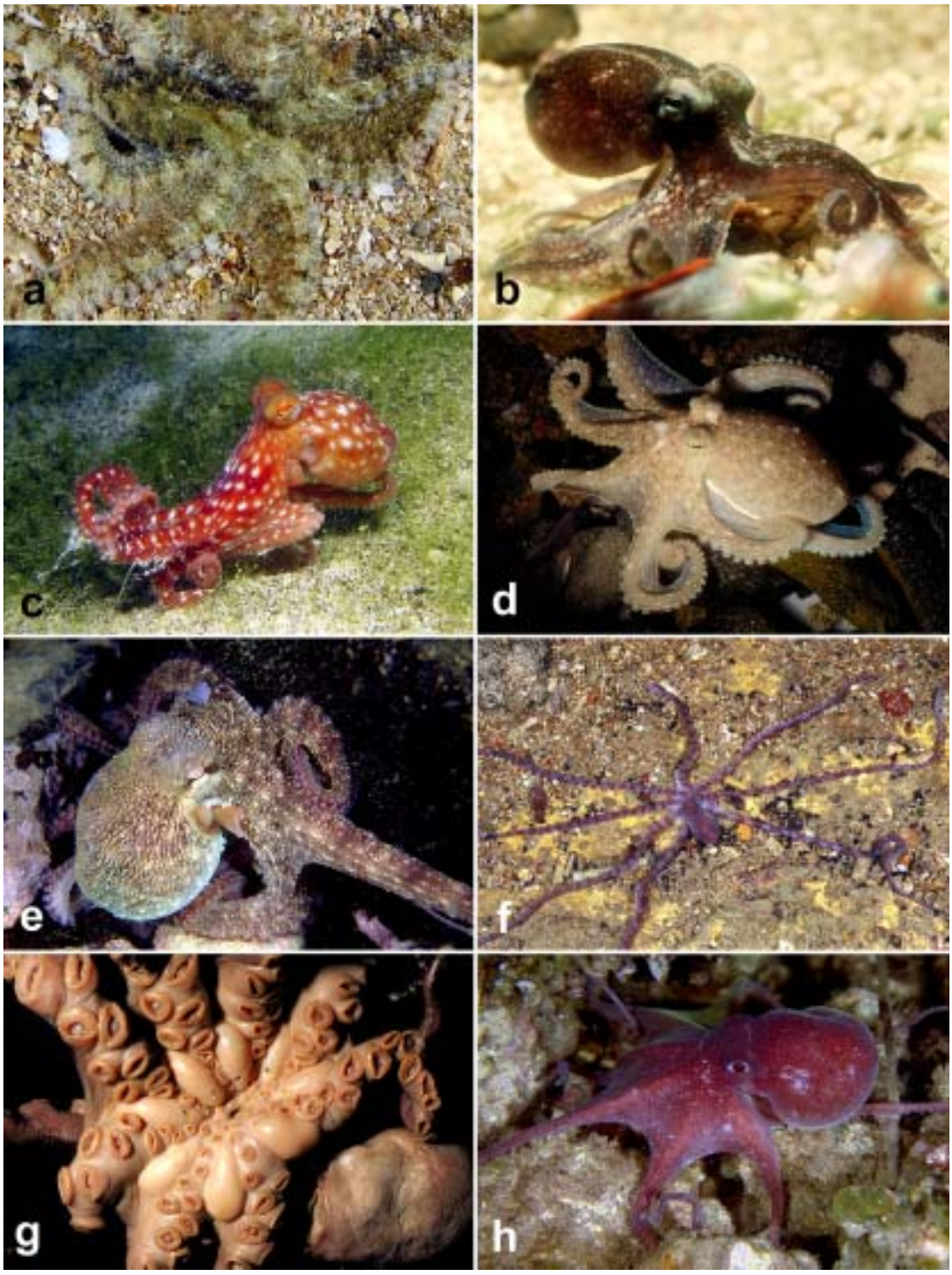

Plate 2.1: Representatives from five *Octopus* species groups (Robson 1929): a. *Octopus aculeatus* (sub-genus *Abdopus*); b. *Octopus aegina* (*O. aegina* group); c. *O. alpheus* (*O. macropus* group); d. *O. australis* (*O. australis* group); e. *O. vulgaris* (*O. vulgaris* group) and three other genera from the subfamily Octopodinae f. *Ameloctopus litoralis*; g. *Cistopus indicus*; h. *Grimpella thaumastocheir* (All photos by Mark Norman).

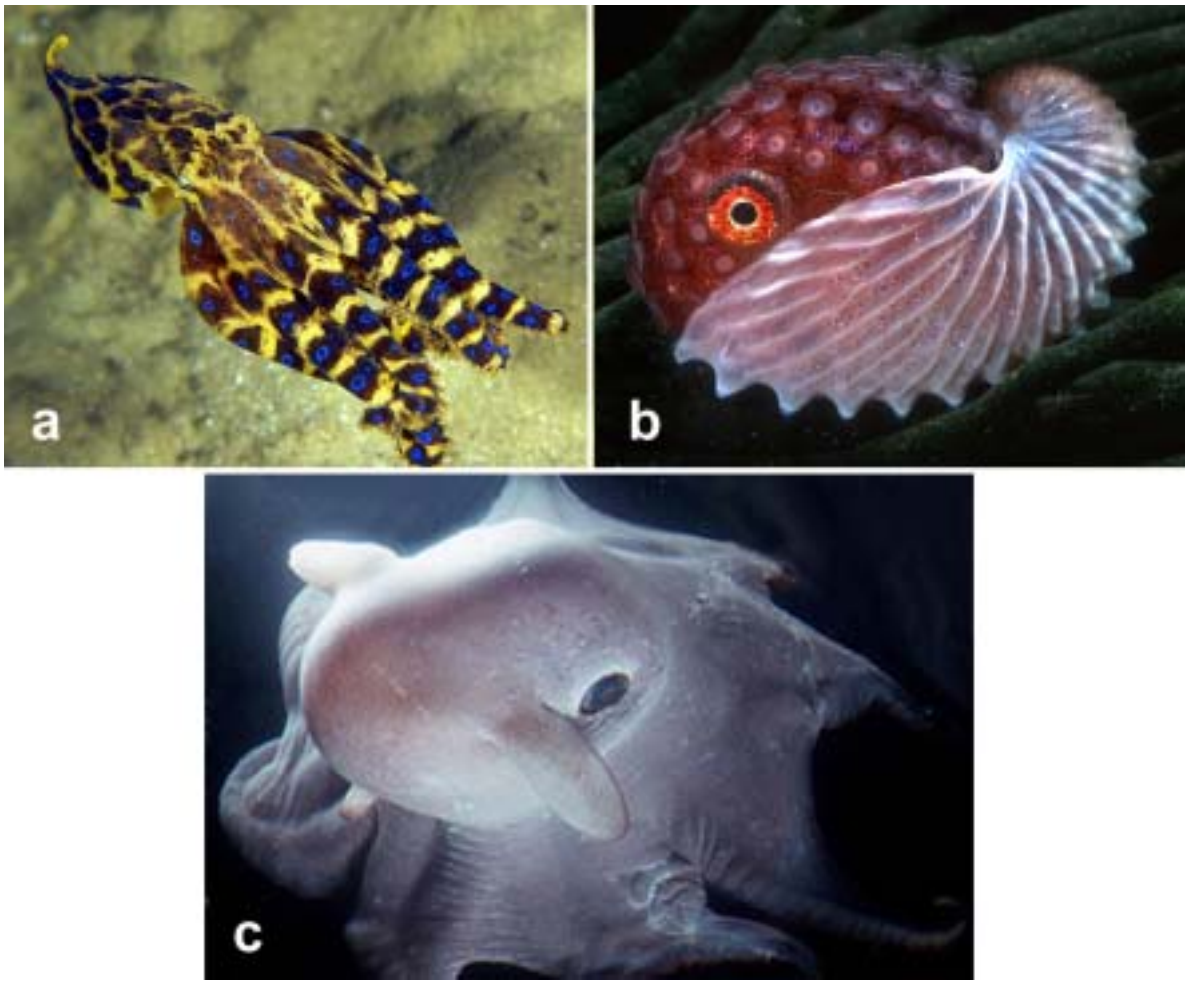

Plate 2.2: Photograph of a representative from the subfamily Octopodinae a. *Hapalochlaena maculosa*; and two phylogenetic outgroup representatives used in this study b. *Argonauta nodosa*; c. *Opisthoteuthis grimaldi* (All photos by Mark Norman).

Evolutionary studies based on morphological data largely avoid the subfamily Octopodinae, which contains the vast majority of benthic octopuses (Voss 1988). Those that do include the sub-family focus on higher level, familial relationships within the Octopoda (Voight 1993, 1997; Young and Vecchione 1996). Interestingly, the single study of 16 Great Barrier Reef octopus species based on morphological data suggests major delineation of taxa into distinct species groups (Norman 1993). Recently, insight into the evolutionary relationships of the Octopodinae has been gained through the availability of molecular sequence data. Mitochondrial DNA sequences (*16S* ribosomal DNA and Cytochrome oxidase subunit I) were used to reconstruct phylogenetic relationships among cephalopod families including a few members of the subfamily Octopodinae (Bonnaud et al. 1996; Boucher-Rodoni and Bonnaud 1996; Bonnaud et al. 1997; Carlini and Graves 1999; Carlini et al. 2001). Furthermore, two studies have somewhat clarified relationships among a small number of *Octopus* species from the Northern Pacific (Sosa et al. 1995) and Atlantic Oceans (Söller et al. 2000) using the mitochondrial DNA gene Cytochrome oxidase subunit III. Like the phylogeny based on morphological characters by Norman (1993), these studies suggest a more complex phylogenetic history than present day taxonomy represents.

A broad-scale phylogeny of the genus *Octopus* and its immediate relatives is otherwise unavailable. To investigate relationships among taxa in this study, phylogeny reconstruction with molecular sequences was used because such data is useful for providing an independent data set that generally reflects true phylogenetic descent (Avise 1994).

Included in this study were 26 *Octopus* species and representatives from four other genera of the subfamily Octopodinae: *Cistopus*, *Ameloctopus*, *Grimpella* (Plate 2.1f-h) and *Hapalochlaena* (Plate 2.2a). These five genera represent taxa from Australia and the broader Indo-West Pacific, but well-known *Octopus* species from all over the world were also included in this study. Members of the genus *Octopus* and its species groups were widely sampled as they represent a group of geographically diverse taxa with species of both highly restricted distribution and extremely cosmopolitan distributions. Care was taken to sample across the range of extant benthic shallow-water octopuses. To infer phylogeny, two mitochondrial DNA (mtDNA) genes Cytochrome oxidase subunit III (*cox3*) and Cytochrome *b* apoenzyme (*cob*), as well as the nuclear DNA

(nDNA) gene Elongation Factor-1α (EF-1α), were partially sequenced. These three genes were chosen according to their expected usefulness in examining recent (*cox3* and *cob*) and ancient (EF-1α) divergences among octopuses. Additionally, both universal and octopus specific primers for these genes were readily available. To reconstruct phylogeny from the sequence data, Maximum Likelihood (ML) and Bayesian approaches were the main types of analysis used.

Maximum Likelihood (ML) is used in phylogenetics as an optimality criterion that estimates the 'best' tree topology from both the discrete character data and a probability model that describes the data in question (Edwards and Cavalli-Sforza 1964; Felsenstein 1981; Huelsenbeck and Crandall 1997; Lewis 2001). Maximum Likelihood estimates the parameters in a model that maximise the probability of observing the data (Edwards 1972). An advantageous aspect of ML in phylogenetic inference is inclusion of increasingly realistic DNA substitution models that accommodate known evolutionary processes in DNA sequences, which in turn improve the chances of estimating the true tree. Alternatively, Bayesian phylogenetic inference is a different form of statistical analysis that uses a posterior probability distribution to infer uncertainty in the tree topologies and parameters of the substitution models (Gelman et al. 1995; Larget and Simon 1999) rather than an optimality criterion to estimate the 'best' tree. Bayesian inference of phylogeny has a basis in likelihood because Bayesian posterior distributions are directly proportional to the product of the prior distribution and the likelihood. For this reason ML models of substitution can be implemented into the Bayesian framework.

Markov chain Monte Carlo (MCMC) simulation (Metropolis et al. 1953; Hasting 1970) has recently been applied to Bayesian inference of phylogeny to search parameter space (Rannala and Yang 1996; Larget and Simon 1999; Mau et al. 1999; Li et al. 2000; Huelsenbeck and Bollback 2001; Huelsenbeck et al. 2001; Lutzoni et al. 2001). The efficiency of MCMC simulation makes computation of Bayesian phylogenetic inference very fast even when complex ML models are implemented, particularly when compared to other methods of phylogeny reconstruction. For this reason a number of authors suggest that this method offers the potential for analysing large data sets with the use of complex substitution models that is much faster than previously possible (Huelsenbeck et al. 2001; Lewis 2001; Whelan et al. 2001; Huelsenbeck et al. 2002). Bayesian

inference has been used effectively in a number of studies (Miller et al. 2002; Whiting et al. 2003) but some problematic issues have been raised, as discussed further below.

The aim of this study was to examine phylogenetic relationships within the genus *Octopus* and its affinities to related genera as a means of testing their present taxonomic classification and inferring evolutionary history among species.

2.2 MATERIALS AND METHODS

2.2.1 Taxon selection

The ingroup comprised 26 *Octopus* species and representatives from four other octopodine genera, *Ameloctopus litoralis, Cistopus indicus, Grimpella thaumastocheir* and *Hapalochlaena* (three species). The pelagic octopus *Argonauta nodosa* and the deep-sea finned octopus *Opisthoteuthis* sp. were sampled as outgroup taxa (see Plate 2.3b-c). For species and collection sites see Table 2.1. Due to difficulties with PCR amplification six species were not sequenced for all genes: four for EF-1α; *C. indicus, O. mototi, O.* sp. 8 and *O. oculifer* and one for *cob*; *O. kaurna*. Overall, 34 species were sequenced for three genes.

Table 2.1: Details of species used in this study. * Denotes species described in Norman (2000).

Species	Collection Site	Museum deposit #
Ameloctopus litoralis Norman 1992	Dudley Point, NT	- [a]
Argonauta nodosa	Port Phillip Bay, VIC	- [a]
Cistopus indicus Gray 1849	Taichung Fish Market, Taiwan	- [b]
Grimpella thaumastocheir Robson 1928	Pt. Victoria Jetty, SA	MV F101627 [a]
Hapalochlaena fasciata Hoyle 1886	Moreton Bay, QLD	MV F101636 [a]
Hapalochlaena sp. 1*	Darwin, NT	MV F101643 [a]
Hapalochlaena maculosa Hoyle 1883	St. Leonards Pier, VIC	- [a]
Opisthoteuthis sp.	Namibia	- [a]
***Octopus aegina* group**		
Octopus aegina Gray 1849	Queen Victoria Market, VIC	- [a]
Octopus exannulatus Norman 1993	Lizard Island, QLD	MV F101641 [a]
Octopus marginatus Taki 1964	Northern Sulawesi, Indonesia	MV F101640 [a]
Octopus mototi Norman 1993	Lismore, Northern NSW	- [a]
Octopus ocellate sp. A Norman 1998	Queen Victoria Market, VIC	- [a]
Octopus cf. *kagoshimensis**	One Tree Island, QLD	MV F82871 [a]
***Octopus australis* group**		
Octopus australis Hoyle 1885b	Port Stephens, NSW	MV F101642 [a]
Octopus berrima Stranks and Norman 1993	Edithburg Jetty, SA	MV F101631 [a]
***Abdopus/ Octopus horridus* group**		

Octopus aculeatus Orbigny 1834	Orpheus Island, QLD	MV F101628 [a]
Octopus sp. 5*	Coconut Wells, WA	MV F101630 [a]
***Octopus vulgaris* group**		
Octopus cyanea[1] Gray 1849	One Tree Island, QLD	MV F101639 [a]
Octopus cyanea[2]	Magnetic Island, QLD	- [c]
Octopus oculifer Hoyle 1904b	Galapagos Islands	- [a]
Octopus tetricus (NSW)[1] Gould 1852	Wallaga Lakes, NSW	MV F101635 [a]
Octopus tetricus (NSW)[2]	Port Stephens, NSW	MV F101637 [a]
Octopus cf. *tetricus* (WA)[1]*	Lucky Bay, WA	MV F101629 [a]
Octopus cf. *tetricus* (WA)[2]	Fremantle Warf, WA	- [e]
*Octopus vulgaris** Cuvier 1797	Port Elizabeth, South Africa	PEM [d]
***Octopus macropus* group**		
Octopus alpheus Norman 1993a	One Tree Island, QLD	MV F101632 [a]
Octopus aspilosomatis Norman 1993a	One Tree Island, QLD	MV F101633 [a]
Octopus bunurong Stranks 1990	St. Leonards Pier, VIC	- [a]
Octopus dierythraeus Norman 1993a	Magnetic Island, QLD	MV F101638 [a]
Octopus graptus Norman 1993a	Townsville, QLD	- [e]
Octopus kaurna Stranks 1990	Port Phillip Bay, VIC	- [a]
Octopus sp. 8*	Rockingham, WA	MV F84227 [a]
Octopus sp. 10*	Exmouth Gulf, WA	MV F101634 [a]
Octopus sp. x (Sth. Africa)	Port Elizabeth, South Africa	PEM [d]
Octopus maorum Hutton 1880	Portsea Pier, VIC	- [a]
***Octopus pallidus* group**		
Octopus pallidus Hoyle 1885b	St. Leonards Pier, VIC	- [a]

Note: Australian states: NSW, New South Wales; NT, Northern Territory; QLD, Queensland; SA, South Australia; VIC, Victoria; WA, Western Australia. Museum abbreviations: MV, Museum of Victoria and PEM, Port Elisabeth Museum. Specimen identification by: [a] Dr. M.D. Norman, [b] Dr. C.C. Lu, [c] J. Finn, [d] Dr. M. Smale and [e] Michelle Guzik.

To ensure that levels of intra-specific sequence divergence were minimal, multiple samples of a single species were sequenced for the following species: *Octopus cyanea* (2x), *O. tetricus* (NSW) (2x) and *O.* cf. *tetricus* (WA) (2x).

2.2.2 Laboratory techniques

2.2.2.1 Tissue sample collection and storage

Samples were collected and either stored fresh frozen (-80°C) or sections placed directly in 100% ethanol for future DNA extraction. Small pieces of tissue were removed from whole frozen animals. Prior to extraction, samples stored in ethanol were dried and placed in TE solution [10mM TrisCl pH 7.5, 1mM EDTA] for up to three hours to remove ethanol.

2.2.2.2 DNA extraction

Samples were placed in liquid nitrogen, and ground in a mortar and pestle and transferred to 1.5ml eppendorf tubes, to which 500µl CTAB buffer [1M Tris-HCL pH8, 0.5M Na_2EDTA pH8, NaCl, Hexadecyltrimethyl ammonium bromide (CTAB)] and 5µl of Proteinase K was added. The homogenate was incubated at 65°C for 2 hours, with regular mixing. Whole genomic DNA was extracted from the homogenate using a modified phenol/chloroform protocol by Doyle and Doyle (1987). The DNA extractions were left to precipitate in 1.5 volumes of 100% isopropanol for about 12 hours at -20°C. Upon precipitation, DNA was centrifuged for 30 minutes at 4°C. The pellet was washed with 500µl of cold 70% cold ethanol. The pellet was resuspended in 50µl of water and stored at -20°C for future use.

2.2.2.3 PCR amplification, primers and sequencing

Due to the variable nature of some regions in octopus *cox3*, a number of primers were used to amplify the 597 base pair region (see Table 2.2). The universal primers CB1 and CB2 were used successfully to amplify a 390 base pair region of *cob* for most samples, in some cases the primers Mlscb1 and Mlscb2 were also used. Most EF-1α target sequences (~600 base pairs in length) were successfully amplified with the primer pair EF0(oct) and EF2(oct) (modified from the universal primers EF0 and EF2 (Palumbi 1996)). These and the internal primers EF1(oct) and EFP3 were used in sequencing reactions to obtain both forward and reverse strands. For primer sequences refer to Table 2.2. All primers were prepared by Sigma-Aldrich. Primer combinations were optimised for each sample using the following protocol.

Table 2.2: Primers (5' to 3') used to amplify three genes.

Name	Direction	Sequence
cox3		
i-F1[1]	F	TAGCTCCAAATATAGATATTG
i-R1[1]	R	GAAAATGATGCTTCTATATATTCT
i-R2[1]	R	TTGAAGGATTGTAAAATAAAATCCTA
CO3a[2]	F	TTATTTATTGCATCAGAAGT
CO3b[2]	R	TCAACAAAGTGTCAGTATCA
Ooc3F[4]	F	CAATGATGACGAGATATTATYCG
Ooc3R[4]	R	CTTCAAATCCAAAATGATGTGA
cob		
CB1[3]	F	TATGTACTACCATGAGGACAAATATC
CB2[3]	R	ATTACACCTCCTAATTTATTAGGAAT
Mlscb1[5]	F	CTTGAGGDCAAATATCWTTTT TVC
Mlscb2[5]	R	ATTGAYCGYAAATHGCATADGC
EF-1α		
EF0[6]	F	TCCGGATGGCAYGGCGAGAAYATG
EF0(oct)[7]	F	TCTGGNTGGCATGGTGATAACATG
EF1[6]	F	GACAACGTTGGCTTCAACGTGAAGAACG
EF1(oct)[7]	F	AGAYAAYGTTGGTTTYAACGTWAAGA
EF2[6]	R	ATGTGAGCAGTGTGGCAATCCAA
EF2(oct)[7]	R	ATRTGAGCRGTGTGGCAATC
EFP3[5]	F	GGCAGAGTCGAGACYGGTRTYTTGAA

Note: [1](Söller et al. 2000), [2](Simon et al. 1994), [3]cited in (Simon et al. 1994), [4]Designed by Yuen Ching Crozier, [5]Designed by the author, [6](Palumbi 1996), [7]Redesigned from (Palumbi 1996) by the author.

PCR reactions in 50µl volumes consisted of 1µl DNA extraction, 1.5mM $MgCl_2$, 1x Mg^{2+} free reaction buffer, 0.04 units of Taq Polymerase (Promega Inc.), 10µM of forward primer, 10µM reverse primer, 0.2mM of each dNTP. Optimal PCR conditions for most samples consisted of a standard amplification protocol for both the mitochondrial and nuclear genes starting with an initial cycle of denaturation at 94°C for three minutes and 30 subsequent cycles of 93°C for 30 seconds (s), annealing

temperatures were sample and primer dependent but typically ranged between 40-50°C for mtDNA gene amplification and 54°C for EF-1α amplification for 30s and 72°C extension for two minutes. PCR was carried out in Perkin-Elmer GeneAmp 9700. Amplification products were electrophoresed on 1.5% agarose gels immersed in 1x TAE buffer to check for fragment size and primer specificity. Remaining PCR reaction was purified with QIAGEN's, QIAquick PCR purification kit or Promega Wizard PCR preps DNA purification columns. Amplified EF-1α fragments were purified using Polyetheylene Glycol (PEG) precipitation. 20% PEG 8000/ 2.5M NaCl solution was added to each PCR reaction, vortexed briefly and incubated at 37°C for 15 minutes then microcentrifuged for 15 minutes at 13000rpm. After removal of supernatant the pelleted DNA was washed (70% cold ethanol) and microcentrifuged at 4°C for 10 minutes. Ethanol was removed and the wash step was repeated. The pellet was vacuum dried and resuspended in ddH_2O. In cases where multiple bands of PCR product were observed, the band of choice, based on expected fragment size, was excised from the gel and purified with QIAGEN's QIAquick gel extraction kit, following manufacturer protocols.

PCR product was directly sequenced from 12µl reactions that consisted of ABI PRISM Big Dye reaction mix (version 1.0), 0.5µM of primer and 30ng of purified DNA. Reactions were cycle sequenced in a Perkin-Elmer GeneAmp 9700 as per the manufacturer's protocol and purified in Centri-Spin 20 columns from Princeton Separations to remove excess primer and dideoxy terminators. Sequences were run on a 377 ABI sequencer.

2.2.3 Sequence alignment

Sequences of *cox3*, *cob* and EF-1α were imported into and aligned in Sequencher™ 3.1 (GeneCodes Corporation). Both 5' and 3' strands of the mtDNA genes were obtained for all taxa. All EF-1α fragments were sequenced in at least one direction, but in some cases complete sequencing of both strands was not possible due to T-rich regions within the introns, which caused abrupt stops when sequencing reactions were electrophoresed. All attempts were made to gain as much overlapping sequence as possible. All sequence chromatograms were cross checked against one another and sequences were pruned to a) ensure the frame started at a first codon position and ended at a third, and

b) remove introns from EF-1α sequences (cut at conserved GT/AG splice sites). All the sequence alignments from Sequencher™ 3.1 (i.e. contigs) were exported as NEXUS formatted files for use in phylogenetic inference programs. No alignment ambiguities were observed within any of the coding gene regions.

Sequences used in this study were deposited in Genbank. For the list of Genbank accession numbers see Appendix 2.

2.2.4 Data Analysis

Nucleotide statistics and genetic distance measures were estimated in MEGA 2.1 (Kumar et al. 2001) and PAUP* 10b (Swafford 2002). Preliminary analyses characterised the individual data partitions *cox3*, *cob* and EF-1α separately. For analyses of individual codon positions the subprogram MOLCODON in MOLPHY 2.3 (Adachi and Hasegawa 1996b) was used to create three separate data sets. For analysis of amino acids, nucleotide sequences of mitochondrial and nuclear genes were translated separately under "*Drosophila* mtDNA" and "Universal" genetic codes respectively, as implemented by MacClade 3.07 (Maddison and Maddison 1989).

2.2.4.1 Test for compositional homogeneity

The TREE-PUZZLE 5.0 (Strimmer and von Haeseler 1996) Chi-square (χ^2) test for compositional homogeneity between sequences was used to test whether sequences deviated significantly from each other in base composition. Each codon position in all three data partitions was tested as well as amino acid sequences.

2.2.4.2 Comparison of amino acid substitution models for mtDNA and nDNA data partitions

Eight models of protein evolution were compared to estimate the 'best fit' model of substitution for both the mtDNA and nDNA amino acid data sets, which would in turn determine whether the data sets could be concatenated. In total, eight models were compared: Dayhoff+F (Dayhoff et al. 1978), JTT+F (Jones et al. 1992), mtREV24+F (Adachi and Hasegawa 1996a) and WAG+F (Whelan and Goldman 2001), plus each of the models with the gamma distribution (+Γ) included.

It is not possible to estimate the number of degrees of freedom for comparison of these models hence, it was intended that a frequency distribution of the -log likelihoods be estimated. 100 random trees were generated for both gene regions from a list of 32 species for both mtDNA genes and 26 species for EF-1α, with the TreeScripts program RANDOMTREE 4.0 (program designed by Agapow, P-M., see Johnson et al. (2003)) some taxa had to be removed from the analysis because MOLPHY 2.3 (Adachi and Hasegawa 1996b) does not tolerate identical sequences, also some sequences were not available for EF-1α). MOLPHY 2.3 (Adachi and Hasegawa 1996b) was used to estimate log likelihoods (ln*L*) for each starting tree (random tree) under all models being tested. It was found that analyses would often become caught on a single tree, searching endless numbers of trees and not moving on to the next starting tree. Suggestive of a flat tree-landscape (Johnson et al. 2003), this problem led to an alternative analysis. Batches of 50 random phylogenies generated with RANDOMTREE were used as starting trees in the subprogram PROTML of MOPLPHY to estimate ML trees using a local rearrangement search (-u-R option) under the mtREV24+F model for the two data partitions. These analyses were run until 100 trees were accumulated for both data partitions. Each of the 100 topologies was then evaluated under each of the chosen models within TREEPUZZLE 5.0 (Strimmer and von Haeseler 1996), which provides ln*L* values for each tree. The difference in ln*L* from mtREV24+F +Γ was calculated for each tree and the difference from 0 (mtREV24+F +Γ) was plotted as a frequency distribution (after Chiotis et al. (2000)).

2.2.4.3 Maximum Likelihood analysis

The mtREV+F +Γ (Adachi and Hasegawa 1996a) model of substitution was identified as the most suitable for both mtDNA and EF-1α data partitions, hence the amino acid sequences were concatenated and treated as a single data set for ML analysis in TREEPUZZLE 5.0 (Strimmer and von Haeseler 1996). This program was used because it has the capability to implement the mtREV24 model and estimate amino acid frequencies and a gamma distribution. For the complete data set, the estimated amino acid frequencies were: A = 0.040, R = 0.025, N = 0.051, D = 0.031, C = 0.010, Q = 0.016, E = 0.041, G = 0.073, H = 0.037, I = 0.082, L = 0.113, K = 0.033, M = 0.040, F = 0.093, P = 0.057, S = 0.075, T = 0.056, W = 0.026, Y = 0.030, V = 0.071 and alpha = 0.20 (S.E. 0.02). Amino acid frequencies and gamma distribution parameter alpha (8

gamma rate categories were used to approximate a continuous gamma-distribution with expectation = 1 and variance = 4.97) were estimated from the data set in an 'approximate' search based on quartet sampling (for substitution process) + Neighbor Joining (NJ) tree (for rate variation).

A ML approach to statistical testing was used to determine the 'best' tree topology from the estimated trees. The Kishino-Hasegawa test (KH-test) (Kishino and Hasegawa 1989) and the Shimodaira-Hasegawa test (SH-test) (Shimodaira and Hasegawa 1999) were used to compare tree topology ln*L*. These tests estimate the difference between likelihoods and the variance of each topology to estimate the probability of fit. More specifically, the KH-test performs a non-parametric likelihood ratio test of topologies, but to be correctly used, *a priori* specified trees are required (Kishino and Hasegawa 1989; Goldman et al. 2000). Alternatively, the SH-test is a more appropriate non-parametric test of best fit among trees selected *a posteriori*, because it compares all topologies and allows for multiple tree comparisons by simultaneous assessment of significance levels for each topology (Shimodaira and Hasegawa 1999; Goldman et al. 2000). These tests were implemented by the most recent version of TREEPUZZLE 5.1 (Strimmer and von Haeseler 1996), and for the non-parametric bootstrap component of the test, the RELL method was used to resample the data set 1000 times.

2.2.4.4 Bayesian analysis

To estimate phylogeny in a Bayesian framework, MrBayes 3.0b (Huelsenbeck and Ronquist 2001) was used. Initially it was intended that the mtREV+F+Γ model of substitution would be implemented, but within MrBayes, mtREV amino acid frequencies override estimated frequency values. Instead, the model prior was 'Fixed' to the 'Equalin' model that allows prior knowledge of amino acid frequencies to be considered. MrBayes also allows implementation of an extra level of complexity to models with a data partitioning function. This was employed and data was partitioned into 'nuclear' and 'mitochondrial' partitions due to the disparate genomic origins of nuclear and mitochondrial gene sequences. Separate character state frequencies for the two data partitions were also applied. The amino acid frequencies estimated in TREEPUZZLE were implemented as 'fixed' state frequency priors for nDNA (A = 0.059, R = 0.050, N= 0.058, D = 0.049, C = 0.004, Q = 0.022, E = 0.071, G = 0.097, H

= 0.016, I = 0.043, L = 0.066, K = 0.077, M = 0.013, F = 0.036, P = 0.088, S = 0.050, T = 0.059, W = 0.014, Y = 0.015, V = 0.113) and mtDNA (A = 0.033, R = 0.015, N = 0.049, D = 0.024, C = 0.012, Q = 0.014, E = 0.029, G = 0.065, H = 0.044, I = 0.097, L = 0.130, K = 0.017, M = 0.051, F = 0.113, P = 0.046, S = 0.084, T = 0.055, W = 0.031, Y = 0.036, V = 0.055) data sets. Furthermore, a continuous gamma distribution for the rate of substitution was estimated with 8 gamma categories (see Appendix 1 for commands). The analysis was run simultaneously on four chains (one cold chain and three incrementally heated chains) for 100,000 generations and every 100th tree was recorded. The number of generations was deemed appropriate after experimental analyses revealed convergence was certainly achieved by the 100,000th tree. In total, 1,000 trees were recorded for estimation of the MAximum Posterior probability (MAP) tree (Rannala and Yang 1996) which, in this case was a majority rule consensus tree showing all compatible partitions (contype = 'allcompat') of the final 720 estimated trees. By plotting ln*L* against the number of generations, it was possible to view graphically that the first 280 trees should be considered 'burn-in' and convergence was attained at about the 28th tree.

Bayesian phylogenetic inference programs that use MCMC simulation have been criticised for overestimating confidence in internal nodes with posterior probabilities when compared to ML bootstrap (Suzuki et al. 2002; Douady et al. 2003). Non-parametric bootstrapping (Efron 1979; Felsenstein 1985b) has been offered as a solution to overconfidence by Douady et al. (2003) and was implemented here to examine support for individual nodes. To generate 100 bootstrapped data sets, SEQBOOT 3.6 of the PHYLIP 3.6 package (Felsenstein 2000) was used to resample the input data set. Individual data sets were split into separate files because the SEQBOOT output is a single file (for MrBayes file formatting Perl Script, see also Douady et al. 2003). Each data set was analysed with MrBayes 3.0b for 60,000 generations with the same embedded commands used to estimate the 'best tree' (see Appendix 1 for commands). The number of generations in the bootstrap analysis was reduced from 100,000 to 60,000 to save time and this number of generations was determined by successively culling results trees to estimate the minimum number of trees required to arrive at the original consensus tree. Douady et al. (2003:249) offered three methods for examining internal node confidence, of which the second was used here; the MAP tree for each bootstrap replicate was estimated from the final 460 trees (i.e. the 'burnin' was up to the

140th tree) with the 'allcompat' consensus method. The Bootstrap Consensus (BC) tree was estimated from 100 MAP trees with CONSENSE 3.6 of the PHYLIP 3.6 package (Felsenstein 2000).

2.2.4.5 Maximum Parsimony analysis

Maximum parsimony analysis was carried out on the complete concatenated data set for protein sequences. The heuristic unweighted parsimony search involved tree-bisection-reconnection (TBR) branch swapping and 10 multiple random addition sequence replicates. The bootstrap analysis comprised 1,000 replicates of a standard heuristic search.

2.3 RESULTS

2.3.1 Nucleotide composition

In total, 1392 base pairs (bp) of coding-region sequence data were obtained for 34 octopus taxa. An A-T% bias of 72.6% and 70.7% was observed in *cob* and *cox3*, while the 405bp EF-1α coding region revealed a base composition of 51% A-T (see Table 2.3). Compositional biases in mtDNA genes are observed widely in mollusc mitochondrial genomes (Hoffman et al. 1992; Boore and Brown 1994; Terrett et al. 1996; Sasuga et al. 1999; Grande et al. 2002), and also octopus mtDNA (Sosa et al. 1995; Söller et al. 2000) and were therefore anticipated.

Table 2.3: Nucleotide composition statistics for three gene partitions.

		Average base frequency (%)						
Marker	**n**	**T**	**C**	**A**	**G**	**Ts**	**Tv**	**Ts/Tv**
Cob	390	45.9	9.9	26.7	17.5	29	24	1.2
cox3	597	39.3	18.2	31.4	11.1	52	53	1
EF-1α	405	24	22.4	27	26.6	32	20	1.6

Nucleotide frequencies of individual codon positions, summarised in Table 2.3, revealed extreme bias against cytosine in *cob* (1%) and *cox3* (15.8%) in third position sites, while a bias in favour of G-C (56%) was observed in first codon positions of EF-1α. The observed transition–transversion ratio (Ts/Tv) at third positions in the mtDNA genes was unusually low (Ts/Tv = 1 for *cob* and 0.8 for *cox3*), while there were close to twice as many transitions than transversions in EF-1α. The mtDNA gene results suggest multiple substitutions (homoplasy) where transversions have accumulated in the most variable positions and the true number of changes is probably no longer discernable (Simon et al. 1994). This finding was reflected in the overall Ts/Tv. Alternatively the Ts/Tv of EF-1α suggests a bias toward transitions overall, particularly in third codon positions (Ts/Tv = 2). It is likely that the homoplasy indicated by the Ts/Tv in the third positions is the confounding factor in the homogeneity of mtDNA sequences.

Variation in base composition was investigated for all three genes, at each codon position, using the χ^2 test for compositional homogeneity between sequences in TREEPUZZLE 5.0 (Strimmer and von Haeseler 1996). All EF-1α sequences were homogeneous, while third codon position sequences in both of the mtDNA genes deviated significantly from the stationarity assumption (9 ingroup sequences in *cox3* and 3 in *cob* were below 5% level of significance). This heterogeneity undermines a basic assumption of ML that from the outset change between sequences is unlikely (Felsenstein 1981), hence ML analysis of amino acid sequences was favoured as the protein sequences of all three genes were found to be homogeneous (>5% level of significance).

2.3.2 Variable and parsimony informative sites

In total 464 amino acids comprised the translated protein sequences that were used to estimate octopus phylogeny. Of these sequences 40.4% of the mtDNA amino acid sequences were variable and 22.8% parsimony informative (Table 2.4), which made the translated data set suitable for phylogenetic analysis. The amino acid sequences of EF-1α were comparable to the mtDNA genes with 23.7% parsimony informative sites.

The combined mtDNA sequence data set of 987 nucleotides comprised 49.9% variable sites and 39.9% parsimony informative sites (Table 2.4), which was also notably higher than that of the amino acid sequences. Similarly, the nucleotide sequences of EF-1α (not including the introns) also had many variable (44.7%) and parsimony informative sites (33.1%), almost matching mtDNA sequences (Table 2.4). This was surprising because the coding regions of EF-1α in other invertebrates are typically much less variable, i.e. percentage of parsimony informative sites in thrips 10.7% (Morris et al. 2001) and moths 8.4% (Cho et al. 1995).

Table 2.4: The number of conserved, variable and parsimony informative sites in three partially sequenced genes.

	Number of sites			
	Total	**Conserved**	**Variable**	**Parsimony informative**
Amino acid				
Cob	130	79	51	26
cox3	199	117	82	49
EF-1α	135	95	40	32
Nucleotide				
Cob	390	207	183	128
cox3	597	293	304	242
EF-1α	405	224	181	134

2.3.3 EF-1a: non-coding and coding regions, and a second copy

EF-1α coding sequences contained two introns that were ~100bp in length (Figure 2.1), but their size varied between species. In accord with invertebrate splice recognition sites (Mount et al. 1992), introns were recognisable by conserved 5' splice sites: intron 1, GAG|GTDRGT and intron 2, CAG|GTATGY and 3', the YAG|G sequence. These Thymine rich introns (intron 1: 58% and intron 2: 47%) had ~20% more A-T than the total coding region, which is consistent with most animal and plant nuclear genomes (Csank et al. 1990). Extreme variation within the intron sequences made alignment difficult. Sequences of closely related taxa were easier to align but overall similarity was low. For analysis of EF-1α, the introns were removed and only the coding regions were used.

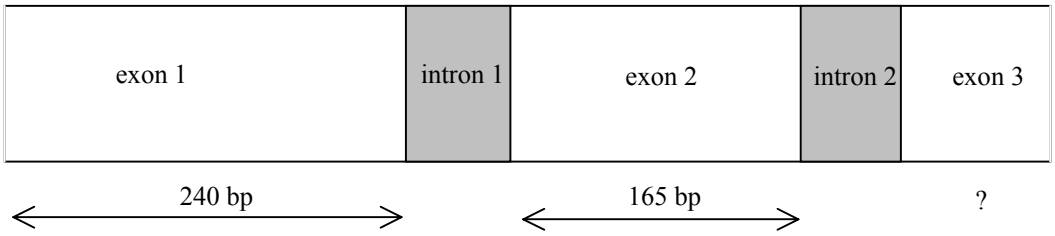

Figure 2.1: Fragment of octopus EF-1a amplified in this study; Intron regions are grey and (?) indicates that minimal data was available for exon 3.

The 405bp coding-region of octopus EF-1α encompassed two exons. A further 20bp of a third exon were also sequenced from some species but not included in the analysis. The translated amino acid sequences readily aligned to sequences of other organisms for which EF-1α has been characterised. A comparison of intron locations within the 1215-1620bp gene region of the crustacean *Artemia* EF-1α (Lenstra et al. 1986), revealed that one of the conserved intron locations described by Danforth and Ji (1998) for the honey bee, *Apis mellifera,* (position 1620bp on the map in Figure 2.2) is also found in octopus EF-1α. These findings suggest the origins of this particular intron may be quite ancient.

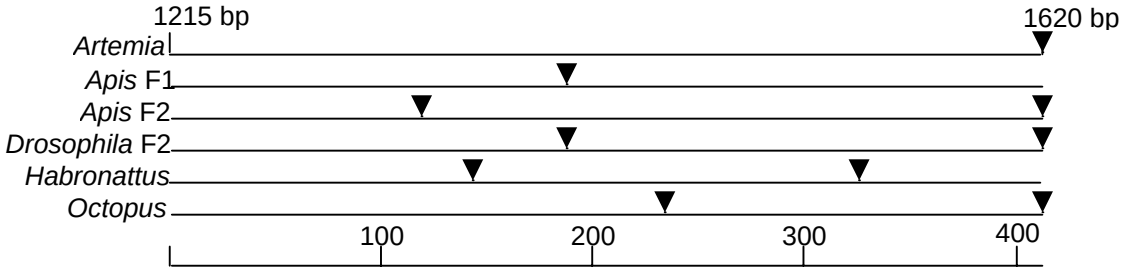

Figure 2.2: Locations of introns (triangles) in an alignment of genomic DNA EF-1a amino acid sequences (after Danforth and Ji (1998)) compared to the homologous region sequenced for octopus.

In addition to the target fragment of ~600bp some PCR reactions also yielded a second smaller band (~400bp). Sequencing of this second fragment for a number of species revealed close homology to the ~600bp fragment if the introns were removed, suggesting that this was a second copy of EF-1α that lacked introns.

2.3.4 Multiply sampled sequences

Sequences of multiply sampled taxa (i.e. *O. cyanea*, *O. tetricus* (NSW) and *O.* cf. *tetricus* (WA)) did not change overall tree topologies, suggesting minimal intra-specific divergence in these genes. For this reason single representative samples were sufficient for phylogeny reconstruction and multiples of sequences were excluded from analysis.

2.3.5 Model comparison

Comparisons of eight models of amino acid substitution revealed the mtREV24 (Adachi and Hasegawa 1996a) model including amino acid frequencies (+F) and gamma-distributed rate heterogeneity (+Γ) to be the most suitable for both data sets (see Figure 2.3). Like simpler models (i.e. JTT (Jones et al. 1992) and DAYHOFF (Dayhoff et al. 1978)), the frequency and replacement parameters of amino acids in the mtREV model are empirical, but mtREV was probably found to be the most suitable model because it accounts for multiple amino acid replacements (Whelan et al. 2001).

2.3.6 Non-parametric bootstrap

The notion that a Bayesian phylogenetic analysis produces results similar to a ML bootstrap has been rejected recently by two studies (Suzuki et al. 2002; Douady et al. 2003), which showed that posterior probabilities of a Bayesian analysis are overconfident when compared to ML bootstrap. For this reason, the non-parametric bootstrap was used here to test support at internal nodes within the Bayesian phylogenetic tree. Results showed extremely conservative support for internal nodes compared to both the original Bayesian posterior probabilities and ML bootstrap. It was found that out of 32 nodes, 11 had support ≥70%, while 18 nodes had <50% support, see Figure 2.4 for the BC tree.

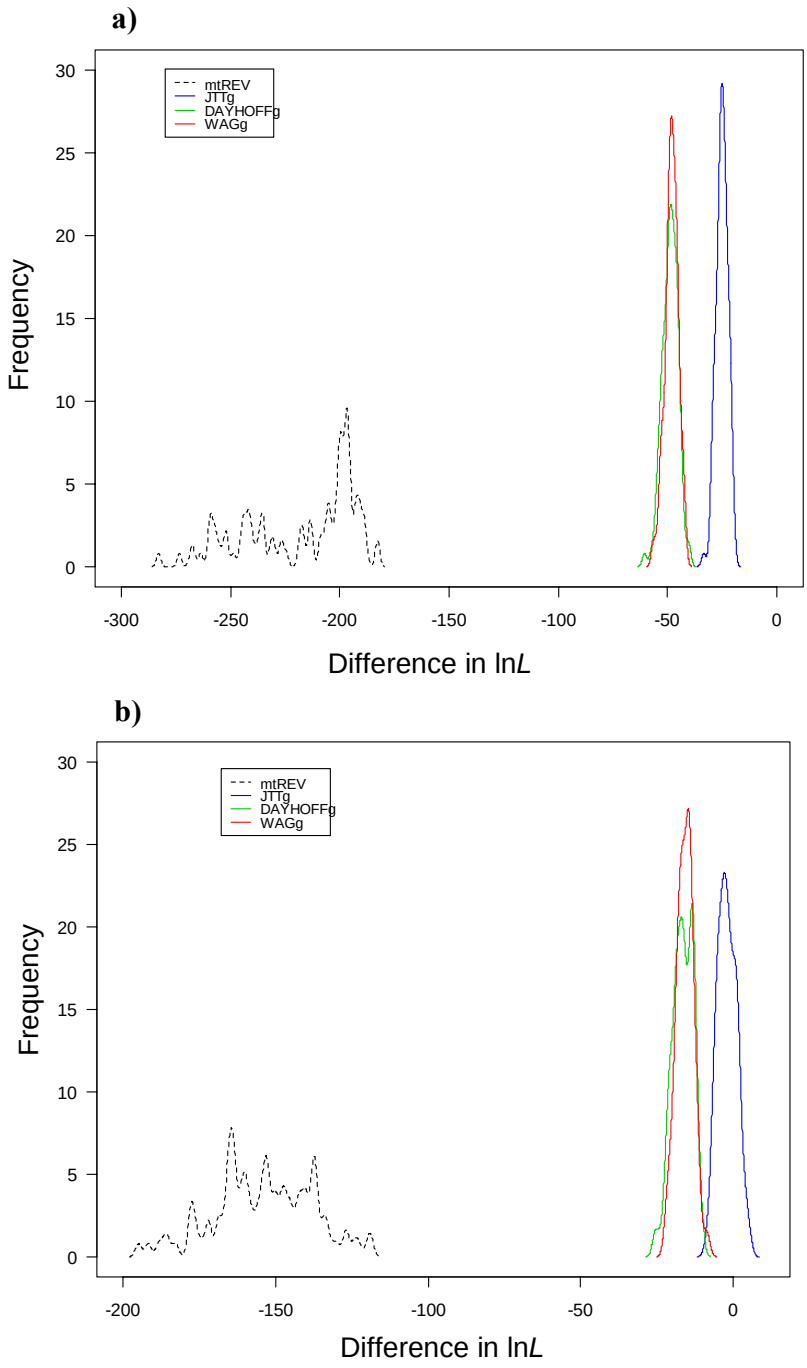

Figure 2.3: Frequency distributions of amino acid models for the combined mtDNA amino acid data (a) and the nDNA gene EF-1a (b). For model comparison, the difference in log likelihood (ln*L*) from 0 (mtREV +Γ model) is represented in blue (JTT +Γ), red (WAG +Γ), green (Dayhoff +Γ) and black (mtREV).

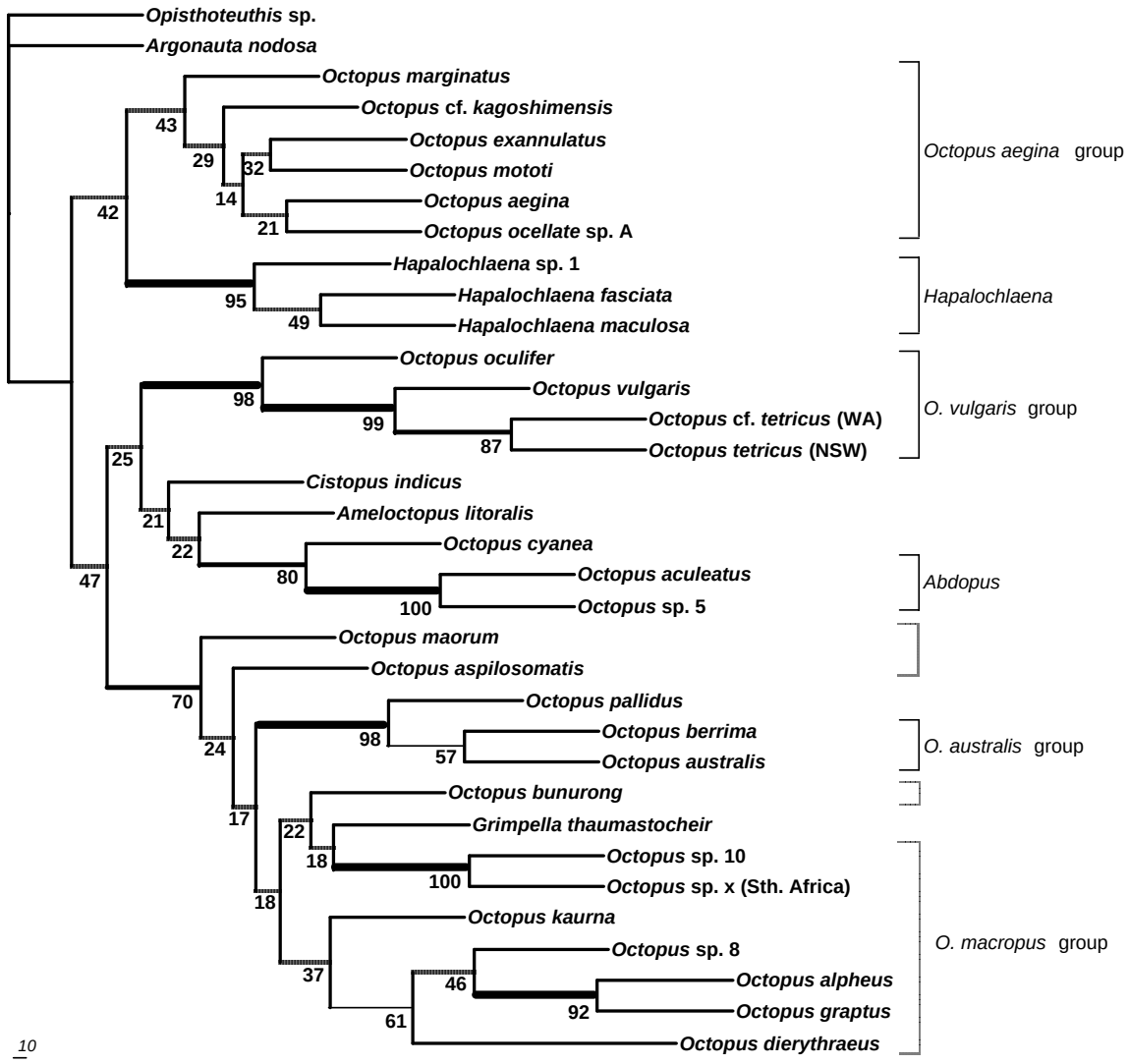

Figure 2.4: Bootstrap Consensus (BC) tree (consensus of 100 non-parametric bootstrap MAP trees). Thick lines indicate bootstrap support ≥ 90%, regular lines, bootstrap support ≥ 70%, thin lines, bootstrap support ≥ 50% and dashed lines, bootstrap support < 50%. Species groups (Robson 1929) are outlined to the right of the tree and dotted lines indicate members of the *Octopus macropus* species group.

2.3.7 Phylogenetic trees

Two main methods of phylogenetic inference were used here, firstly, ML which estimates the most likely tree according to the probability of the data based on an empirical model of evolution (Felsenstein 1981). Secondly, a Bayesian phylogenetic inference framework was applied, which evaluates the probability of a ML model of evolution given the data to find the tree with maximum posterior probability (referred to earlier as the MAP tree). Results revealed that the ML tree (see Figure 2.5) was not fully resolved but distinguished between three main groups of taxa, whilst the Bayesian analysis (MAP tree) recovered a well-resolved tree topology (see Figure 2.6). The MAP tree and BC tree were similar, differing, only in placement of the *O. vulgaris* and *Abdopus* + *O. cyanea* clades. Finally, the MP analysis found 728 equally parsimonious trees. The 50% majority rule consensus tree from these 728 equally parsimonious trees (shown in Figure 2.7) revealed support for the main clades and relationships among closely related terminal taxa. The resultant MP topology was very similar to the trees reconstructed using Bayesian and ML methods and showed strong support among groups of closely related species.

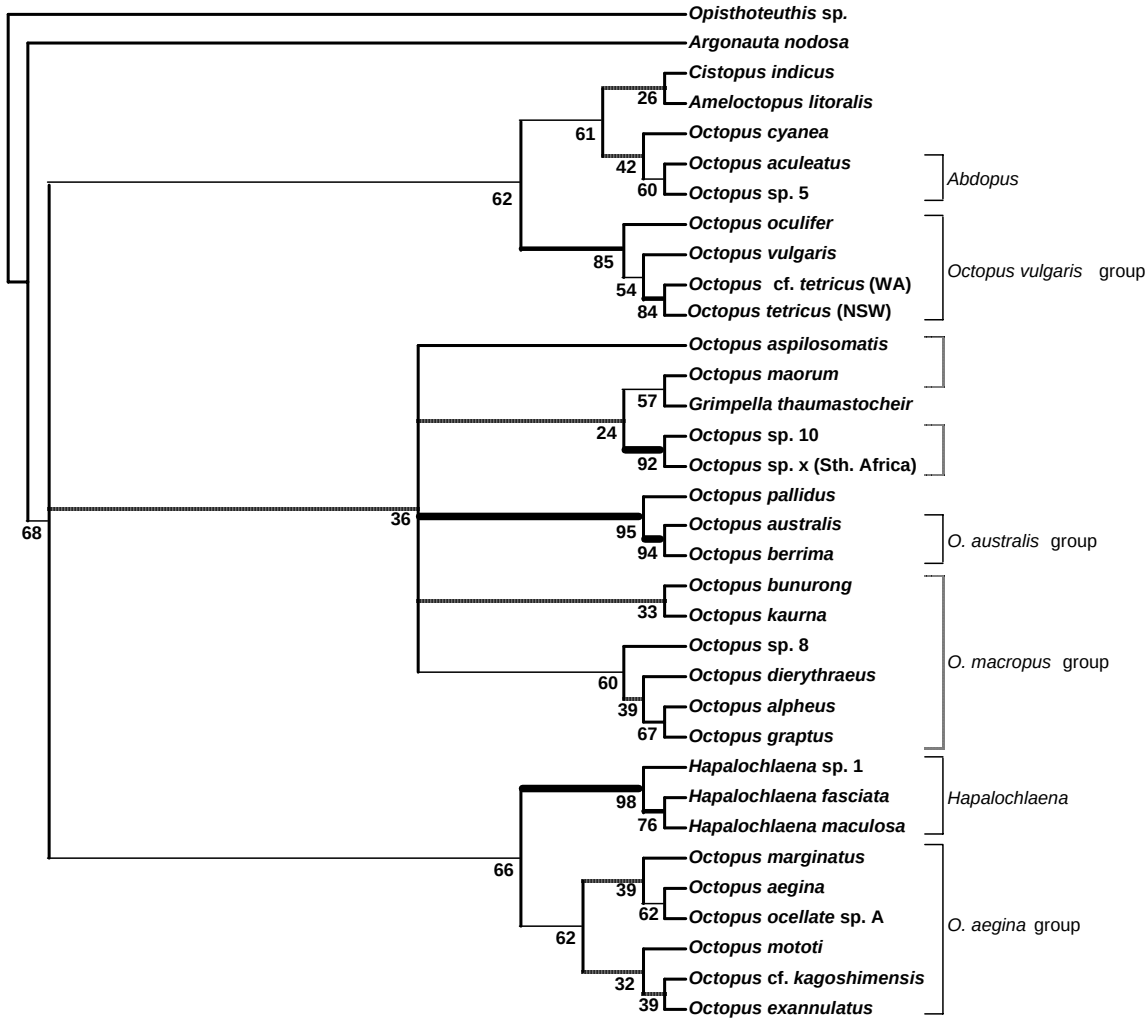

Figure 2.5: Maximum Likelihood (ML) tree. Thick lines indicate bootstrap support ≥ 90%, regular lines, bootstrap support ≥ 70%, thin lines, bootstrap support ≥ 50% and dashed lines, bootstrap support < 50%. Species groups (Robson 1929) are outlined to the right of the tree and dotted lines indicate members of the *Octopus macropus* species group.

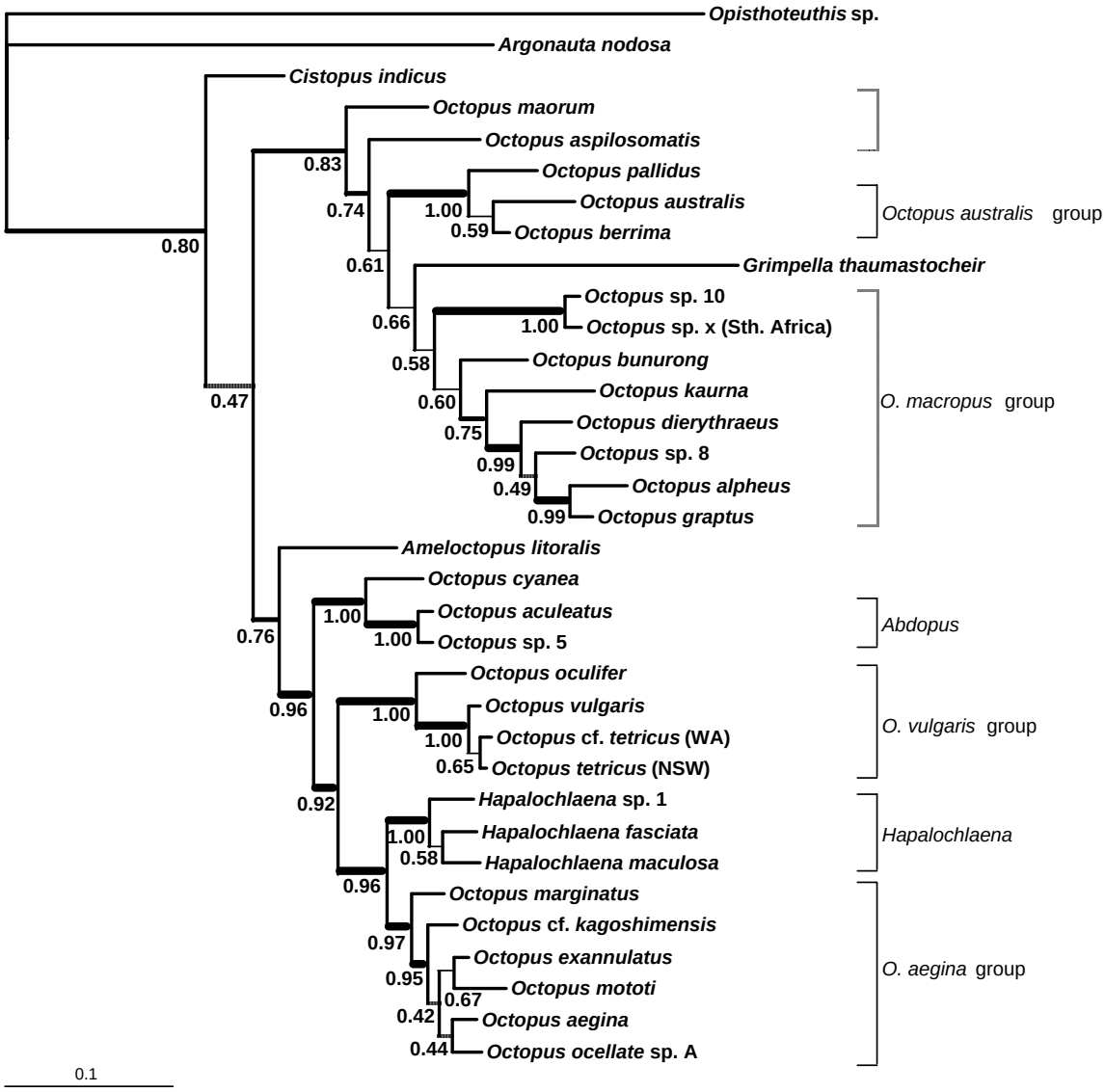

Figure 2.6: MAximum Posterior probability (MAP) tree. Thick lines indicate bootstrap support ≥ 90%, regular lines, bootstrap support ≥ 70%, thin lines, bootstrap support ≥ 50% and dashed lines, bootstrap support < 50%. Species groups (Robson 1929) are outlined to the right of the tree and dotted lines indicate members of the *Octopus macropus* species group.

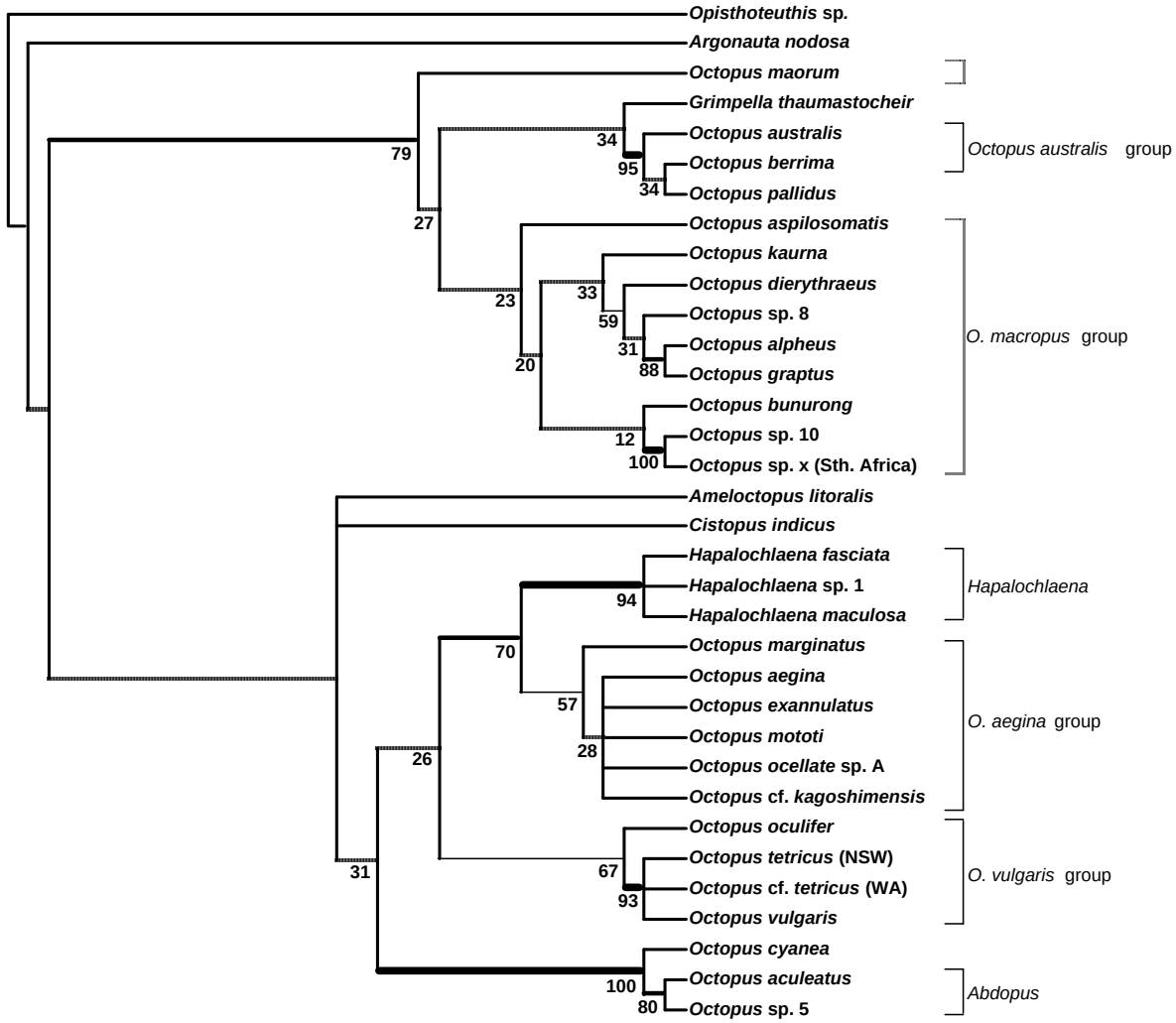

Figure 2.7: Maximum Parsimony (MP) 50% majority rule consensus tree of 728 equally parsimonious trees. Thick lines indicate bootstrap support ≥ 90%, regular lines, bootstrap support ≥ 70%, thin lines, bootstrap support ≥ 50% and dashed lines, bootstrap support < 50%. Species groups (Robson 1929) are outlined to the right of the tree and dotted lines indicate members of the *Octopus macropus* species group.

2.3.8 Comparison of user trees

To investigate whether the aforementioned trees were statistically different, the one-sided KH-test (without clock) (Kishino and Hasegawa 1989) and SH-test (Shimodaira and Hasegawa 1999) were used to compare between ln*L* for each topology and to estimate probability of difference from the best tree for all other trees. Both tests found the BC tree to be the 'best' topology compared to the MAP, ML and MP trees, but the KH-test indicated only the ML tree was significantly worse than the best tree, while none of the alternative topologies were significantly worse under the SH-test (see Table 2.5).

Table 2.5: Comparison of user trees with KH- and SH- tests, MAP = MAximum Posterior Probability (Bayesian) tree, ML = Maximum Likelihood tree, BC = Bootstrap consensus tree, MP = Maximum Parsimony tree. The - denotes a significant difference in log likelihood (Δ ln*L*) and, the + indicates a non-significant difference from the best tree.

Topology	ln*L*	Δ ln*L*	S.E.	*p* -1sKH	*p* - SH
MAP	-4390.37	8.21	14.35	0.2770 +	0.5010 +
ML	-4410.11	27.95	13.74	0.0203 -	0.0870 +
BC	-4382.16	0.00	← best	1.0000 +	1.0000 +
MP	-4393.80	11.64	15.14	0.2190 +	0.3880 +

Four well-supported clades that contained closely related taxa were consistently retrieved in all topologies: (1) *Hapalochlaena* clade, (2) *Octopus australis* clade, (3) *Abdopus* + *O. cyanea* clade and (4) *O. vulgaris* clade (see Figure 2.4). Resolution of deeper divergences, particularly between clades and among genera was poor and unstable in all analyses but support for the four main clades was consistent. Bootstrap values >80% supported the *Hapalochlaena*, *O. australis, O. vulgaris* groups and the *Abdopus* + *O. cyanea* clade (see Table 2.6). Additionally, a number of other clades were commonly observed. The *O. aegina* group and genus *Hapalochlaena* usually grouped together. Likewise, the *O. macropus* and *O. australis* species groups clustered together within a larger clade. This large clade also contained *Grimpella thaumastocheir*, the only representative of the monotypic genus *Grimpella*

and overall support for the clade was ≥70% in all trees except the ML tree (see Table 2.6). The *O. vulgaris* group and the *Abdopus* + *O. cyanea* clade were commonly grouped together but their placement within trees was unstable and varied between trees.

Table 2.6: Bootstrap (%) support for major octopus clades within phylogenetic trees. The – indicates the node was not supported in that tree topology.

Species group or genus	BC	MAP	ML	MP
O. macropus	61	99	60	59
O. vulgaris	98	100	85	67
O. australis	98	100	95	95
O. aegina	43	97	62	52
Abdopus + *O. cyanea*	80	100	42	100
Hapalochlaena	95	100	98	94
O. macropus + *O. australis*	70	83	36	79
O. vulgaris + *O. horridus*	25	-	62	-
O. aegina + *Hapalochlaena*	42	96	66	70

2.4 DISCUSSION

2.4.1 Effectiveness of the genetic markers used in octopus phylogeny reconstruction

Overall, the mtDNA genes *cob* and *cox3* were found to be useful for reconstructing phylogeny among the shallow-water octopuses, particularly among closely related species. These genes were not as effective in resolving deep divergences between clades and distantly related genera and there are a number of explanations for this result. Firstly, characterisation of the nucleotide sequences indicated that the mtDNA genes are evolving quite quickly. This was demonstrated by both the deviations in nucleotide sequence homogeneity by a number of ingroup taxon sequences and the high levels of sequence saturation at the third codon position as indicated by the Ts/Tv. The characters at these sites were also shown to be strongly informative but it is likely that the phylogenetic signal of these sites was obscured by multiple mutations. Secondly, an Adenine-Thymine (A-T) bias was also observed in the nucleotide sequences, which is consistent with findings of Sosa et al. (1995) that suggest octopus mtDNA genes are under directional selection pressure toward A-T. This kind of directional selection pressure has been demonstrated in the mtDNA gene *cob* in a number of organisms (Sueoka 1962; Osawa et al. 1992; Jermiin et al. 1994). Under these circumstances, the directional bias towards A and T can reduce the resolution of deeper nodes in a tree because it can have the effect of increasing the level of multiple mutations at individual sites (sequence saturation) and in turn obscuring the phylogenetic signal (Brower and DeSalle 1994; Blouin, et al. 1998). Finally, there may have been difficulty reconstructing deep divergences if a burst of rapid species group evolution or speciation had occurred historically. Short internal branches separating species groups and long branches to terminal taxa within the trees, as suggested by Leys et al. (2000), are an indicator of rapid speciation. Particularly short branches were observed within the *O. aegina* group suggesting that divergence of this group was probably very recent making it very difficult to resolve the deeper divergences.

Amino acid sequences were primarily used to reconstruct phylogeny here because unlike nucleotide sequences the amino acid sequences were shown to be homogeneous across all taxa. Use of amino acid sequences was beneficial as it avoided the problematically high levels of homoplasy that were indicated in the nucleotide analysis,

which have the potential to obscure phylogenetic signal. These sequences were also more conservative than the nucleotide sequences because amino acid sequences are known to be subject to strong selection pressures to maintain their composition. However, the amino acid sequences were still useful in resolving recent divergences among taxa. Hence, use of the amino acid sequences over the nucleotide sequences was a favourable approach as they reflected an accurate phylogenetic signal.

The phylogenetic signal of individual genes was found to be similar. Both the *cox3* and *cob* amino acid data sets provided the most resolution between species and clades while and EF-1α provided some resolution at nodes of medium depth, but provided very little information at the inter-specific level (see below for explanation). Groupings within the species groups were consistently estimated with all the genes and it is expected that disparities between tree topologies in this study arose from differences in phylogenetic signal between the nuclear and mitochodrial genes that may be the result of biases within the mtDNA genes. Missing data for some gene regions in a number of species was also likely to have added a level of ambiguity to the phylogenies.

2.4.2 EF-1a gene evolution

EF-1α was chosen to examine deep divergences among benthic octopuses because it is known in many organisms for its conserved DNA sequences within the coding gene region (Friedlander et al. 1992; Brower and DeSalle 1994; Cho et al. 1995; Belshaw and Quicke 1997; Mitchell et al. 1997; Reed and Sperling 1999). Unexpectedly, EF-1α was found here to be a quickly evolving gene in octopuses and a second copy of EF-1α was also amplified in a number of species. Multiple copies of EF-1α have been found in bees (Danforth and Ji 1998), shrimp (Duda and Palumbi 1999; France et al. 1999; Williams et al. 2001), frogs (Djé et al. 1990; Abdallah et al. 1991) and fruit fly (Walldorf and Hovemann 1990). Similar to the present study, an intron-less second copy of EF-1α has also been described in spiders (Hedin and Maddison 2001), whereas Cho et al. (1995) found that moth EF-1α sequences did not contain introns at all. The second copy of EF-1α in the octopus nuclear genome is likely to be a processed pseudogene as suggested by Hedin and Maddison (2001) for EF-1α in the spider genus *Habronattus*. The two characteristic features of pseudogenes (i.e. intron absence and stop codon presence) were observed in some of the sequences of the second EF-1α

copy, therefore supporting this hypothesis for octopuses (Li and Graur 1991; Page and Holmes 1998).

The exchange of genetic information between duplicated genes through conversion, where the independent evolution of each copy is constrained from divergence, is known as concerted evolution (Ohta 1983, 1990; Walsh 1987). This process can homogenise similar sequences and reduce divergence between loci, but it can also introduce mutation to otherwise conserved genes (Bettencourt and Feder 2002). Concerted evolution between the pseudogene and the true gene may be the reason that EF-1α in octopuses is less conserved than it is in other invertebrates. In this study, concerted evolution in EF-1α appears to have influenced the ability of this genetic marker to resolve deeper divergences in octopuses and related genera compared to other invertebrates such as moths (Cho et al. 1995; Mitchell et al. 1997) and thrips (Morris et al. 2001) because new mutations may have been introduced. Other studies of invertebrate EF-1α, where a second locus was observed, have also proposed concerted evolution between the two copies (France et al. 1999; Hedin and Maddison 2001). These studies also report strong concerted evolution of the pseudogene, as observed here.

Comparison of coding gene sequences that contained an intron (intron+), and non-coding sequences where introns were absent (intron-) revealed high similarity within species. Phylogenetic analysis of both loci in a number of species showed greater similarity between copies within species than between the same copies of the same locus among species (data not shown). It is widely accepted that mutational constraints on non-functional pseudogenes are usually relaxed due to a lack of selection pressure and such sequences commonly accumulate mutations. In observing high similarity between the intron+ and intron- loci, it is concluded that the concerted evolution has had an homogenising effect on both genes, but has reduced the number of mutations in the pseudogene, whilst accelerating the mutation rate in the coding gene. The mechanism by which this has occurred was not clear from the present data.

Partial sequencing of the coding region in EF-1α revealed two introns and three exons. The introns were extremely A-T rich and highly variable to the extent that only

sequences of very closely related species could be aligned and have potential use as population level genetic markers (Sanchis et al. 2001). Comparison of octopus intron positions to those of *Drosophila*, *Apis*, *Artemia* and *Habronattus* (see Figure 2.2) showed the location of intron 2 (see Figure 2.1) is a highly conserved intron position among the first three organisms (after Danforth and Ji (1998)). It is likely therefore that insertion of this intron into EF-1α occurred prior to the divergence of Molluscs, Annelids and Arthropods. The first intron in octopus EF-1α was not shared with any other organism, suggesting that insertion of this intron sequence occurred independently of, and probably more recently than intron 2.

The paralogous copy of EF-1α in octopuses is believed to be a processed pseudogene and, in agreement with Hedin and Maddison (2001), that gene duplication in EF-1α probably occurred independently and quite recently. Given the present data, however, it is impossible to tell when the duplication occurred. As sequence data from the EF-1α gene becomes available it is increasingly obvious that multiple copies of this gene occur in many animal genomes. It is strongly suggested that care be taken when considering EF-1a for reconstruction of a higher-level phylogeny, particularly if a second copy is observed. The reasons for this are, firstly that the gene may not be as informative as previously recommended due to concerted evolution of sequences and secondly, the results could be misleading with regard to gene history and lineage. The present results showed similar phylogenetic signal indicating a correct evolutionary history.

2.4.3 Phylogenetic methods

Maximum Likelihood analyses of nucleotide sequences were ruled out here because the χ^2 test for compositional homogeneity between sequences showed that both mtDNA genes did not pass the 5% level of significance, while all amino acid sequences passed. For this reason, only amino acid sequence data was used for phylogenetic analysis. Amino acid sequences are generally considered to be conservative markers because character state change is constrained by the functional requirements of the protein they encode. For this reason nucleotide sequence data is usually preferred when examining inter-specific relationships. In situations where nucleotide change is so excessive that it no longer reflects phylogenetic history, amino acid sequence data should be used, which was the case in this study. It was found here that amino acid sequences reconstructed

species level phylogeny as well as nucleotide sequences (data not shown) and there was no improvement on higher-level phylogenetic relationships. It is expected that the phylogenies reconstructed from amino acid sequences are the best hypothesis of octopus evolutionary history to date.

To compare between reconstructed topologies statistically, the KH- and SH- tests were implemented. It was observed that the BC tree was the 'best' topology, but all alternate topologies, excluding the ML tree under the KH-test, were not significantly different than the 'best' according to both tests. A number of authors have warned against using the KH-test on multiple *a posteriori*-specified trees (Shimodaira and Hasegawa 1999; Goldman et al. 2000; Whelan et al. 2001; Strimmer and Rambaut 2002). These authors suggest the KH-test is appropriate only in situations where two *a priori* specified trees are being examined. Two alternatives to the KH-test are the parametric bootstrap and the Shimodaira-Hasegawa-test (SH-test) (Shimodaira and Hasegawa 1999). Although favoured, the parametric bootstrap was not implemented in this study. This was because the large amount of bootstrapping with the Bayesian phylogenetic inference computer program, MrBayes, required more computational memory and time than was available to the author at that time. Therefore, the one-sided KH-test and the SH-test were used to test between topologies. Overall, it is concluded that the best estimate of shallow-water benthic octopus phylogeny is the BC tree, but according to the ML statistical tests, the alternate topologies; MAP, MP and ML tentatively, cannot be rejected as possible hypotheses of phylogeny.

As expected, posterior probability support for internal nodes of the Bayesian tree was high compared to results of the non-parametric bootstrap (noted on MAP tree, Figure 2.6) and the ML and MP bootstraps. Results overall showed high (≥80%) support for the *Hapalochlaena*, *O. vulgaris*, *Abdopus* + *O. cyanea* and *O. australis* clades across all bootstrap methods, but deep divergences were not well supported, particularly by non-parametric bootstrap values (see Table 2.6 for a summary of bootstrap values). Exceptionally high posterior probabilities estimated for internal nodes are problematic (Suzuki et al. 2002; Douady et al. 2003) and although these authors suggest the problem may be overcome with non-parametric bootstrap there are also difficulties associated with this method. Non-parametric bootstrapping (Efron 1979) was first applied to phylogeny estimation by Felsenstein (1985b) and Penny and Hendy (1985). This

method has been used broadly to infer confidence in phylogenetic tree topologies and provides a robust measure of confidence in a phylogenetic analysis (Felsenstein and Kishino 1993; Efron et al. 1996). Critics of the method have demonstrated that it provides overly conservative estimates of node reliability compared to ML bootstrap (Hillis and Bull 1993; Wilcox et al. 2002). Here we observed that the non-parametric bootstrap estimates were extremely conservative even in comparison to ML bootstrap but they also provided a balance to the strong support of the Bayesian posterior probabilities.

Among all tree topologies, there was considerable agreement regarding key clades. The 'best' tree, or the non-parametric bootstrap consensus (BC) tree, supported four main lineages with bootstrap values >70%: *O. vulgaris* (98%), *Abdopus* + *O. cyanea* clade (80%), *Hapalochlaena* (95%), and the greater *O. macropus* + *O. australis* clade (70%) (see Table 2.6). These groupings as well as similar bootstrap support were also observed in the other tree topologies presented. In addition to these groups though, the *O. aegina* group and the genus *Hapalochlaena* were shown to form a further clade that was supported by bootstrap values >65% in the MAP, ML and MP trees (see Table 2.6). This grouping was also frequently observed in preliminary phylogenies reconstructed from the untranslated nucleotide data of this study (not shown). It is expected that closer investigation and/or additional data may find the *O. aegina* group and the genus *Hapalochlaena* to be sister groups although any conclusions made with regard to this clade are tentative at present.

The key methods of ML and Bayesian phylogeny estimation proved appropriate for reconstruction of octopus phylogeny, considering some of the difficulties associated with the data. The computer programs presently available for ML analyses of amino acid sequences are still quite restrictive compared to those available for nucleotide sequences. The MOLPHY package (Adachi and Hasegawa 1996b) does not offer all ML models that are available, whilst the TREEPUZZLE (Strimmer and von Haeseler 1996) package only uses a quartet puzzling approach and output of branches with support less than 50% are collapsed, which makes determination of tree structure difficult. Implementation of the chosen models of substitution was therefore easier to carry out with a Bayesian approach because this method searches a range of parameters at each tree estimate rather than fitting the data to the model for verification of tree

topology (as in ML). The findings of this study reinforce recommendations of Suzuki et al. (2002) and Douady et al. (2003) that confidence inferred by posterior probabilities be regarded cautiously and should be verified with ML and/or non-parametric bootstrap.

2.4.4 Taxonomic implications

2.4.4.1 Validity of the genus Octopus

As it stands, the genus *Octopus* is polyphyletic and contains a number of distinct and divergent clades. Evidence supporting the hypothesis that *Octopus* is not monophyletic (Carlini et al. 2001) was the observation that two distinct octopodine genera *Hapalochlaena* and *Grimpella* consistently nested within clades containing mainly *Octopus* species. In particular, consistent bootstrap support ≥70% for the clade containing *Grimpella thaumastocheir* was observed in all topologies except the ML topology (36%), which is considered conclusive evidence of non-monophyly. These findings have direct implications for systematics and taxonomy of the shallow-water octopuses. Clearly *Octopus* has been treated as a "catch all" genus and taxa should be reassigned to a number of distinct genera. Many of the distinct clades recognised in this study are appropriate to be raised to the generic or sub-generic level.

Phylogenetic analysis of shallow-water octopuses using the mtDNA markers *cox3* and *cob* and the nuclear gene EF-1α, revealed strong groupings between certain taxa. In the Bayesian, ML and MP analyses of the amino acid data sets, four monophyletic groups were supported by bootstrap values ≥80%: *O. vulgaris* group, *Abdopus* + *O. cyanea* clade, *O. australis* group and *Hapalochlaena* (see Table 2.6). Each of these groups reflects the groupings of Robson (1929) and a number of subsequent authors (i.e. Stranks and Norman 1992; Norman 1993; Norman and Sweeney 1997; Norman and Finn 2001). A further, larger clade comprised the *O. australis* group, *G. thaumastocheir* and the following *Octopus* taxa: *O. alpheus*, *O. bunurong*, *O. dierythraeus*, *O. graptus, O. kaurna*, *O. maorum, O.* sp. 8, *O.* sp. 10 and *O.* sp. x (Sth. Africa). Named the *O. macropus* + *O. australis* clade, bootstrap support was ≥70% (except for ML, 36%). Relationships within this large clade were poorly resolved and within it many taxa were unusually grouped. Overall, it is thought that the larger *O. macropus* group + *O. australis* group clade probably comprises a number of

distinct of species lineages including the true *O. macropus* group, *O. maorum* and its relatives, the *O. australis* group and *Grimpella thaumastocheir* whose closest relatives remain unknown. Based on this phylogeny these relationships are indiscernible and their morphologies, which are distinctively different among groups, do not offer any clear insights. Further phylogenetic work would be required to investigate these relationships. Here, a discussion of specific terminal nodes among closely related taxa and the implications of our results for *Octopus* taxonomy and phylogeny are presented.

2.4.4.2 Octopus vulgaris *group*

This species group was coined by Robson (1929) to incorporate physically robust and muscular species, many of which are large. Defining features of the group include a bilaterally symmetrical web of moderate depth, uneven arms with the lateral pairs the longest and a small copulatory organ (1.5-5.5% of arm length). This group contains a large number of species including *O. vulgaris* (the type species of the genus *Octopus*) *O. tetricus*, *O. oculifer* and *O. cyanea*, which were all represented in this study. The present results strongly support the existence of this species group, excluding *O. cyanea* for which no phylogenetic evidence of a relationship to the *O. vulgaris* group was observed, as previously proposed by both Norman (1993) and Robson (1929). According to Robson's treatment of the *O. vulgaris* group, the features that *O. cyanea* shares with this group included: large muscular body and arms, longer lateral arms than dorsal arms, enlarged suckers on arm pairs 2 and 3, a tiny copulatory organ and simple linear terminal organ (penis) with small diverticulum. Here *O. cyanea* was observed to have a closer affinity to the sub-genus *Abdopus* (Norman and Finn 2001) than to *O. vulgaris*. This is a new hypothesis that has not previously been considered by other authors and offers a new insight into the phylogeny of *Octopus* species. *O. vulgaris* is the type species of the genus *Octopus*, it is therefore recommended that treatment of this group as the true representatives of the genus in the strict sense (*sensu strictu*) is appropriate.

2.4.4.3 Sub-genus Abdopus

Norman (1993) originally coined the *O. horridus* group, which contained small, long armed octopuses that have the ability to autotomise arms (detach at the base) as a decoy to predators. Members are intertidal animals that have unequal arm lengths where

lateral pairs are longer than dorsal arms and lateral webs are deeper than the dorsal web. Subsequently, Norman and Finn (2001) raised this group to a sub-genus called *Abdopus*, which contains species where mature males have enlarged suckers on arms 2 and 3, the hectocotylised arm is long and the terminal organ has a simple small diverticulum. The group contains the species *O. horridus, O. abaculus, O. aculeatus, O. capricornicus, O. tonganus, O.* sp. 2 (Norman and Sweeney 1997) and *Octopus* spp. 3-5 (Norman 2000; Norman and Finn 2001). The two representatives of *Abdopus* used in this study, *O. aculeatus* and *O.* sp. 5 (Norman 2000) consistently grouped together. Also, *O. cyanea* had a close affinity to these species. It is likely therefore, that the morphological similarities that *O. cyanea* shares with the *O. vulgaris* group may be the result of a convergence of form and niche rather than recent common origin. Further insight into ancestry would be gained by further sampling of *Abdopus* taxa. The strength of phylogenetic support for this group indicates that it is monophyletic and raising this taxon to generic rank is supported.

2.4.4.4 Octopus australis *group*

Defined by Stranks and Norman (1992), this group contains taxa with long and subequal arms, a broadly ovoid mantle and fine skin sculpture of rounded tubercles over dorsal surfaces. Representatives of this group include *O. australis, O. berrima, O. campbelli* and *O. warringa*, all of which have distributions restricted to southern Australia and New Zealand waters. The geographic restriction of all four species in this group to temperate Australia and New Zealand suggests regional origins or affinities. Similar geographic distributions are observed in some temperate Australian fishes which are considered "palaeoaustral" taxa, potentially linked to the long isolated drift north of the Australian continent following break up of Gondwana (~40 million years ago) (Wilson and Allen 1987).

The results of this study support a strong relationship between *O. australis* and *O. berrima* and also support inclusion of the previously unplaced taxon, *O. pallidus* and (Stranks 1988b; Norman 2000). Based on these results it would be valid to raise this group to generic rank. The observation that *O. australis* group members had affinities to the larger *O. macropus* group clade is presently inconclusive and tentative because of overall morphological disparities among these groups.

2.4.4.5 Octopus aegina *group*

The *Octopus aegina* group was coined by Robson (1929) and further revised by Norman (1993, 2000) and Norman and Sweeney (1997). Member species are characterised by two short front arms, while arms 2 and 4 are long, the front web is shorter than others and a diamond of short skin ridges is present on the upper body. Results of the present study grouped *O. aegina* member species in all analyses but resolution of relationships between species was limited. Short branch lengths in the MAP tree suggest species are very closely related and have experienced a rapid divergence during their evolution. Further resolution of these very close relationships would require sequence data from a fast evolving gene.

Morphologically these small, short-armed octopuses share a range of distinct traits including a deeply incised dorsal web sector. Should greater resolution and support be gained for this species group, the generic name *Amphioctopus* Fischer, 1882 is available to represent this group.

2.4.4.6 Genus Hapalochlaena

Collectively known as the blue-ring octopuses, this genus comprises 13 toxic (potentially fatal) species. These octopuses are restricted to the Western Pacific Ocean from Southern Australia to Japan. They are identifiable by their small size, dramatic colour patterns of iridescent blue rings and/or lines over the body and arms, enlarged salivary glands and an extended posterior mantle forming a pixie-cap shape (Norman 2000). Voss (in Roper (1983:19)) has previously questioned the validity of this genus on the grounds that there was only a single diagnostic character proposed at that time: the absence/reduction of the ink sac, even though some member species possess a functional ink sac (Norman 2000). Results of this study support the validity of this genus as a monophyletic group that is distinct from the genus *Octopus*.

2.4.4.7 Octopus macropus *group*

This species group was coined by Robson (1929) and incorporates medium to large octopuses. Characteristics of the group are: an elongate-ovoid shaped mantle, a long and robust first pair of arms, a hectocotylised arm that is shorter than its opposite arm and a web that is deepest on the dorsal side. Taxa have two other distinctive features,

multicuspid radula (teeth) and red and white skin colouration and patterning. Robson included nine species into the group, but subsequent studies have increased the number of member taxa. Revision by Norman (1992a, 2000) and Norman and Sweeney (1997) included new species such as: *O. alpheus*, *O. aspilosomatis*, *O. dierythraeus, O. graptus*, *O*. sp. 8 (Norman 2000) and *O*. sp. 10 (Norman 2000), all of which were represented in this study and were found to group together within most tree topologies.

Relationships among these taxa were unstable and poorly resolved in all phylogenetic reconstructions even though they all consistently grouped together. Further sampling of species from this group would be required to improve resolution of these relationships. Should future research support raising the *O. macropus* group to generic rank, the genus name *Callistoctopus* (Taki 1964) is available although Voss (1981) has proposed that Taki's name is a synonym of *Octopus*. Furthermore, Norman (1993) resurrected Robson's (1929) name *Macroctopus* to define *O. macropus* group species including the large and robust species *O. maorum*. Greater phylogenetic resolution is required to revise this species group, its components and generic status.

2.4.5 Other genera

The positions of the monotypic genera *Ameloctopus, Cistopus* and *Grimpella* were not clearly resolved in this study. The two former taxa fluctuated in position depending on the tree and their affinities to *Octopus* taxa of this study are indiscernible based on the present data. To further investigate these relationships, a highly conserved gene would be required for phylogenetic reconstruction in addition to further sampling of additional species.

2.4.6 False eyespots and the origins of the blue-ringed octopuses

False eyespots ("ocelli") are observed widely in the animal kingdom and best known from the Lepidoptera as body patterning components used in defense to intimidate and deflect predators (Blest 1957). False eyespots often exist as pairs and their colours and components are often quite striking. In certain octopuses these distinctive eye spot patterns comprise a dark spot and/or iridescent blue ring and are present as a pair on the lateral webs, one on each side, between the base of arms 2 and 3 (see *O. mototi*, Plate 2.3a). Species that possess such patterns are known as "ocellate octopuses" and the

spots are displayed when the animal flares the arms and webs in alarm displays (Hanlon and Messenger 1996) (for examples see Plate 2.3). Ocellate octopuses are known from a number of species groups of benthic shallow-water octopuses suggesting that ocelli have evolved independently in octopodid evolution (Norman 1993). Table 2.7 lists known ocellate octopuses by species group. The most dramatic form of this character exists in the blue-ringed octopuses, genus *Hapalochlaena,* where the entire body is covered in iridescent blue rings/lines (see Plate 2.3b-d). Interestingly, for many *Hapalochlaena* species the largest ring occurs in exactly the same location as the ocellus of the ocellate octopuses.

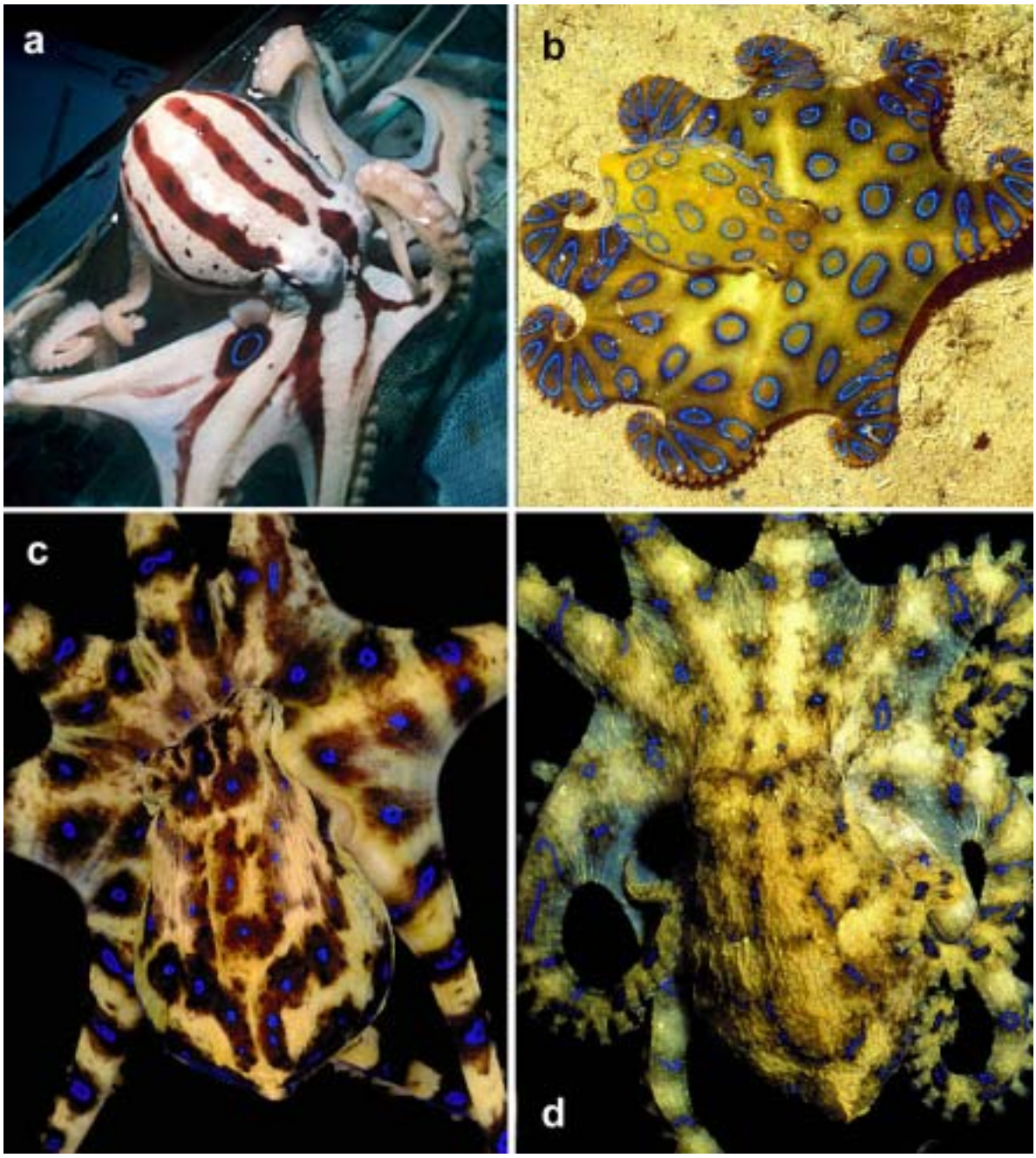

Plate 2.3: Photographs of ocellate octopuses a. *Octopus mototi* (photo Mark Norman) and representative blue-ring octopuses b. *Hapalochlaena* sp. 1 (Northern Territory) (photo by Clay Bryce); c. *H. maculosa* (photo by David Paul); d. *H. fasciata* (photo by Mark Norman).

Table 2.7: List of ocellate octopuses from the genus *Octopus* and the nature of their false eyespots. Iridescent rings are present (+) or absent (-).

Species	Iridescent	Ocellus	Reference
Octopus sensu strictu			
O. bimaculatus	+	Blue ring of open spoked links	1
O. bimaculoides	+	Blue ring of closed chain links	2
O. filosus	+	Simple blue ring inside	3
O. maya	-	Central light spot in ocellus	4
O. oculifer	-	Dark ocellus with thin outer ring	5
***Octopus aegina* group**			
O. fangsiao	+	Simple gold-green ring inside	6
O. siamensis	+	Simple white ring inside	7
O. rex	+	Simple pink-purple ring inside	7
O. neglectus	+	Simple blue-purple ring inside	7
O. mototi	+	Simple blue ring inside	8
O. polyzenia	+	Simple blue ring inside	9
O. exannulatus	-	Simple and dark	8
O. robsoni	+	Simple white, blue or pink ring	10
O. varunae	+	Iridescent ring, colour not reported	11
Unplaced taxa			
O. cyanea	-	Dark spot with dark outer ring	9
O. micropyrsus	-	Simple white spot	12
O. parvus	-	Simple white spot	13

Note: 1, (Verrill 1883); 2, (Pickford and McConnaughey 1949); 3, (Howell 1867); 4, (Voss and Solis Ramirez 1966); 5, (Hoyle 1904); 6, (Orbigny 1835-1848); 7, (Nateewathana and Norman 1999); 8, (Norman 1992b); 9, (Gray 1849); 10, (Adam 1941); 11, (Oommen 1971); 12, (Berry 1953); 13, (Sasaki 1917).

Results of this study provide insights into the origins of blue-ring octopuses and their complex skin colouration and patterning. Some evidence for the *Octopus aegina* species group being the closest relative of the toxic blue-ringed octopuses (genus *Hapalochlaena*) was observed. The *O. aegina* group contains a large number of ocellate octopuses (see Table 2.7) and *O. mototi* has been reported by the local peoples

of Rapa Island in the South Pacific to be poisonous (Norman 1992b). The ocelli of *O. mototi* are also the most dramatic alarm display form within the *O. aegina* group consisting of dramatic maroon stripes over a white base with highlighted iridescent blue ocelli (see Plate 2.4a). Because dramatic colouration and patterning are often associated with toxicity in marine organisms, it is possible that the ancestor to the *Hapalochlaena* was *O. aegina*- or *O. mototi*-like with iridescent blue rings and some toxicity. Subsequent selection for increased toxicity associated with the duplication of blue rings may have led to development of a complex warning display and defense mechanism resulting in the displays observed in the *Hapalochlaena*. Based on morphological character data, *Hapalochlaena* is thought to be a derived group because in some species the ink sac, which is an ancestral character, is reduced (Voss 1988). The reduction of this important octopodid defense mechanism (i.e. the ink sac) suggests that being poisonous, as advertised by numerous blue rings, is an effective means of defense that is probably selectively advantageous and possibly more effective than the ancestral ink sac.

2.4.7 Conclusions and future directions

The data presented in this study suggest the genus *Octopus* is not monophyletic and it is clear that the systematics of the subfamily Octopodinae requires major revision. The present phylogeny revealed strong relationships among closely related species and some information on divergences at the species group level, however deep relationships among the Octopodinae remain unresolved. In particular, the *Abdopus* and *O. vulgaris* groups and representatives from genera other than *Octopus* did not find a stable position within the phylogenies. It is thought that the genes used in this study were better suited to examination of recent phylogenetic relationships, which has helped us to understand relationships among closely related taxa, rather than deeper divergences among genera and species groups. Based on these results, it is strongly recommended that care be taken when choosing nuclear genes for resolving divergences among genera and species groups particularly if evidence of a second copy of the gene of choice is observed. To further improve the resolution among distantly related octopodines, it is suggested that more sequence data be obtained from conserved genes. Two possible gene markers that have been used in elucidating such relationships in other studies are the *Wingless* (Regier and Shultz 2001) and *Pol II* (Shultz and Regier 2000) genes.

Our understanding of phylogeny and systematics of the Octopodinae has benefited from this study. A better knowledge of the subfamily could be attained by sampling further species from within the genus *Octopus*, more genera within the Octopodinae and representatives of other subfamilies within the Octopodidae.

CHAPTER 3 Evolution of reproductive strategies in the benthic shallow-water octopuses (Cephalopoda: Octopodinae).

3.1 INTRODUCTION

3.1.1 Evolution of reproductive strategies in benthic marine invertebrates

Embryonic and post-embryonic life history traits directly affect fitness (Futuyma 1986). The existence of high inter- and intra-specific variability in these traits is an indicator of the potential selective forces involved in trait evolution. Many marine invertebrate congeners have evolved disparate life history strategies where the strongly associated traits of egg and clutch sizes differ dramatically (e.g. Perron 1986; Lessios 1990; Hart 1995; Wellington and Robertson 2001). Additionally, these species usually diverge in juvenile morphology and juvenile habitat occupation (Thorson 1950; Strathmann 1978; Strathmann 1985; Emlet 1995). The two key life history strategies that are commonly observed among closely related species comprise 1) small eggs, large clutch sizes and planktotrophic juveniles and 2) large eggs, small clutch sizes and benthic juveniles.

Life history theory predicts that the evolution of such dichotomous reproductive strategies involves a fecundity-survival trade-off because of the influence of size on juvenile survivorship (e.g. Bagenal 1969; Vance 1973; Smith and Fretwell 1974; Christiansen and Fenchel 1979; Ferguson and Fox 1984; Sinervo 1990). In turn, egg size is thought to reflect a balance between fecundity and mortality risk (Sinervo 1990; Hart 1995) because high rates of juvenile mortality intensify selection on early life stages (Vance 1973; Sibly and Calow 1985; Fox 2000). The rationale for this hypothesis in benthic marine invertebrates is that the strong relationship between egg size and juvenile type reflects the high level of constraint that total energy invested into each egg imposes on embryonic development time and juvenile type (Emlet et al. 1987; Sinervo and McEdward 1988; Emlet 1995; Hart 1995). Furthermore, under certain environmental conditions (i.e. heterogeneous environments), production of many small, defenseless progeny may be catastrophic to subsequent generations particularly in semelparous organisms. In these situations strong, well-developed young are often at

an advantage compared to smaller juveniles (Sibly and Calow 1986; Calow 1987; Stearns 1992).

The main factors involved in the evolution of early life history traits of benthic marine invertebrates are environmental temperature and food availability (e.g. Thorson 1950; Vance 1973; Christiansen and Fenchel 1979; Levitan 1993; Havenhand 1995; McEdward 1997; McEdward and Miner 2003), and maternal size and the level of maternal investment per egg (Parker and Begon 1986; Sinervo 1990; Poulin 1995). Temperature and food availability directly affect growth rates while maternal size and level of investment by the mother into each egg directly determine the size of eggs and progeny.

Among benthic marine organisms it has been observed that juvenile types vary with differences in latitudinal gradient (Thorson 1950; Vance 1973; Christiansen and Fenchel 1979). This variation is usually attributed to food and temperature effects which differ between geographic regions (Thorson 1950). Growth and development in poikilotherms (animals unable to regulate their internal body temperature), in particular, is influenced by these factors such that growth can be extended or shortened depending on the environmental conditions (Van Heukelem 1979). Theoretically, planktotrophic juvenile types predominate in tropical environments, even though this planktonic environment is highly competitive and risky, due to the generally constant nature of this environment. The long periods of warmth, good light and consistent resource availability (in the plankton) that characterise the tropics promote fast growth, short life cycles and high plankton population turn over (Lowe-McConnell 1977; Nybakken 1996) and favour high fecundity reproductive strategies. Conversely, temperate coastal regions are characterised by large seasonal fluctuations in resource availability and temperature, particularly in the plankton (Menge et al. 1997). For many species living in these latter environments, a common juvenile strategy is to have prolonged egg incubation, larger eggs and progeny whose degree of development at time of hatching is advanced. In producing competitive benthic juveniles, organisms are able to avoid the temperate planktonic realm and reduce the high risk of juvenile mortality in this highly fluctuating environment.

In the marine environment, temperature and food vary according to latitude, season and water depth (Forsythe and Van Heukelem 1987). It has therefore been hypothesised that macro-environmental differences that vary with latitude can exert strong selective forces on egg size and juvenile type (Thorson 1950; Christiansen and Fenchel 1979; Kingsford 1995; Nybakken 1996). Many empirical studies of egg size have demonstrated this association at the intra-specific level (e.g. Hancock 1998; Moltschaniwskyj and Martinez 1998; Fox and Czesak 2000; Forsythe et al. 2001; Pecl 2001; Cardillo 2002; Jackson and Moltschaniwskyj 2002). The inference from empirical studies of selection is that egg size is adaptive, particularly under adverse conditions (Parker and Begon 1986; Sibly and Calow 1986; Sinervo and McEdward 1988; Azevedo et al. 1996; Fox 2000). In this study, latitude, represented by the midpoint of a species distributional range, was used to represent a gradient of macro-environmental variation.

In addition to examining the influence of environmental variation on egg size, understanding the influence of maternal body size on egg size is also important to any study of egg size evolution, particularly when examining animals with a semelparous life cycle. This is because survival of future generations in semelparous organisms generally requires optimal investment into progeny by parents (Calow 1987; Roff 1992; Stearns 1992).

Egg size is widely defined as a maternal trait because the amount of energy that a mother invests in each embryo mediates the quality and quantity of offspring (Lack 1968; Smith and Fretwell 1974; Sinervo 1990). An important maternal factor that influences total energy investment per egg is size at maturity (Stearns 1992) because large females are thought to have greater energy reserves to allocate than small females (Parker and Begon 1986; Simpson 1995; Fox and Czesak 2000; García-Barros 2000). Generally, species that have large female size lay larger eggs than species with smaller body sizes (Fox and Czesak 2000). Interestingly though, larger bodied species usually invest a smaller proportion of their resources into each egg and lay more eggs than small bodied species. The main reason for this is that selection generally favours high fecundity (large clutch sizes) in large females but as the number of eggs increases the amount of resources available to the progeny, and in some cases, the size of eggs decreases (Roff 1992; Stearns 1992). Therefore, when the total amount of resources

available to a mother to invest into progeny is constant, the trade-off between egg size and egg number results in a decrease in egg size as maternal fecundity increases.

Examining patterns of trait evolution is difficult to do empirically because studies of present day taxa and their traits do not take historical processes or phylogenetic relationships into account (Harvey and Pagel 1991; Hart et al. 1997; McHugh and Rouse 1998). A few studies have examined egg size and juvenile type at the inter-specific level by incorporating phylogeny into comparative analyses. Findings from these studies suggest that egg size may respond adaptively to environmental pressures (Poulin 1995; Levitan 2000), but the degree to which change occurs is constrained by phylogeny (Lessios 1990). Results such as these contradict the widely accepted tenet that egg size is an evolutionarily stable trait that evolves towards one end of a continuum depending on selective pressures present (Vance 1973).

Selection pressures associated with body size and macro-environmental variation were examined here to investigate the evolution of egg size and juvenile type, and consequently reproductive strategy, using a comparative phylogenetic approach.

3.1.2 Reproductive strategies of the Octopodinae

In direct contrast to most cephalopod species, the Octopodinae belong to a family of octopuses (Octopodidae) that have two reproductive strategies: "small egg - planktonic juvenile type" and "large egg- benthic juvenile type" (Boletzky 1977, 1978, 1987b, 1992). As a rule, the variation between these strategies is inter-specific (Voight 1998) and the differentiating factors between the strategies are egg size, clutch size, degree of juvenile development at hatching and juvenile niche preference (Young and Harman 1988). Juvenile type is associated with the negatively correlated traits: egg size and clutch size (Calow 1987). In this study the juvenile types and reproductive strategies are not directly known for most species. However, due to the relationship between juvenile type, reproductive strategy and egg size, these traits could be inferred from egg size. This was achieved by using the ratio of egg size to adult body size (i.e. mantle length). Eggs that are ≤10% of adult mantle length are indicative of a 'small egg type' (Boletzky 1992). Those species that produce small eggs usually have very large clutch sizes and typically have planktonic young. This type of reproductive strategy is described here as a "small egg - planktonic juvenile" strategy. Alternatively, species

that produce eggs of a 'large egg type', that is >10% of the adult mantle length (Boletzky 1992), usually produce very few young that have a benthic mode of life at hatching (Boletzky 1977). This type of reproductive strategy is described here as a "large egg - benthic juvenile" strategy.

There are three distinguishing features of a "large egg - benthic juvenile" reproductive strategy in octopuses. Eggs are large and yolky, developing over a number of months. At hatching, juvenile development is direct and these progeny are extremely dexterous and capable of surviving in highly competitive benthic environments (Boletzky 1987a). As a result of their large size, relatively few progeny are produced in a single clutch. This reproductive strategy is considered a derived character state (Boletzky 1987a; Engeser 1990) and potentially an adaptation to adverse environmental conditions such as cold temperatures (Boletzky 1994) and/or deep-sea environments (Voss 1988) due to the robust nature of its embryonic and post-embryonic stages. The benthic strategy is common in temperate shallow-water species such as *Octopus australis*, *O. berrima* (Stranks and Norman 1992), *O. pallidus* (Stranks 1988b), *O. kaurna*, *O. bunurong* (Stranks 1990), *Grimpella thaumastocheir* (Stranks 1988a), *Hapalochlaena maculosa* and *H. fasciata* (Stranks 1998). However, some tropical and equatorial species like *O. graptus* (Norman 1992a) also have medium to large sized eggs and a benthic juvenile strategy.

In direct contrast, the "small egg - planktonic juvenile" strategy is characterized by minimal energy investment into each egg and thousands to hundreds of thousands of eggs are produced per clutch. The time to hatching for these progeny is much shorter than benthic species and the post-embryonic stage comprises a period of growth and development in the plankton during which juveniles are highly energetic and actively hunt prey prior to settlement (Rees 1950; Nixon and Mangold 1996). At settlement, planktonic juveniles develop quickly to become as well equipped as their benthic counterparts are at hatching (Voight 1994; Nixon and Mangold 1996). Planktonic juvenile survival after hatching is not well documented but is expected to be low, given the highly competitive and risky planktonic environment in which they live at this time and the levels of mortality observed in other marine invertebrates with a planktonic developmental stage (Gosselin and Qian 1997). Small species with a planktonic phase dominate the tropics, but variation in egg sizes, female size and geographic distribution

is high. Compared to many species that live in the tropics, temperate species such as *O. vulgaris* and *O. maorum* (Smale and Buchan 1981; Mangold 1987; Grubert and Wadley 2000) are distinctive because they have large body sizes, tiny eggs relative to their body size, and planktonic juveniles.

Potentially high flexibility in the evolution of these strategies is observed in differences in strategy between recently divergent taxa (e.g. sympatric species). For example, the sibling species *O. bimaculatus* and *O. bimaculoides* probably diverged following the closure of the Isthmus of Panama (Voight 1988) and show little difference in morphology but are clearly distinguished based on a number of features including their opposing juvenile life history traits (Pickford and McConnaughey 1949). The disparity in these traits between closely related taxa is attributed to habitat (Pickford and McConnaughey 1949) and environmental temperature differences in the geographic distributions of the species (Ambrose 1988). These simple observations of differences in juvenile types between closely related taxa suggest the switch from one juvenile type to the other may have occurred on numerous occasions in evolutionary history (Boletzky 1987a) and their divergence could be the product of encountering new environmental conditions. It is likely that these reproductive strategies are sufficiently variable genetically to change according to different selection pressures.

Life history theory predicts that when two alternative strategies are maintained among species, like those of the Octopodinae, both strategies have the potential to maximise fitness. The maintenance of one strategy over another in a species is thought to be influenced by the environmental niche that the species occupies. It has been suggested by Boletzky (1987a) that the planktonic juvenile type is an ancestral character among the Octopodinae and that evolutionary transitions in juvenile type have always been in the direction of planktonic to benthic juvenile type among taxa. This suggests that the benthic juvenile type in octopuses is a derived trait that may be an adaptation to some environmental conditions (Boletzky 1987b). The objective of this component of the project was to examine the pattern of evolution in these juvenile types as well as the potential factors that have maintained a dichotomy in egg sizes and juvenile types among species and influenced the evolution of these reproductive strategies. It was hypothesised that if transitions in juvenile types have evolved predominantly in the direction of planktonic to benthic, as suggested by Boletzky, benthic juvenile type and

large egg sizes may be an adaptation to certain selection pressures. In this way it was of interest to examine what these factors might be.

A comparative phylogenetic approach was used to examine four primary aims to this component of the study. The first aim was to identify the pattern of evolution in egg size and juvenile type among species. Using a comparative phylogenetic approach it is possible to investigate whether evolutionary shifts in these traits have been frequent or conserved and whether these transitions have been associated with differences in environment type. The final aim was to identify whether body size has influenced egg size and juvenile type evolution. As described above, the underlying hypothesis of this approach was that phylogenetic inertia has been minimal, with egg size and reproductive strategies responding swiftly to ecological factors.

Octopuses are useful for examining the evolution reproductive of life history strategies in a comparative phylogenetic framework for a number of reasons. Firstly, they posses extreme inter-specific variation between the adult and juvenile life-style. This feature is distinctly different to most cephalopods and permits a comparison of life history strategies between species. Secondly, there is a strong association between egg size and juvenile type in these animals, which allows tentative inference of juvenile type from egg size. Thirdly, newly hatched generations are extremely sensitive to environmental variation and the semelparous life cycles of octopuses indicate that the strategies of extant taxa are selectively advantageous under recent historical processes within the environment they occupy. Finally, there is distinct variation in habitat preference and geographic distributions among species, which permits an examination of covariation between the traits and macro-environment types. For practical reasons octopuses are suitable because species distributions and life cycle characteristics of described taxa are well documented. Furthermore, a suitable phylogeny is available (Chapter 2) which permits the analysis of reproductive strategies to be performed in a phylogenetic context.

Egg size and its derivatives were primarily used in analyses of association among traits. There were two assumptions that permitted egg size to be used as an indicator of juvenile type. Firstly, the well-documented relationships between both egg size and clutch size, and egg size and juvenile type (Boletzky 1977, 1978, 1987b, 1992).

Secondly, egg size as a percentage of body size provides an index that permits inference of juvenile type using the 10 percent rule of Boletzky (1992).

There are two main factors that have the potential to influence analyses of egg size and latitude. They are 1) body size and 2) species that have adapted to the productivity and temperature gradients of deep-sea environments rather than latitudinal gradients. To account for the effect of body size on egg size in analyses of latitude and egg size, the residuals from the regression between egg size and body size were used to incorporate the effect of egg size independent of body size into the analyses. To further ensure that latitudinal effects alone were studied, analyses were restricted to benthic shallow-water octopus species (subfamily Octopodinae), which are exposed to selective forces of surface temperature and productivity, to avoid the confounding effects of species adapted to the very different conditions of deep-sea habitats.

3.2 MATERIALS AND METHODS

3.2.1 Inter-specific variation in life history traits

To explore the influence of latitude and body size on inter-specific variation in both egg size and juvenile type a series of tests were initially carried out (see section 3.2.2 for definition of juvenile type). These correlation tests assume that individual taxa are independent of one another and therefore do not account for phyletic relationships between the taxa. This exploratory investigation was based on an earlier study into the influence of latitude on reproductive strategies of shallow-water octopuses by Voight (1998), which was expanded upon here with new data. A large number of shallow-water octopus species were included in this analysis to gain insight into trends that exist across a wide range of species. Tests for association included the following comparisons: a) egg size and latitude, b) juvenile type and latitude, and c) egg size and body size. Adult body size has the potential to play a role in the evolution of egg size. To ensure that any relationship between egg size and latitude was not influenced by an allometric relationship between egg size and adult body size, a further test for correlation was estimated for the residual variation in egg size after accounting for body size, and latitude. In this way it was possible to account for the influence of body size on any relationship between egg size and latitude.

3.2.2 Data

Egg size, body size and species distributions were obtained from the published data set of Voight (1998) and references cited therein (Hochberg et al. 1992; Norman 1992a, 1992b; Mangold 1998; Stranks 1998; Toll 1998; Toll and Voss 1998; Voss and Toll 1998). Data for additional taxa were also obtained from references cited in Appendix 3b. All raw data used in this study is listed in Appendix 3a and 3b.

Latitude was used here as an indicator of macro-environmental variation. For each species, latitude was calculated as the average of the northern- and southern-most species distributions reported in the literature. Generally, species distributions are reported in the literature as localities of where a species is found and/or the boundaries of its distribution. The latitudinal degrees and minutes of these localities and boundaries were estimated for the furthest northern and southern locations reported in

the literature. Latitudes for species whose distributions are wide-ranging or geographically disparate were estimated in the same way. Data for all species was cross-referenced between old and new references of species distribution to ensure the information was up to date (see Appendix 3b for references). For all analyses, the average latitudes were represented as degrees from the equator (i.e. absolute latitude) to ensure a continuous environmental gradient was represented.

Estimates of egg size were obtained from the literature (see Appendix 3b for references) and the longest reported egg lengths (mm) were recorded. Species were excluded from the analyses if the only data available on egg size was from immature egg data to eliminate ambiguity in egg sizes. The measure of adult body size (i.e. body size) used here was the longest mantle length (mm) reported for each species. Both egg size and body size variables were $\log_e$ (*ln*) transformed for all analyses in order to stabilise the mean-variance relationship.

The evolution of life history traits in shallow-water octopuses was examined in this study, particularly the traits of egg size and juvenile type. The term "reproductive strategy" is used here to describe the two traits of octopus life history that are crucial to species fitness: egg size and juvenile type. Egg size by definition, describes the overall size and quality of progeny at the embryonic stage whereas juvenile type is the phenotype of offspring immediately after hatching, that is, the post-embryonic stage. In octopuses, juvenile type is strongly associated with egg size because the size and degree of development of an individual juvenile at hatching depends on the size of its egg and the amount of nutrients that are available for it to absorb during its embryonic stage.

As discussed in the Introduction to this chapter (section 3.1.2), there are two reproductive strategies amongst the benthic shallow-water octopuses that encompass the relationship between egg size and juvenile type. They are: the "small egg - planktonic juvenile strategy" and the "large egg - benthic juvenile strategy". In general, information on egg size is readily available in the literature for described species but data on juvenile types is mainly known for only a small number of well-studied species. To infer the juvenile type of a species, the phenotype (i.e. character state) of egg size in a species was used here as an indicator of juvenile type, using the 10% rule of Boletzky (1992). This rule is based on the relationship between egg size, adult body size and

juvenile type so that eggs ≤10% of adult mantle length are of a small egg type and indicate a planktonic juvenile type, while species with eggs >10% of adult mantle length, indicate a large egg type and benthic juvenile type. To determine the percentage of egg size to body size, the Egg Length Index (EgLI) was calculated for each species, where egg length (i.e. egg size) as a percentage of adult mantle length (i.e. body size) is estimated. Therefore, those species with EgLI ≤10% were classified as 'small egg type' species with an inferred 'planktonic juvenile type', while species with EgLI >10%, were categorized 'large egg type' species and a 'benthic juvenile type'. These juvenile types were subsequently used as the binary character states in an analysis of the pattern of evolution and ancestral character state reconstruction in juvenile types.

To remove the influence of body size from the relationship between egg size and latitude, residuals from the regression of *ln*(egg size) on *ln*(body size) were calculated for both the complete data set (Appendix 3a and 3b) and for the subset of species that were used in phylogenetic comparative analyses (see Table 3.1 for a list of species and section 3.2.4 for analysis details). These residuals provided a measure of "residual egg size" after accounting for body size, which were used in correlation analyses.

3.2.3 Tests for correlation between traits not adjusted for phylogeny

To test for associations between traits, 73 benthic shallow-water octopus species were compared using Pearson's product-moment correlation test. This tests for linear association between two variables and the correlation coefficient (r) indicates the degree of association between variables. Scatter plots were used to display relationships among pairs of variables. Additionally, data points were coded according to the estimated juvenile type in the plot of latitude against egg size. This provided a visual interpretation of the distribution of the juvenile types over the latitudinal gradient with egg size.

3.2.4 Evolutionary analysis of reproductive strategy traits in the Octopodinae

Overall, the primary interest of this study was to examine the evolutionary history of egg size and juvenile type and whether these traits covary with environmental variability as represented by latitudinal gradient. In order to investigate ancestral states and patterns of inter-specific variation in these traits in the context of their evolution,

both ancestral state reconstruction and correlation analyses that incorporate the influence of phylogeny were conducted. Phylogenetic relationships were not known for all species used in the correlation analyses described in the previous section, therefore a subset of species was used from the complete data set for which phylogenetic relationships among taxa are known (Chapter 2). Using a comparative phylogenetic analysis approach it was possible to gain an historical insight into evolutionary transitions of juvenile types in octopuses and factors that have influenced this evolution.

The Bayesian Consensus (BC) phylogeny estimated in Chapter 2 was used to represent the phylogenetic relationships in these analyses because it is the best hypothesis of octopodine phylogeny to date. A number of taxa were excluded from the present analyses because egg sizes are unknown for these species. These include, *Hapalochlaena* sp. 1, *Octopus* sp. 5, *O.* sp. 8, *O.* sp. 10, *O.* sp. x (Sth. Africa), *O.* cf. *kagoshimensis* and *O. oculifer*. A further three species, *O. alpheus*, *O. aspilosomatis* and *O. dierythraeus*, were also excluded because the only data presently available on egg sizes for these taxa is based on immature eggs. The two outgroup species *Opisthoteuthis* sp. and *Argonauta nodosa* were also excluded from analyses because present hypotheses pertained specifically to the evolutionary history of traits among shallow-water octopuses. These outgroup taxa were potentially confounding to the analysis because they both occupy different niches and they are likely to have experienced selection pressures disparate to those of the shallow-water octopuses during their history. *Opisthoteuthis* sp. is a deep-sea finned octopus and its life history strategies are strongly adapted to life at abyssal ocean levels (Voss 1988). Likewise, *A. nodosa,* a pelagic octopus, is more likely to be subjected to selective forces associated with the pelagic realm rather than the shallow-water realm that is of interest here. *A. nodosa* was used in the analysis of ancestral state reconstruction for the purpose of rooting the tree because it is a close ancestor to the benthic shallow-water octopuses. In total, 22 taxa were used in the analysis of trait evolution (see Figure 3.1 for the phylogenetic tree used here). Data on body size, egg size and latitude were obtained from the cited references (see Table 3.1). The EgLI was also estimated for each species as described in section 3.2.2.

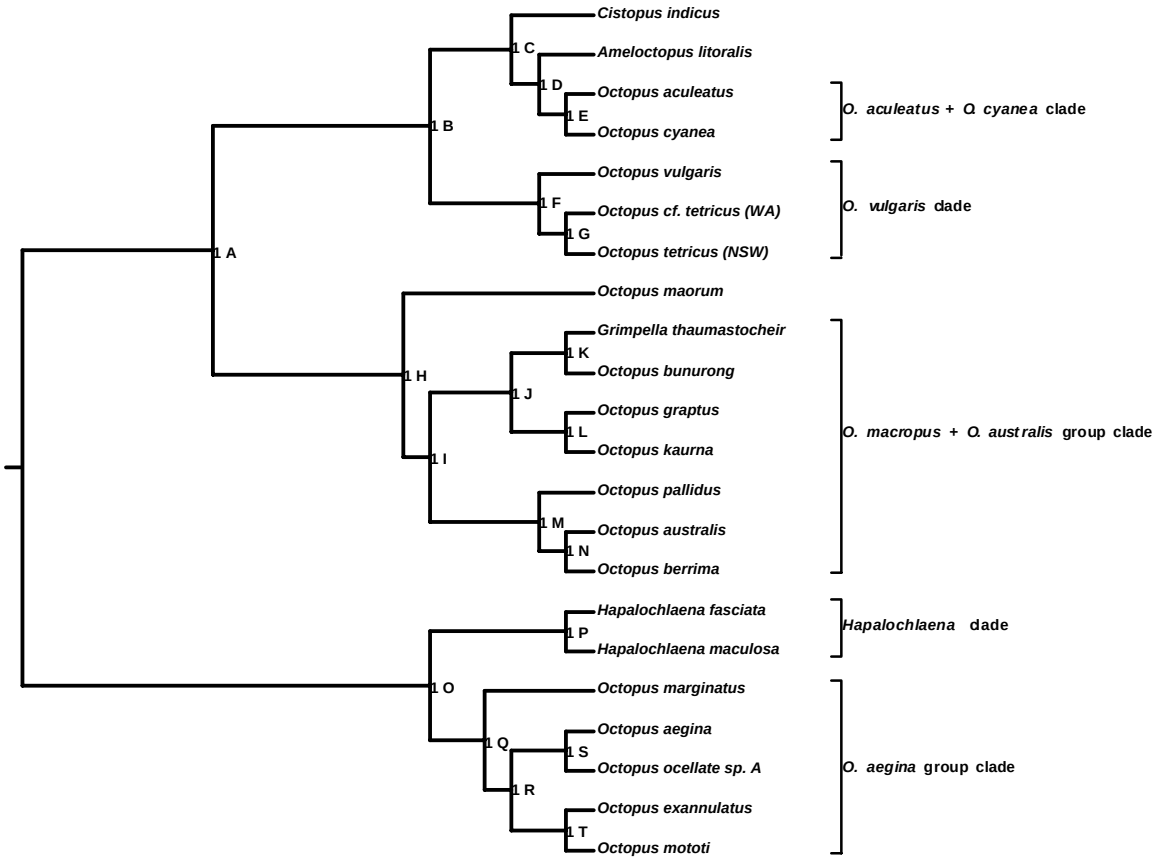

Figure 3.1: Phylogeny of the shallow-water octopuses used to determine the phylogenetic distance among species used in comparative analyses of association among traits and variables. Alphanumeric codes describe individual nodes on the tree. Clades are described on the right according to their species groups (as described in Chapter 2, by Robson (1929)).

Table 3.1: List of taxa and estimates of their respective latitude, body size (i.e. mantle length), egg size and residual egg size. † Indicates binary characters for inferred juvenile types, planktonic juvenile type (EgLI ≤10%) = 0 and benthic juvenile type (EgLI >10%) = 1. *Denotes species used in ancestral character state reconstruction analysis only.

Species	Latitude	Body size	Egg size			Residual egg size	Source
	(degrees)	(mm)	(mm)	(EgLI)	†	*ln*(mm)	
*Argonauta nodosa**	-	100	1.5	1.5	0	-	a
Ameloctopus litoralis	21.00	30	10.0	33.3	1	0.27	b, c, d
Cistopus indicus	15.00	86	5.0	5.8	0	-0.17	d, e, f, g
Grimpella thaumastocheir	35.75	40	15.0	37.5	1	0.74	h
Hapalochlaena fasciata	32.00	45	9.0	20	1	0.26	c, d
H. maculosa	36.25	57	9.0	15.8	1	0.32	c, g
Octopus aculeatus	0.50	58	3.0	5.2	0	-0.77	i, d, e
O. aegina	4.00	62	2.0	3.2	0	-1.16	d, e, f, g
O. australis	32.00	72	12.0	16.7	1	0.67	c, d, j
O. berrima	37.75	105	14.0	13.3	1	0.91	c, j
O. bunurong	36.25	95	10.0	10.5	1	0.55	c, m
O. cyanea	2.00	172	3.0	1.7	0	-0.51	d, e, f, l
O. exannulatus	6.00	50	3.9	7.8	0	-0.55	e, d, l
O. graptus	14.50	190	28.0	14.7	1	1.75	d, k
O. kaurna	36.25	85	11.0	12.9	1	0.62	c, m
O. maorum	41.75	255	6.0	2.4	0	0.28	c, g, h
O. marginatus	8.50	80	3.0	3.8	0	-0.69	d
O. mototi	0	100	3.2	3.2	0	-0.58	d, l
O. ocellate sp. A	1.00	60	3.0	5.0	0	-0.77	d, f
O. pallidus	37.75	73.5	11.0	15.0	1	0.58	c, h, n
O. tetricus (NSW)	30.50	140	3.0	2.1	0	-0.56	d
O. cf. *tetricus* (WA)	29.75	250	2.4	1.0	0	-0.64	g
O. vulgaris	7.50	200	2.7	1.4	0	-0.57	g, o

Note: a, (Norman 2000); b, (Norman 1992c); c, (Stranks 1998); d, (Norman 1998); e, (Norman and Sweeney 1997); f, (Nateewathana 1997); g, (Hochberg, et al. 1992); h, (Stranks 1988a); i, (Norman and Finn 2001); j, (Stranks and Norman 1992); k, (Norman 1992a); l, (Norman 1992b); m, (Stranks 1990); n, (Stranks 1988b); o, (Mangold 1998).

When examining covariation between traits, historical non-independence among taxa may confound analyses if relationships between traits reflect common phylogenetic descent rather than adaptation (e.g. Clutton-Brock and Harvey 1984; Felsenstein 1985a; Harvey and Pagel 1991; Harvey and Purvis 1991). It is possible to test and reconstruct historical events of evolution among extant species using information on their character states and phylogeny (Pagel 1999a). Maximum likelihood inference of trait evolution estimates the likelihood of the observed data given a model of character state evolution (Edwards 1972; Pagel 1999a). Such estimation methods of character state evolution have been developed for both discrete (Schluter et al. 1997; Pagel 1999a) and continuous characters to examine ancestral states, and covariation between traits and variables among species.

3.2.4.1 Discrete character analysis

Methods of testing for covariation between discrete characters (i.e. binary character states that represent the phenotypes of a trait) in a Maximum Likelihood framework largely employ two models of trait evolution (Pagel 1994, 1999b; Schluter et al. 1997; Maddison 2000; Lewis 2001). These models describe 'Independent' and 'Dependent' patterns of evolution to estimate the likelihood that the observed character states have evolved independently or by some other process such as a dependent covariation with another variable.

To explore the pattern of evolution in the trait "juvenile type", the method of Pagel (1999a) was employed to reconstruct the ancestral states among the discrete character states "planktonic juvenile type" and "benthic juvenile type". The ancestral state for each internal node was estimated in a Maximum Likelihood framework (Calculate Fossil Likelihood command) in DISCRETE 4.0 (Pagel 1994, 1997, 1999a). This method estimates the likelihood of each character state being ancestral at a given node under the Independent model of trait evolution and then compares both values to infer the ancestral state. The likelihood ratio test was then employed to estimate which character was most likely based on the likelihood ratio test statistic, an indicator of difference between two models that can then be compared to a χ^2 distribution to test for statistical significance (Pagel 1994). Estimation of the ancestral state at each node

allowed the pattern of evolution in both traits to be traced over the phylogeny. The parameters of the model were left free to be estimated for each node and a 'Local' likelihood estimation method was used to calculate the likelihoods. Under this search method the support for each ancestral state hypothesis is estimated for each of the discrete character states at a given node (Pagel 1999a).

3.2.4.2 Continuous character analysis

Methods that incorporate phylogenetic information into the analysis of covariation among continuous character states of a trait include phylogenetic Generalised Least Squares (GLS) (Pagel 1997) and the Independent Contrasts (IC) method (Felsenstein 1985a; Harvey and Pagel 1991; Martins and Garland 1991; Garland et al. 1992; Pagel 1992). Both GLS and IC methods incorporate branch lengths and fit a model of trait evolution to identify non-independence between traits. Under the GLS method non-independence between species is controlled for by referring to a matrix of expected co-variances among species (Pagel 1997, 1999a). A number of parameters may also be estimated regarding the evolution of traits, including; kappa (κ), which describes punctual versus gradual trait evolution, delta (δ), which defines accelerated or slowed evolution over time, and lambda (λ), which estimates the level of phylogenetic inertia observed in the traits. Alternatively, IC analysis corrects for phylogenetic non-independence in traits by estimating independent contrasts between species (i.e. tree tips) and nodes for each trait that indicate differences in these traits between taxa and groups of taxa.

The GLS approach was implemented with the program CONTINUOUS 1.0d13 (Pagel 1994, 1997), to test whether the evolution of egg size and juvenile type were associated with variation in latitudinal gradient and/or body size. Under the GLS method, a phylogenetic regression is estimated where a variance parameter of trait evolution is estimated by fitting a 'Random Walk' model, i.e. one that describes a drift-like pattern of trait evolution, to the data (Pagel 1997; Schluter et al. 1997). This method controls for non-independence between species by referring to a matrix of expected covariances among species (Pagel 1997, 1999a) and is particularly useful in analyses where species are closely related. Two models are available within the CONTINUOUS package but only Model A, a one-parameter random-walk model that accounts for instantaneous

variance of evolution was employed here. Model B, a two-parameter directional random-walk model that accounts for both instantaneous and directional changes was not used because it is not suitable for analyses where phylogeny is described by an ultrametric tree. Nested hypothesis tests were used to test whether the traits covary. The Null Model was set to Model A, and the covariances among pairs of comparisons (i.e. between nodes and tips of the phylogeny) were constrained to be 0 when calculating the likelihood. The Alternative Model was also set to Model A but the covariances were left free to be estimated. In this way, the null hypothesis is nested within the alternative model and provides a test of whether the correlation between two traits is greater than 0. An estimated probability less than 0.05 is indicative of covariation between the traits after the influence of phylogeny has been removed. The model parameters, κ and δ were left at the defaults of the program while λ was set to 1 (exploratory analyses estimated the value of λ to be 0.97). The reason that only λ was changed according to the estimated value of the parameter, was that very little is known of the application of the parameters κ and δ, and the procedure of only implementing estimated λ by Freckleton et al. (2002) was followed.

Using the phylogenetic GLS approach, covariation of *ln* egg size with two independent variables, latitude and *ln* body size was investigated. To examine whether observed covariation between egg size and latitude was influenced by an allometry between egg size and body size, estimated residual egg size (after accounting for body size) was tested against the independent variable, latitude. Covariation between the inferred binary juvenile type characters and latitude was also tested.

To examine whether egg size, body size and latitude were influenced by the phyletic relationships among taxa and whether phylogenetic correction was required for these data (Pagel 1999a; Freckleton et al. 2002), the program CONTINUOUS was used to estimate the parameter lambda (λ), which is a measure of phylogenetic inertia and non-independence among species. This was examined here by setting λ to 0.0 in the Null Model A, then estimating λ under the Alternative Model A, and then performing a likelihood ratio test to determine the model that the data fits best.

Phylogenetic IC and GLS approaches are known to be functionally identical (Garland and Ives 2000), although GLS methods additionally allow estimation of a number of phylogenetic parameters (as described above). IC analysis methods were used to estimate contrasts in trait values between adjacent taxa. IC analysis corrects for phylogenetic non-independence in traits by estimating independent contrasts. These are the value of a trait at each node of a phylogeny subtracted from the value of its sister node so that each species is compared to its nearest included relative (Felsenstein 1985a). Each character is treated separately and the contrasts are statistically independent of evolution in other parts of the phylogeny. To estimate a common variance, each independent contrast is divided by its standard deviation, which is calculated to include branch length information, and a "Brownian Motion" model of character evolution is assumed (Harvey and Pagel 1991). The regression through the origin of these contrasts estimates the relationship between the traits having accounted for phyletic relationships. These IC and the fitted regression line can be plotted to aid in the visual interpretation of the relationship between traits estimated by these analyses. Additionally, the residuals from the regression through the origin of the IC are available to determine that linear model assumptions are met and to examine regression diagnostics for outliers. These residuals and the option to examine model assumptions are presently unavailable under the GLS approach in the CONTINUOUS program.

The computer program PDTREE (Garland and Ives 2000) was implemented to estimate IC confidence intervals, regression residuals and plot diagnostic graphs. The regression diagnostics obtained to examine the influence of observations on the regression results included: Studentised residuals (also called leave-one-out, or jackknifed, residuals, these are standardised residuals where the fitted value and standard deviation are calculated omitting the current observation) that identify observations with large residuals; estimates of leverage that identify observations with potentially large influence on regression coefficients; Cook's Distance and DFFITS that combine information on the residual and leverage to provide general measures of influence (Rousseeuw and Leroy 1987; Barnett and Lewis 1994). Where these diagnostics indicated outlying values, observations were flagged for further investigation.

Formal tests for potential outliers in the residual values of the linear regression, through the origin, between IC were examined using Grubbs test for outlier identification (Grubbs 1950, 1969). This test estimates the Z ratio statistic as the difference between the largest (or smallest) residual and the mean of all residuals divided by the standard deviation of the residuals. This statistic indicates how far the most extreme residual is from the other points and can then be compared to a table of critical values given the sample size, to determine whether the point is a significant outlier. Outlying data points are of concern in statistical analyses because they can be highly influential, having the effect of obscuring underlying patterns or trends, particularly if a data point has been influenced by processes different to those of other taxa in the data set. The manual of the CONTINUOUS program (Pagel 2002) warns against the potentially strong effects that outliers can have on model results. The definition of an outlier used here is that of Barnett and Lewis, "an observation which appears to be inconstant with the remainder of that set of data" (Barnett and Lewis 1994:7).

3.3 RESULTS

3.3.1 Tests for correlation between traits not adjusted for phylogeny

Correlation coefficients and the results of tests for correlation are shown in Table 3.2. A strong relationship was observed between both egg size (mm) and latitude and *ln* egg size and latitude (Table 3.2, Tests 1 and 2 $p \leq 0.0001$, see also Figures 3.2 and 3.3a respectively). This relationship held when the influence of body size was removed from egg size (Table 3.2, Test 4, and Figure 3.4). A non-significant correlation was found between *ln* body size and *ln* egg size (Table 3.2, Test 5, $p = 0.11$ and Figure 3.3b). Similarly, there was no relationship between latitude and body size (Table 3.2, Test 3, $p = 0.39$). Two outlier species, *O. graptus* and *O. conispadiceus* were observed in the test for correlation between egg size and latitude (see noted species in Figure 3.2). To examine whether these taxa influenced the correlations, Tests 1, 2 and 3 were recalculated after removing these taxa. Results showed the linear relationship between egg size and latitude was slightly strengthened (latitude versus egg size r = 0.50, t = 4.85, d.f. = 69, $p = <0.0001$ and latitude versus *ln* egg size r = 0.53, t = 5.21, d.f. = 69, $p = <0.0001$) and the relationship between *ln* egg size and *ln* body size did not dramatically change (r = 0.1, t = 0.79, d.f. = 69, $p = 0.43$).

Table 3.2 Results of tests for correlation not adjusted for phylogeny. Correlation coefficient (r), the t-test (t) and probability (*p*).

Test	Independent variable	Dependent variable	r	t	d.f.	*p*
1	Latitude	Egg size	0.43	4.04	71	0.0001
2	Latitude	*ln* Egg size	0.51	5.06	71	<0.0001
3	Latitude	*ln* Body size	0.10	0.86	71	0.39
4	Latitude	Residual egg size	0.50	4.92	71	<0.0001
5	*ln* Body size	*ln* Egg size	0.18	1.61	71	0.11

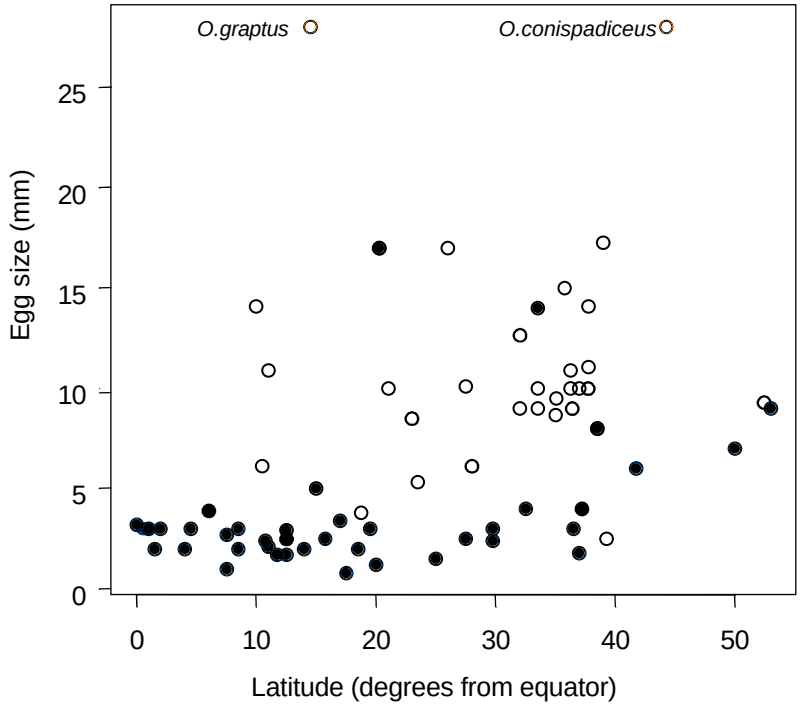

Figure 3.2: Scatter plot of association (not adjusted for phylogeny) between latitude and egg size (mm). Circles represent 73 individual species and are coded according to their inferred juvenile types. Closed circles indicate species with a "planktonic juvenile type" and open circles indicate a "benthic juvenile type".

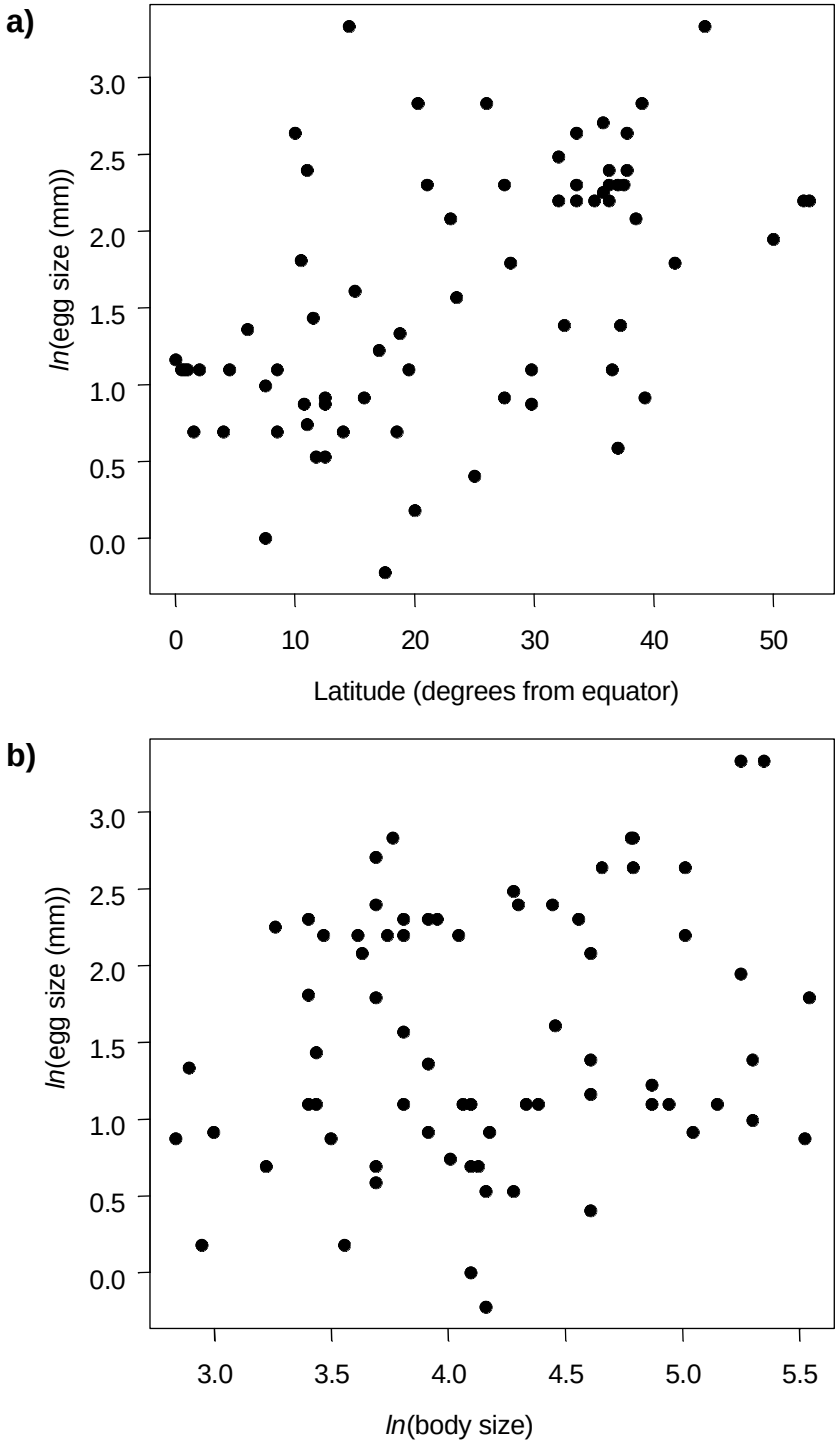

Figure 3.3: Scatter plot of association (not adjusted for phylogeny) between the variables a) latitude and *ln* egg size and b) *ln* body size and *ln* egg size.

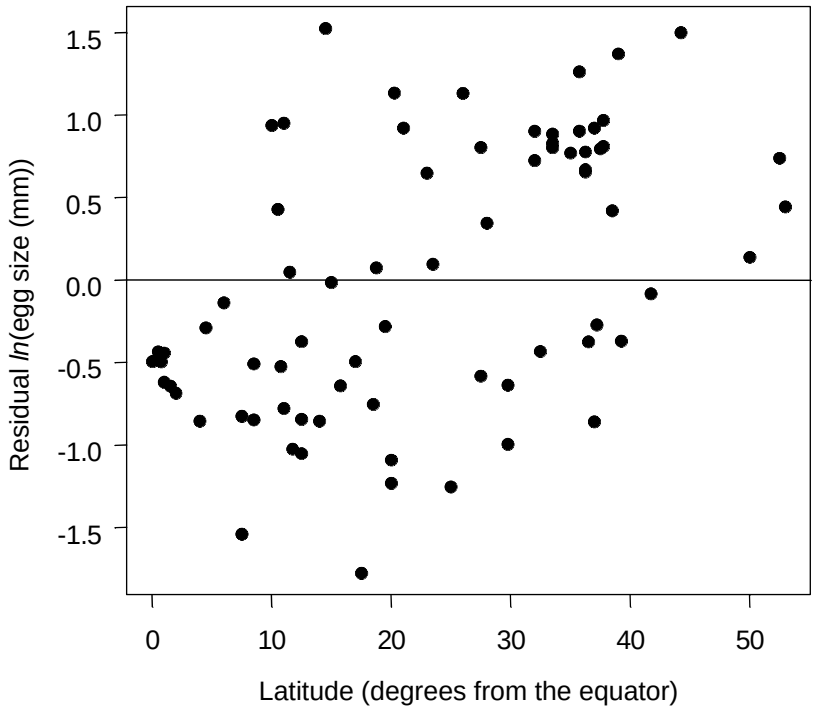

Figure 3.4: Scatter plot of association (not adjusted for phylogeny) between latitude and residual egg size after adjusting for body size.

The scatter plot (not adjusted for phylogeny) of latitude and egg size (Figure 3.2) showed a general positive trend of association between these traits. Separating these points according to their inferred juvenile type showed the distribution of expected juvenile types of taxa relative to their egg size and latitude. A large percentage (76%) of all species with low average latitudes (i.e. less than absolute 23.5˚) had a planktonic juvenile type (closed circles), while 63% of species with high average latitudes (greater than or equal to absolute 23.5˚) had a benthic juvenile type (open circles).

3.3.2 Pattern of evolution in discrete juvenile types

The pattern of evolution in the trait 'inferred juvenile type' was examined by estimating most likely discrete ancestral character state for each node in a phylogeny of shallow-water octopuses. Figure 3.5 shows results of ancestral state reconstructions for each node. For all nodes the difference in log Likelihood ($\Delta \ln L$) between the two possible character states was $\Delta \ln L \geq 2$, which provided an estimate of the best-fit character state at each node. A difference in log likelihood greater than or equal to two is often used as evidence that the likelihoods between the two hypotheses are significantly different and can be used to favour one character over the other as the most likely state (Edwards

1972; Pagel 1999b). The ancestral states reconstructed for each node under the 'Independent' Maximum Likelihood model showed the planktonic juvenile type to be the reconstructed state for all major ancestral nodes, providing evidence for this to be the ancestral state among these taxa. Among the taxa examined there were three transitions from planktonic juvenile type to the benthic juvenile type; at the terminal branch of *Ameloctopus litoralis* and at nodes 1P (*Hapalochlaena* clade) and 1I (*O. macropus* + *O. australis* group clade) (see Figure 3.5). After these transitions, the benthic juvenile type was ancestral among nine taxa in total, at nodes 1P (*H. fasciata and H. maculosa*) and 1I (*Octopus pallidus, O. australis, O. berrima, O. bunurong, Grimpella thaumastocheir, O. kaurna* and *O. graptus*). No subsequent reversals to planktonic juvenile type from benthic juvenile type were observed.

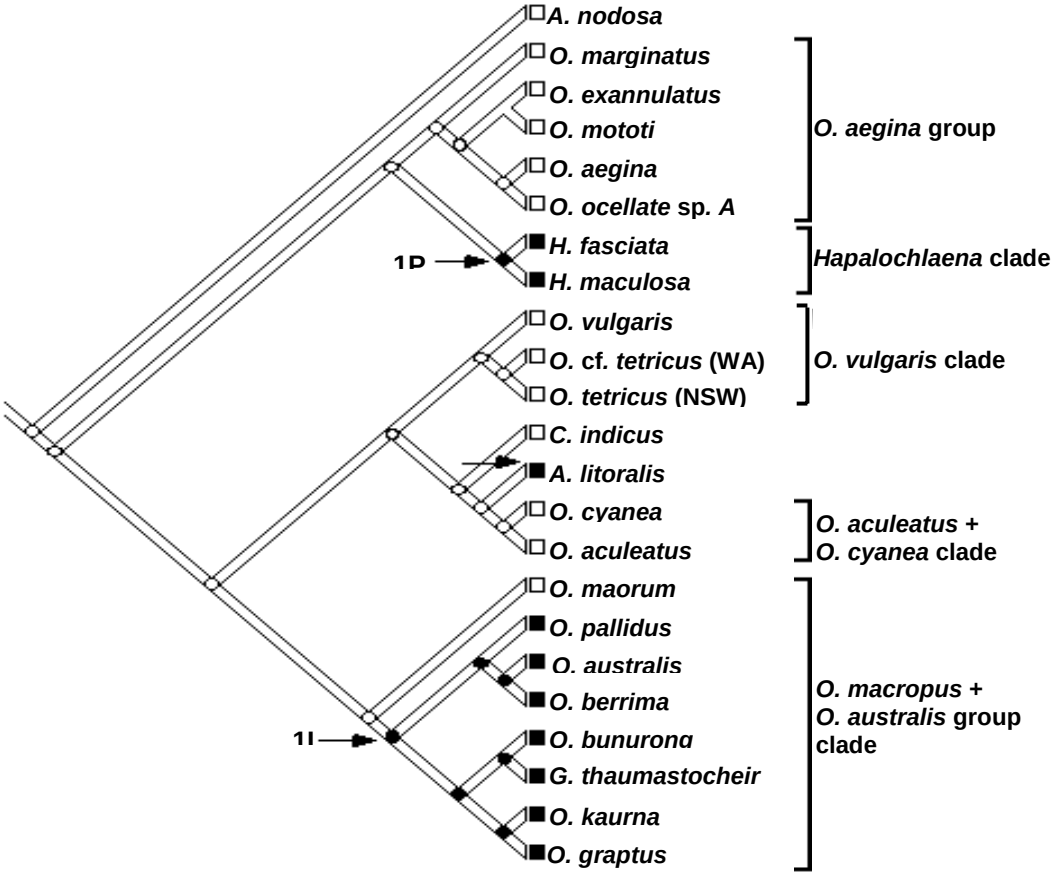

Figure 3.5: Reconstructed ancestral character states by node. Dots represent the best-fit state reconstruction for inferred juvenile type. Open circles and squares indicate planktonic juvenile type, closed circles and squares indicate benthic juvenile type. Arrows and alphanumeric codes indicate a transition from one character state to another at that node and/or tip.

3.3.3 Phylogenetic inertia

The parameter λ is an estimate of whether phylogenetic inertia is operating on the characters under investigation. A value of 0 indicates no phylogenetic correction is required for that trait (i.e. changes in value of this trait are independent of phylogeny) and a value of 1 suggests strong phylogenetic inertia is operating on the trait. To test whether $\lambda > 0$ for egg size, body size and latitude, a likelihood ratio test was employed to examine evidence against the null hypothesis $\lambda = 0$. A significant probability ($p \leq 0.05$) that $\lambda > 0$ was observed for both egg size and latitude, while body size had an estimated $\lambda = 0.34$ which is not significantly different from 0 ($p = 0.40$). These results show that egg size and latitude are not phyletically independent, indicating a need for

phylogenetic correction when examining covariation among traits and latitude in this study. In contrast, body size does appear to be phyletically independent.

3.3.4 Covariation between egg size and body size

A test for covariation between *ln* body size and *ln* egg size using the phylogenetic GLS approach revealed a non-significant association between these traits (see Table 3.3 Test 1, $p = 0.11$). Visual inspection of the scatter plot of *ln* body size against *ln* egg size supports this result (Figure 3.6a). However, further examination suggests some evidence for a negative association between these traits: smaller species tend to have larger eggs and larger species tend to have smaller eggs. The exception to this trend is *O. graptus*, which is a large species that has very large eggs (Figure 3.6a).

Table 3.3: Results of likelihood ratio tests for trait covariation corrected for phylogeny between continuous and binary (B) variables represented by the difference in log likelihood ($\Delta \ln L$) for Model A and associated probability (p). Grubbs Z ratio statistic for outlier detection is also shown. Critical Z values for Grubbs test were 2.73 for n = 21 and 2.71 for n = 20. Bold type denotes $p \leq 0.05$, † denotes removal of *O. graptus* from the analysis and # are analyses where residual egg size was recalculated to exclude *O. graptus*.

Test	Character variables tested			Model A			Z ratio	
	Independent	Dependent		$\Delta \ln L$	d.f.	p =	n	
1	*ln* Body size	*ln* Egg size		1.32	1	0.11	21	**2.76**
2			†	4.84	1	**0.002**	20	2.23
3	Latitude	*ln* Egg size		0.42	1	0.36	21	**2.97**
4			†	4.38	1	**0.003**	20	2.34
5	Latitude	Residual egg size		0.17	1	0.56	21	**3.46**
6			† #	5.95	1	**0.0006**	20	2.36
7	Latitude	Juvenile type (B)		1.69	1	0.07	-	-
8			†	2.53	1	**0.02**	-	-

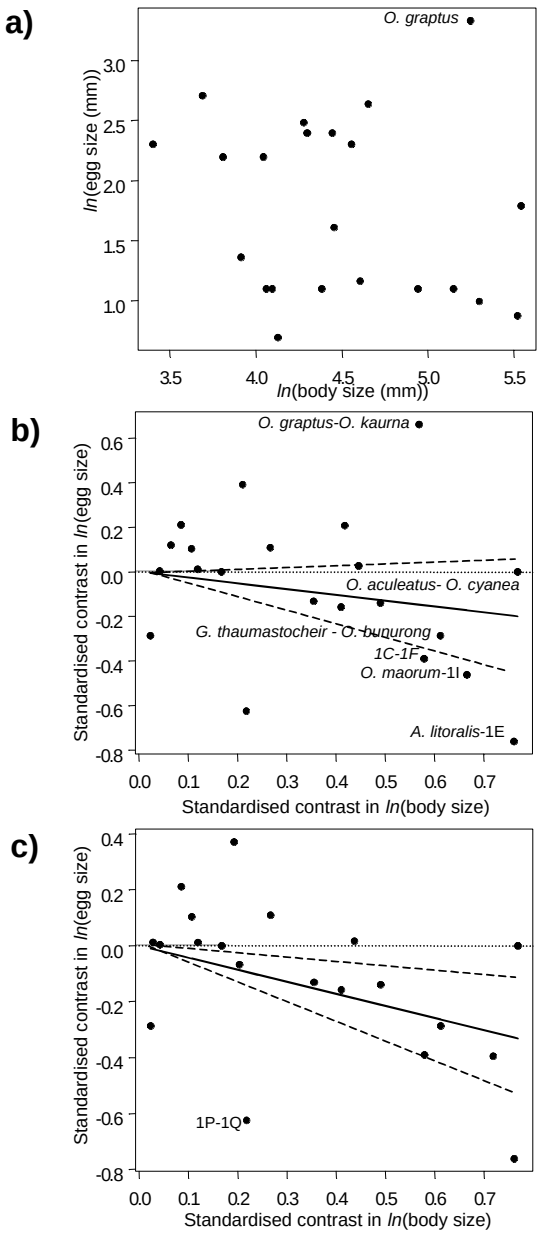

Figure 3.6: Comparison of *ln* egg size plotted against *ln* body size for 22 species used in a comparative phylogenetic analysis. Scatter plots are displayed for (a) *ln* body size and *ln* egg size, (b) standardised independent contrasts for these traits for all taxa (standardised contrasts in body size are also positivised) and (c) as for (b) excluding *O. graptus.* Species names and alphanumeric codes for comparisons between nodes are noted. The solid line represents the estimated regression line through the origin between traits, dashed lines are 95% confidence intervals and dotted lines are the zero reference lines.

To further examine the relationships between these traits among compared taxa, an Independent Contrasts (IC) analysis was performed. Figure 3.6b shows the results of the regression through the origin of the contrasts of standardised *ln* egg size and standardised, positivised contrasts of *ln* body size (*x*- axis), calculated for each node of the phylogeny. The advantage of this approach to the analysis was that the relationship between the traits, having removed any effect of phylogeny, could be displayed. The correlation between the trait contrasts is –0.34 and the test for covariation between the traits was non-significant (and exactly equivalent to the GLS analysis reported above). Further, the non-significant relationship between these traits was evident by the confidence intervals for the regression line encompassing the zero (*y*-axis) reference line (Figure 3.6b).

Individual contrasts represent the relative differences in egg size and body size between closely related taxa. Any observed association between traits therefore, indicated that the association was not an artefact of phylogenetic lineage effects among the traits. The contrasts that contributed most to the direction of association between the traits were those contrasts representing large differences in body size, i.e. those comparisons between smaller and larger species (or clades of species). For most contrasts with a large observed difference in body size to their nearest relatives, larger body sizes were associated with smaller egg sizes. Contrasts exemplary of this pattern included: *Octopus aculeatus* versus *O. cyanea, Grimpella thaumastocheir* versus *O. bunurong, O. maorum* versus node 1I (*O. macropus* + *O. australis* group clade), node 1C (*Cistopus indicus*, *Ameloctopus litoralis* and the *O. aculeatus* + *O. cyanea* clade) versus node 1F (*O. vulgaris* clade) and *A. litoralis* versus node 1E (*O. aculeatus* + *O. cyanea* clade) (see Figure 3.6b for the contrasts and Figure 3.1 for nodes on the phylogeny). One contrast that also represented a large difference in body size revealed the opposite pattern where larger body size was associated with a larger egg size. Here, *Octopus graptus* was shown to have a larger body size and larger egg size compared to its closest relative *O. kaurna* (this contrast is noted in Figure 3.6b). This observation indicates *O. graptus* is much larger in body size than *O. kaurna* and associated with this larger body size are also larger egg sizes relative to *O. kaurna*. This opposes the relationship observed among other taxa with larger body sizes because their larger body sizes are associated with smaller egg sizes compared to their nearest relatives.

The contrast between *O. graptus* and *O. kaurna* was of particular interest because of the direction of difference in egg size and body size between these phyletically related species compared to most other closely related taxa. The *O. graptus* versus *O. kaurna* contrast was found to contribute to a relationship that was distinctly opposite to that of most other contrasts. As a result the *O. graptus* versus *O. kaurna* contrast had a large leverage effect on the analysis and this was supported by various regression influence diagnostics that were examined (see Methods section 3.2.4.2). A formal test of outlier detection on the residuals of the IC analysis showed that the contrast between *O. graptus* and *O. kaurna* was a significant outlier ($Z = 2.76$, $p < 0.05$; Table 3.3, Test 1).

The observed egg size to body size relationship between *O. graptus* and *O. kaurna* was of interest here as it indicated that an increase in body size has been associated with an increase in egg size in *O. graptus* and also, this contrast was highly influential on the analysis. I was also interested in examining a possible relationship between egg size and body size among the other taxa used in the analysis. In order to explore this relationship between traits, *O. graptus* was excluded from the data. An exclusionary approach is statistically justifiable on the basis that this species had a strong contrasting influence on the fit of the data to the regression model, but it was solely carried out for the purpose of investigating whether a relationship exists among other octopus taxa. A complete discussion of covariation in egg size and body size in the octopuses will consider all the findings, including the relative egg sizes and body sizes observed between *O. graptus* and *O. kaurna*.

A test for covariation between *ln* body size and *ln* egg size having removed *O. graptus* (indicated by † in the GLS analyses, Table 3.3) using the phylogenetic GLS approach revealed a highly significant association between these traits (Table 3.3 Test 2, $p = 0.002$). The IC analysis, excluding *O. graptus*, showed the correlation between the trait contrasts is –0.61 and the test for covariation between the traits is significant (and exactly equivalent to the GLS analysis reported above). Further, the significant relationship between these traits is evident by the confidence intervals for the regression line excluding the zero (*y*-axis) reference line (Figure 3.6c). The same contrasts as described above drive this relationship (Figure 3.6c). A formal test of outlier detection

on the residuals of the IC analysis did not reveal any outliers ($Z = 2.23$, $p > 0.05$; see Table 3.3, Test 2).

Another contrast that was found to be distinct compared to the general trend in egg size and body size among contrasts was node 1P (*Hapalochlaena* clade) versus node 1Q (*O. aegina* group clade). The species of these clades were found to be similar in body size but members of the *O. aegina* group have small eggs relative to their nearest relatives, species of the genus *Hapalochlaena* (noted in Figure 3.6c). This is highlighted here as a deviation from the general trend.

3.3.5 Covariation between egg size and latitude

A test for covariation between latitude and *ln* egg size using the phylogenetic GLS approach revealed a non-significant association (Table 3.3 Test 3, $p = 0.36$), which contrasted strongly with the results of the non-phylogenetic analysis between these traits (Figures 3.2 and 3.3a). Visual inspection of the scatter plot of latitude against *ln* egg size supports this result (Figure 3.7a). However, closer examination suggests there is evidence for a positive association between egg size and latitude: species with high latitude distributions tend to have larger eggs and species with low latitude distributions tend to have smaller eggs. The exception to this trend is again *O. graptus*, which is a species that has a low latitude distribution and very large eggs (Figure 3.7a).

To further examine the relationships between these traits among compared taxa, an Independent Contrasts (IC) analysis was again implemented. Figure 3.7b shows the results of the regression through the origin of the contrasts of standardised *ln* egg size and standardised, positivised contrasts of latitude (*x*- axis), calculated for each node of the phylogeny. The correlation between the trait contrasts was 0.19 and the test for covariation between the traits was non-significant (and equivalent to the GLS analysis above). The non-significant relationship between egg size and latitude was shown by the confidence intervals for the regression line that encompasses the zero (*y*-axis) reference line (Figure 3.7b).

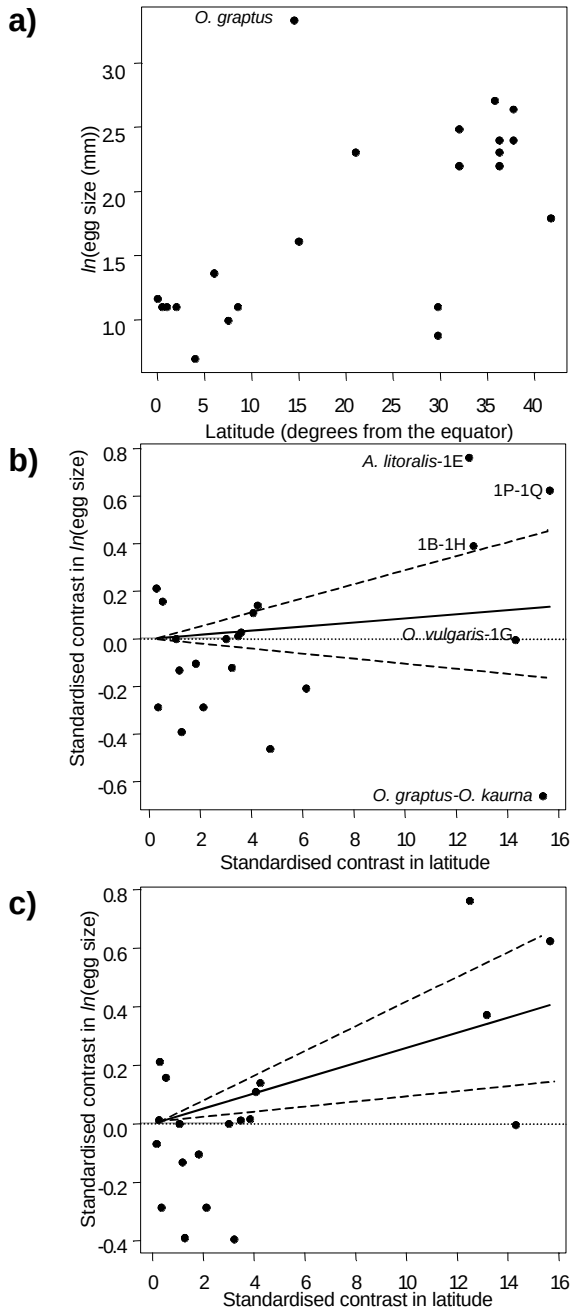

Figure 3.7: Comparison of *ln* egg size plotted against latitude for 22 species used in a comparative phylogenetic analysis. Scatter plots are displayed for (a) latitude and *ln* egg size, (b) standardised independent contrasts for these variables for all taxa (standardised contrasts in latitude are also positivised) and (c) as for (b) excluding *O. graptus.* Species names and alphanumeric codes for comparisons between nodes are noted. The solid line represents the estimated regression line through the origin between variable, dashed lines are 95% confidence intervals and dotted lines are the zero reference lines.

Individual contrasts represent the relative differences in egg size and latitude between closely related taxa (i.e. tips of the tree) and nodes. The contrasts that contributed most to the direction of association between egg size and latitude were those contrasts representing large differences in latitude, that is, those comparisons between lower latitude and higher latitude distributed species (or clades of species). For most contrasts with a large observed difference in latitude, higher latitude distributed species were associated with larger egg sizes. Contrasts exemplary of this pattern include: *A. litoralis* versus node 1E (*O. aculeatus* + *O. cyanea* clade), node 1P (*Hapalochlaena* clade) versus node IQ (*O. aegina* group clade), *O. vulgaris* versus node 1G (*O. tetricus* species) and node 1B (large clade containing *C. indicus, A. litoralis*, the *O. cyanea* + *O. aculeatus* clade and the *O. vulgaris* clade) versus node 1H (*O. maorum* and the *O. macropus* + *O. australis* group clade) (see Figure 3.7b for the contrasts and Figure 3.1 for nodes on the phylogeny). A single contrast representing a large difference in latitude revealed the opposite pattern where a low latitude distribution was associated with large egg size. Here, *Octopus graptus* was shown to have a lower latitude distribution and larger egg size compared to its closest relative *O. kaurna* (this contrast is noted in Figure 3.7b). This observation indicated that *O. graptus* has a much lower latitude distribution than *O. kaurna* and associated with this lower latitude distribution were larger egg sizes relative to *O. kaurna*. This contrast opposes the relationship observed among other taxa that also have lower latitude distributions relative to their nearest relatives, but their lower latitude distributions are associated with smaller egg sizes compared to their nearest relatives.

The *O. graptus* versus *O. kaurna* contrast was found to contribute to a relationship that was distinctly opposite to that of most other contrasts. As a result the *O. graptus* versus *O. kaurna* contrast had a large leverage effect on the analysis and this was supported by various regression influence diagnostics that were examined. A formal test of outlier detection on the residuals of the IC analysis showed that the contrast between *O. graptus* and *O. kaurna* was a significant outlier ($Z = 2.97$, $p < 0.05$; Table 3.3, Test 3).

The observed egg size to latitude relationship between *O. graptus* and *O. kaurna* indicated that a lower latitude distribution has been associated with a relative increase in egg size in *O. graptus*. The potential relationship between egg size and latitude among other taxa used in the analysis was of interest here. In order to explore this relationship

between traits, *O. graptus* was excluded from the data. The complete discussion of covariation in egg size and latitude in the octopuses will consider the relative egg sizes and latitudinal distribution observed between *O. graptus* and *O. kaurna*.

A test for covariation between latitude and *ln* egg size having removed *O. graptus* (indicated by † in Table 3.3) using the phylogenetic GLS approach revealed a highly significant association between these traits (Table 3.3 Test 4, $p = 0.003$). The IC analysis, excluding *O. graptus*, showed the correlation between the trait contrasts to be 0.58 and the test for covariation between the traits was significant (and exactly equivalent to the GLS analysis reported above). Further, the significant relationship between these traits is evident by the confidence intervals for the regression line excluding the zero (*y*-axis) reference line (Figure 3.7c). The same contrasts as described above drive this relationship (Figure 3.7c). A formal test of outlier detection on the residuals of the IC analysis did not reveal any outliers ($Z = 2.34$, $p > 0.05$; see Table 3.3, Test 4).

3.3.5.1 Covariation between egg size independent of body size and latitude

As described above, phylogenetic analyses that eliminated *O. graptus* and in turn the contrast between *O. graptus* and *O. kaurna* showed that both body size and latitude covary with egg size independently of any phyletic relationships. In such situations caution must be taken regarding the nature of these relationships as the positive relationship between two variables (i.e. egg size and latitude) can be confounded by a correlation with a third variable (such as body size) (Harvey and Keymer 1991; Garland and Janis 1993). To further investigate whether the relationship between egg size and latitude was influenced by the relationship between egg size and body size, residuals from the regression of *ln*(egg size) on *ln*(body size) were calculated to gain a measure of residual egg size after accounting for body size. The contrast between *O. graptus* and *O. kaurna* was again found to be an outlier in the IC analysis (Figure 3.8a), and this was indicated by Grubbs test for outlier detection (Table 3.3, Test 5, $Z = 3.46$).

To exclude of *O. graptus* from the analysis residual egg sizes were recalculated for the data set excluding *O. graptus* (indicated by # † in Table 3.3) prior to reanalysis to ensure that the variances associated with this taxon had not unduly influenced estimation of residuals. A test for covariation using the phylogenetic GLS approach

between latitude and residual egg size having removed *O. graptus,* revealed the relationship between *ln* egg size and latitude held true, indicated by a highly significant association (Table 3.3, Test 6, $p = 0.0005$). The IC analysis, excluding *O. graptus*, showed the correlation between the residual egg size and latitude contrasts was 0.66 and the test for covariation between the traits was significant (and exactly equivalent to the GLS analysis reported above). Further, the significant relationship between residual egg size and latitude was also represented by the confidence intervals for the regression line excluding the zero (*y*-axis) reference line (Figure 3.8b). A formal test of outlier detection on the residuals of the IC analysis did not reveal any outliers ($Z = 2.36$, $p > 0.05$; see Table 3.3, Test 6). As mentioned above, these findings were consistent with the results of the *ln* egg size and latitude comparison (Table 3.3, Test 4).

3.3.5.2 Covariation between juvenile type and latitude

A test for covariation between latitude and the binary character juvenile type using the phylogenetic GLS approach revealed an association that was non-significant (Table 3.3 Test 7, $p = 0.07$). The species *O. graptus* was removed from the analysis for the purpose of examining whether a relationship between these traits exists among the other species in the study. A significant relationship between latitude and inferred juvenile types (Table 3.3 Test 8, $p = 0.02$) among these 21 species was observed.

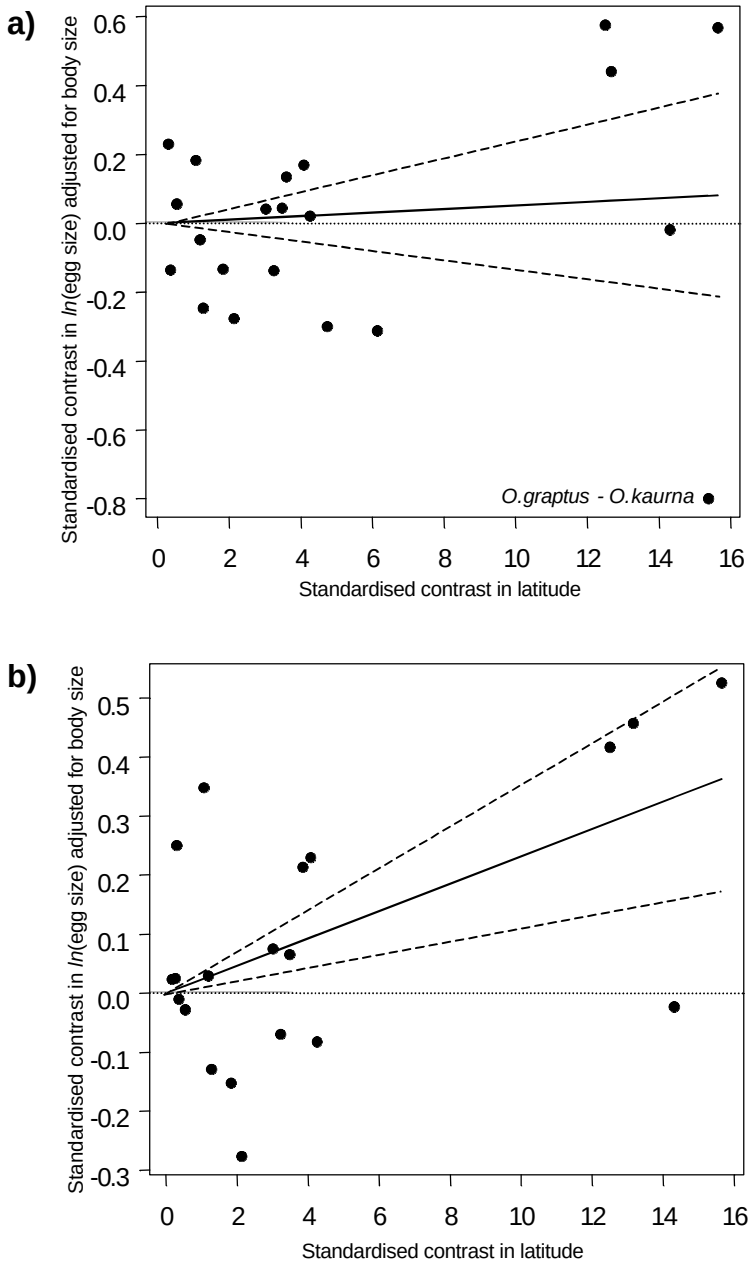

Figure 3.8: (a) Standardised independent contrasts for latitude and residual egg size adjusted for body size for all taxa (standardised contrasts in latitude are also positivised) and (b) as for (a) excluding *O. graptus.* Species names and alphanumeric codes for comparisons between nodes are noted. The solid line represents the estimated regression line through the origin between variable, dashed lines are 95% confidence intervals and dotted lines are the zero reference lines.

3.4 DISCUSSION

Results of the present study provide substantial evidence that the evolution of juvenile types in the benthic shallow-water octopuses has been conservative. Among species, selective pressures associated with variation in both body size and latitudinal gradients have influenced evolution of egg size indicating that transitions in this trait may be adaptive responses to macro-environmental variation. Interestingly though, the evolution of egg size has been constrained by phylogenetic lineage.

3.4.1 Pattern of evolution in juvenile types

The reconstructed pattern of evolution in inferred juvenile types among benthic shallow-water octopuses showed the planktonic juvenile type was ancestral among 22 species. Three independent evolutionary transitions to the benthic juvenile type were subsequently observed among taxa. These results support traditional hypotheses of life history evolution in the Octopodinae. Boletzky (1987a, 1987b) and Engeser (1990) hypothesised that a planktonic juvenile phase is an ancestral character, probably originating at a time when most cephalopods were pelagic (Young et al. 1998), whereas the benthic juvenile mode is derived. A polyphyletic pattern of evolution in juvenile type was observed in this study with three independent transitions from planktonic juvenile type to benthic juvenile type observed among these 22 octopus species. This result is consistent with the hypothesis of Boletzky (1987a) that differences in developmental patterns do not reflect a single phylogenetic shift. These three transitions indicate that selective pressures have favoured the benthic juvenile type at three separate times during the evolution of these species.

The transitions from planktonic juvenile type to benthic juvenile type have been relatively infrequent, with no subsequent reversals in character state after a change from planktonic juvenile type to benthic juvenile type. This is exemplified by the common ancestral nodes to the blue-ring octopuses, *Hapalochlaena fasciata* and *H. maculosa*, and to the *Octopus macropus* + *O. australis* clade, which have each undergone a transition from planktonic juvenile type to benthic juvenile type. Further, all of the descendents of both nodes shared the derived state benthic juvenile type. A further independent change from planktonic juvenile type to benthic juvenile type was observed in *Ameloctopus litoralis*. Conclusions regarding this taxon are tentative

because the placement of *A. litoralis* within the phylogeny was not well supported (see Chapter 2). An absence of close relatives to *A. litoralis* within the phylogeny also made it difficult to discern whether this was a single independent change in character state or a change within an entire clade. These transitions in character state indicate this trait is somewhat evolutionarily labile and that transitions from one strategy to another have occurred independently on a number of occasions in octopus evolutionary history. However, an absence of reversals in trait evolution among these taxa indicates that these transitions in juvenile type among octopuses have also been conservative and may be subject to phylogenetic inertia.

The absence of reverse transitions subsequent to a benthic juvenile type change at the common ancestral nodes to *H. maculosa* and *H. fasciata* clade and the *O. macropus* and *O. australis* clades, is consistent with the findings of Lieberman et al. (1993) who hypothesised that non-planktonic lineages (i.e. that have advanced and complex characters) rarely revert to a planktonic larval mode in turritellid gastropods. Reversals in these traits among benthic shallow-water octopuses should not be discounted altogether considering that *H. lunulata*, a close relative of *H. maculosa* and *H. fasciata*, is known to have small sized eggs, and until its phylogenetic relationships to other blue-ring octopuses are examined, this cannot be discounted as a reversal in character state. However, there does appear to have been a strong phylogenetic constraint operating among species, particularly once a change from planktonic to benthic strategy has taken place.

3.4.2 Covariation between life history traits and environmental variation

The evolution of egg size in the benthic shallow-water octopuses was examined to determine whether latitudinal variation and/or body size have influenced variation in egg sizes among 22 species of octopus. Results of analyses where the influence of phyletic relationships among taxa was removed showed no strong evidence for covariation between these factors and egg size. Interestingly, closer inspection of the data revealed a species, *O. graptus*, with strongly disparate life history strategies compared to other taxa. For the purpose of exploring underlying relationships among the other species in the study this outlying species, *O. graptus*, was excluded from the subsequent set of analyses. Here a discussion of the results is presented that will firstly examine the trends that were observed among 21 species, not including *O. graptus*,

between egg size and body size, egg size and latitude, and egg size independent of body size and latitude. Furthermore, the relationship between these traits in *O. graptus* relative to other octopuses will be discussed. There is no intent to imply that the results observed for the complete data set (i.e. including *O. graptus*) should be removed or ignored, but for the purpose of clarity of discussion, these results and their implications for the potential selection pressures operating on species will be discussed separately.

3.4.2.1 Influence of body size on egg size evolution

Results showed that egg size covaries negatively with body size such that species with larger body sizes were found to have smaller egg sizes compared to smaller body sized species. These comparisons indicate that some of the variation observed in egg size is attributable to evolutionary changes in body size. A negative relationship between egg size and body size has been observed in other animals such as lizards (Bauwens and Díaz-Uriarte 1997) and butterflies (Wiklund et al. 1987). However, a positive relationship between egg size and adult size is generally observed at the species (García-Barros 2000) and population levels (Parker and Begon 1986; Simpson 1995; Fox and Czesak 2000), where large bodied species, or individuals, have larger eggs compared to smaller species. This type of relationship reflects a larger amount of stored resources available to larger females for investment into eggs than smaller species such that egg sizes are scaled according to the body sizes of adults (Schmidt-Nielsen 1984).

In the study by Wiklund et al. (1987) the negative covariation in egg size relative to body size in pierid butterflies was suggested to be the product of selection on females to maximise fecundity, where selection pressures favour a decrease in egg size and an increase in clutch size. The trait clutch size is used here to infer differences in fecundity between species. Under this hypothesis larger females tend to produce larger clutches of offspring in response to selection pressures that favour increased fecundity (Parker and Begon 1986). Due to the trade-off between clutch size and egg size, large clutch sizes (i.e. higher fecundity) coincide with reduced egg sizes compared to species that produce smaller clutches of eggs (Simpson 1995). It is suggested that some large octopus species experience selection to maximise fecundity and therefore adaptive transitions towards smaller egg sizes are favoured (Calow 1987; Mangold 1987). The present findings suggest that larger body sized species are influenced by selection pressures that favour smaller egg sizes.

These results require further discussion due to a number of conflicting observations in the analysis. The negative correlation between egg size and body size (adjusted for phylogeny) disagreed with the tests for correlation (not adjusted for phylogeny) that provided no evidence for a relationship between body size and egg size. This is an unusual result as analyses that adjust for the influence of phylogeny generally either give support to (e.g. Thompson 2001), or refute, significant covariation between traits among taxa where phylogenetic relationships are not accounted for (e.g. García-Barros and Munguira 1997; Poulin 1997; Poulin et al. 2003). After the effect of phylogeny is accounted for, observation of a significant relationship between traits is therefore unexpected. Furthermore, a test phylogenetic inertia revealed body size has been influenced little by phylogenetic constraint ($\lambda = 0.34$, $p = 0.40$ that λ is different from 0), suggesting that body size evolves relatively independently of phylogeny. The implication is that the influence of phylogeny may not need to be taken into account in analyses of body size, which would lead to the alternative inference of the raw correlation that there is no evidence of a relationship between egg size and body size.

The observed relationship between egg size and body size may therefore, be an artefact of the species and the phylogeny that were used in this comparative analysis. The most influential contrasts on the negative relationship between egg size and body size were those species that had larger body size and smaller egg size compared to their closest relatives, i.e. *Grimpella thaumastocheir* versus *O. bunurong, O. maorum* versus the *O. macropus* + *O. australis* group clade, the clade containing *Cistopus indicus*, *Ameloctopus litoralis, O. aculeatus, O. cyanea* versus the *O. vulgaris* clade and *A. litoralis* versus the *O. aculeatus* + *O. cyanea* clade. The latter contrast was particularly influential in the analysis due to the larger body sizes and smaller egg sizes that *O. aculeatus* and *O. cyanea* have compared to *A. litoralis*. However, the placement of *A. litoralis* within the best estimate of phylogeny among the octopuses was not well supported (see Chapter 2) and close relatives of this taxon were not represented within the phylogeny (they are also presently uncertain (Norman 1992c)). It is difficult to discern therefore, firstly, whether *O. aculeatus* and *O. cyanea* are immediate relatives of *A. litoralis* and, secondly, if the differences between these taxa (and consequently their independent contrast) are valid. Hence conclusions regarding this contrast must be treated tentatively because these species and their inferred phylogeny may have biased

the results of the relationship between egg size and body size because their inferred phylogenetic relationships may be incorrect.

It is difficult to determine whether the relationship between egg size and body size is an artefact of the strong influence that the contrast between *A. litoralis* and the *O. aculeatus* + *O. cyanea* clade has had on this analysis. However, the present results based on the other influential contrasts in this analysis are consistent with the observed negative association between traits discussed above. Furthermore, their phylogenetic relationships, as reconstructed in Chapter 2, are more likely to be accurate than those of *A. litoralis* versus the *O. aculeatus* + *O. cyanea* clade, hence the present data indicates that a negative relationship exists among taxa. To resolve these issues, information on a larger set of species and their phylogenetic relationships is required. This would help to increase the number of phylogenetic comparisons among closely related species and provide a more complete estimate of whether a relationship between egg size and body size exists among the shallow-water octopuses.

3.4.2.2 Influence of latitude on egg size evolution

Results showed that egg size covaries with latitudinal gradient in 21 benthic shallow-water octopus species. These findings, which were based on continuous character data, support models of life history evolution in benthic marine invertebrates (Thorson 1950; Vance 1973; Christiansen and Fenchel 1979; Levitan 1993; Havenhand 1995; McEdward 1997). This is also new evidence that, evolutionarily, egg size is an adaptive trait that responds to macro-environmental variation and associated selection pressures.

Results of the tests for correlation between egg size and latitude, not adjusted for phylogeny, were concordant with phylogenetic comparative analyses, providing evidence for a relationship between egg size and latitude. This result was observed in tests for association between egg size and latitude (adjusted for phylogeny) among 22 species of octopus and in a test for correlation (not adjusted for phylogeny) among 73 octopus species. The concordance between the analyses indicates it may be possible to generalise about the applicability of this trend to other octopuses. For instance the test for covariation between egg size and latitude (adjusted for phylogeny) indicated that this association is likely to exist independent of phylogeny. Furthermore, the

association between these traits observed among 73 benthic shallow-water octopuses provides evidence that this association may apply generally to other benthic shallow-water octopuses. This finding could be clarified in a comparative phylogenetic analysis that encompassed more taxa.

Here it was shown that species (or clades of species) with higher latitude distributions had larger egg sizes compared to lower latitude species. Contrasts that were most influential to these results were the *Hapalochlaena* clade compared to the *O. aegina* group clade, the *O. macropus* + *O. australis* group clade compared to the clade containing *C. indicus, A. litoralis*, *O. cyanea, O. aculeatus* and the *O. vulgaris* clade, and *A. litoralis* compared to the *O. aculeatus* + *O. cyanea* clade (Figure 3.7b and Figure 3.1). Again the contrast between *A. litoralis* and the *O. aculeatus* + *O. cyanea* clade should be treated with caution due to the uncertainty associated with the phylogenetic relationships of *A. litoralis*. However, these results show that egg sizes and latitudinal variation covary and potentially environmental differences between closely related taxa may mediate change in egg sizes among octopuses.

Latitude was used here as an indicator of macro-environmental variation because the primary components of environmental variation along latitudinal gradients are temperature and food availability (Forsythe and Van Heukelem 1987). These factors are known to directly influence growth and survivorship of both eggs and juveniles (Thorson 1950; Christiansen and Fenchel 1979; Kingsford 1995; Nybakken 1996). Similarly, experimental studies within species have shown that environmental temperature and food variation are major factors in the evolution of egg size (Sibly and Calow 1986; Azevedo et al. 1996; Fox 2000) and that egg sizes adapt to variation in these factors. Covariation of egg sizes with latitudinal variation in these octopuses is indicative of egg size in octopuses being an adaptive trait, responding to natural selection associated with macro-environment types. It is likely therefore that variation in this trait may be mediated by a fecundity-survival trade-off.

Egg size in benthic marine invertebrates is thought to reflect a balance between fecundity and juvenile mortality risk (Vance 1973; Smith and Fretwell 1974; Sibly and Calow 1985; Sinervo 1990; Hart 1995; McEdward 1997; McEdward and Miner 2003). This trade-off is often dependent on the environmental niche that an organism occupies.

Under this hypothesis, the present day egg size of a species reflects the phenotype that maximises fitness in a particular environment, where fitness is defined as reproductive success (McEdward and Miner 2003). This relationship is based on three underlying assumptions. Firstly, egg size and juvenile type are tightly related traits where juvenile type is determined by the total amount of energy invested into each egg (Emlet et al. 1987; Sinervo and McEdward 1988; Emlet 1995; Hart 1995). Secondly, a trade-off exists between the number and the size of offspring that a female can produce, where a large number of small eggs that each require minimal investment is balanced against having a small number of large, nutrient rich eggs (e.g. McEdward 1997). Finally, high rates of juvenile mortality intensify selection on early life stages, including the embryonic stage, i.e. eggs (Vance 1973; Sibly and Calow 1985; Fox 2000).

Under the fecundity-survival trade-off hypothesis, fecundity is balanced against juvenile survival among species. Fecundity is defined by the number of eggs a female produces and is highest when eggs are small and numerous. High fecundity strategies (i.e. small eggs and planktonic juvenile types) are generally favoured when food levels and temperature are high (McEdward and Miner 2003). Juvenile survival though, is strongly influenced by egg size in general. Larger offspring often have a higher survival advantage than small offspring in conditions of higher juvenile mortality risk (e.g. environments that are nutrient poor and/or cold), hence large egg strategies are selectively advantageous in these conditions (Bagenal 1969; Ferguson and Fox 1984; Perron 1986). The present results suggest that egg sizes in octopuses are therefore adaptive, responding to differences in environment types during evolution and that large egg sizes are favoured in difficult and particularly cold environment types. This observation is consistent with the long held prediction that large eggs in octopuses are adaptations to cold temperatures and/ or deep-sea realms (Voss 1988; Boletzky 1994).

The semelparous life cycle of the Octopodinae requires optimal investment into progeny to ensure species fitness (Calow 1987; Roff 1992; Stearns 1992). The present results indicate that the dichotomous nature of egg sizes in octopuses is maintained by a fecundity-survival trade-off and species fitness may be maximised according to the macro-environmental niche that it occupies. Interestingly though, it was also observed that evolution of egg sizes in octopuses is phylogenetically constrained as indicated by an estimate of the parameter lambda that was significantly different from 0 (Pagel

1999a; Freckleton et al. 2002). Phylogenetic relationships among related taxa can constrain variation in life history traits through associations with physiological or environmental attributes within lineages (Harvey and Pagel 1991; Stearns 1992). Such species have a tendency to retain the egg sizes of their ancestors and, in turn, other members within the same lineage. The implication is that evolutionary transitions from small egg sizes, which are assumed to be the ancestral character state, to larger egg sizes are probably rare but as suggested above this trait has the capacity to adapt to environments that have higher juvenile mortality risks associated with them. These conclusions are consistent with the findings of Lessios (1990) who showed that echinoderm egg sizes are influenced by geographic productivity differences on either side of the Isthmus of Panama but the extent to which egg size could change was also strongly influenced by phylogenetic relationships between species. This may indicate a commonality among benthic marine invertebrates, such that egg size is an adaptive trait but its evolution is also constrained by phyletic relationships among taxa.

3.4.2.3 Covariation between egg size independent of body size and latitude

Independent of phylogeny, body size was found to covary with egg size, and latitude covaries with egg size. Caution must be taken regarding the nature of these relationships, as a positive relationship between two variables (e.g. egg size and latitude) can be confounded by a correlation with a third variable (e.g. body size) (Harvey and Keymer 1991; Garland and Janis 1993). To investigate whether the relationship between egg size and latitude was influenced by the relationship between egg size and body size, the variation in egg size attributable to body size was removed by calculating the residuals from the regression of *ln*(egg size) on *ln*(body size). A positive relationship between latitude and residual egg size, where the influence of phylogeny was adjusted for, was consistent with the covariation between *ln* egg size and latitude, indicating that any association between latitude and egg size is independent of the association between body size and egg size. These results provided further evidence that environmental variation has influenced the evolution of egg size among benthic shallow-water octopus species.

3.4.2.4 Alternative trends in covariation among traits and variables

In preceding sections, major findings of analyses of covariation between both egg size and body size, and egg size and latitude, were discussed. However, one taxon, *Octopus graptus,* was observed to differ significantly from to the majority of other taxa in the analysis. The contrast between *O. graptus* and its closest relative *O. kaurna,* based on the present phylogeny, showed an association between these traits that was opposite to the general trends observed for the majority of other contrasts (as above). *Octopus graptus* was shown to have a larger body size and larger egg size relative to those of *O. kaurna* (contrast is noted in Figure 3.6b) and a lower latitude distribution and larger egg sizes relative to those of *O. kaurna* (Figure 3.7b). The differences between *O. graptus* and *O. kaurna* among these traits were large and their contrast therefore, was strongly influential on the phylogenetic comparative analyses. Formal tests of outlier detection indicated that the influence of the independent contrast between *O. graptus* and *O. kaurna* on the analyses was significant.

Based on the phylogeny presented here, *O. kaurna* is the nearest relative to *O. graptus*, but in fact, the species *O. alpheus, O. dierythraeus* and *O*. sp. 8 are the nearest relatives of *O. graptus* (Chapter 2, Figure 2.4). Unfortunately, these taxa were not included in the present analyses because data on their mature egg sizes are presently unknown. Based on immature egg sizes though, these species too have large-sized eggs and low latitude distributions (Norman 1992a, 2000). Hence, in these analyses, *O. kaurna*, which is an outgroup to this clade of species, was the closest relative to *O. graptus*. It is likely that comparison of *O. kaurna* to any other of these taxa would reflect the same kinds of results as those observed when *O. kaurna* was compared to *O. graptus*. *Octopus kaurna* has a similar body size, egg size and latitudinal distribution to other members of the *O. macropus* species group (the group to which both *O. graptus* and *O. kaurna* belong phylogenetically according the phylogeny presented in this study). The distinct difference of *O. graptus* compared to its relatives (i.e. those of the broader *Octopus macropus* species group) in both egg size and body size, and egg size and latitude, is of interest because it suggests a divergent evolutionary history and/or alternate selective pressures for this taxon (and its more closely related species such as *O. alpheus* and *O. dierythraeus*) compared to other octopuses. It is presently suggested that *O. graptus* has been subjected to historical processes and selective pressures that

are different to those operating on many other shallow-water octopuses and this may be the basis for the distinct life history strategy that exists in this species (and perhaps in closely related species such as *O. alpheus* and *O. dierythraeus*).

The eggs of *O. graptus* are twice the size of most other shallow-water octopuses, it also has a tropical geographic distribution that is restricted to the northern Australian coastline (Norman 1992a). The present findings suggest that *O. graptus* has both a latitudinal distribution and body size relative to its egg size that is quite different to those of other species. It is proposed that these large differences may be explained in terms of a significant change in niche during the evolution of the species (and/or a clade of species) while past life history strategies have been retained.

A number of possible scenarios of niche change in *O. graptus* may explain its differences to other taxa. Firstly, its difference may be associated with known oceanic and climatic historical changes to the Australian continent and its surrounding marine environment. *O. graptus* shares a geographic distribution with another remarkable, unrelated, member of the subfamily Octopodinae, *Ameloctopus litoralis* (Norman 1992c), which has a morphology and biology that is distinctively different to many other shallow-water octopuses. Species such as *O. graptus* and *A. litoralis* are possibly relics from a palaeoaustral (cool-temperate) environment and may therefore possess life history strategies adapted to cooler environments. About 40 million years ago, the Australian continent broke away from the Gondwanan Continent and slowly drifted north in isolation. Coastal marine taxa carried along with this land mass as it moved into warmer latitudes have been described as "palaeoaustral" (Wilson and Allen 1987). The land mass eventually joined the Indo-West Pacific region such that northern Australia became a tropical realm (Knox 1979; Wilson and Allen 1987). This may have resulted in a significant change in niche for *O. graptus*, but not in life history traits. Alternatively, *O. graptus* may have historically lived in deeper waters. Larger egg sizes and body sizes are thought to be adaptations to deep-sea environments (Voss 1988). It is possible that *O. graptus* or its ancestors had occupied a deeper water realm but subsequently moved back into a shallow-water habitat. These suggestions are tentative, but it seems that *O. graptus* has experienced selective pressures that favour both larger body size and larger egg size compared to other species that live in lower latitude environments.

To investigate the factors that have influenced the evolution of egg size in this species it would be desirable to examine the relationship between egg size and body size and egg size and latitudinal variation in other close relatives of *O. graptus*. This would provide an insight into whether an alternative trend exists among these taxa and if alternate present day selection pressures are operating on these species compared to other species. Alternatively, as suggested here, some past processes may have influenced the life history traits that these species presently possess.

Another contrast that was observed to differ from the general trend observed among taxa between egg size and body size was the *Hapalochlaena* clade compared to the *O. aegina* group clade. Little difference in body size was observed between the groups but members of the genus *Hapalochlaena* used in this study (i.e. *H. fasciata* and *H. maculosa*) have larger eggs relative to members of the *O. aegina* species group. This same contrast showed the *Hapalochlaena* species to have a larger egg size and higher latitudinal distribution relative to members of the *O. aegina* group clade. Overall, it is more plausible that egg sizes in *Hapalochlaena* are adapted to their present day temperate macro-environmental niches (i.e. cool-temperate waters of southern Australia) rather than to selection pressures imposed by body size. It is thought that they were found to have a disparate egg size versus body size relationship compared to other taxa because of the their difference in latitudinal distribution. Another species to consider is *H. lunulata*, which is a tropical species that has small sized eggs. Its relationship to the other blue-ringed octopuses is presently unknown but it would also be of interest to examine the comparative relationships among these closely related species.

3.4.2.5 Covariation between inferred juvenile type and latitude

An indication that the association between macro-environmental variation and egg size may also apply to juvenile types was observed. A formal test for covariation between inferred juvenile type and latitude (Table 3.3) indicated that transitions in juvenile type might be consistent with changes in latitude. Additionally, a descriptive graphical representation (i.e. scatter plot, Figure 3.2) where egg size was plotted against latitude and individual species were coded according to their inferred juvenile type showed that species with benthic juvenile types are most prevalent at high latitudes (greater than

absolute 23.5˚). Species with planktonic types were shown to be most prevalent at low latitudes (less than or equal to absolute 23.5˚). It should be noted though, that without measured juvenile types for all species (i.e. as demonstrated by morphology of juveniles and the amount of time they spend in the plankton) any conclusions regarding the adaptability of juvenile types are tentative and solely based on inference.

3.4.3 Evolution of reproductive strategies in the Octopodinae

The phenotype of both egg size and juvenile type in a species are contributing factors in defining its reproductive strategy. Among the octopuses two key strategies are known, the small egg and planktonic juvenile type strategy and the large egg and benthic juvenile type strategy. This study examined factors that influence the evolution of egg sizes as well as reconstructing the pattern of evolution in inferred juvenile types among species. Here I discuss both of these life history traits to make some inference about the evolution of reproductive strategies in the benthic shallow-water octopuses.

Based on the present results, and the assumption that egg size and juvenile type are tightly related traits, an hypothesised pattern of evolution among reproductive strategies in benthic shallow-water octopuses is proposed. Small egg sizes and the planktonic juvenile type are thought to be the ancestral character state and subsequent evolutionary transitions towards larger egg sizes and benthic juvenile types have been adaptive responses to specific environmental conditions. However, transitions from one strategy to another are thought to be rare due to a tendency for egg sizes to be constrained by phylogeny, retaining the strategies of their ancestors and other members within their own lineage while responding less to natural selection. Despite this constraint, evidence that a dichotomy in egg size has been maintained throughout the evolution of octopus species indicates that the adaptive transitions in egg size and perhaps juvenile type have been an advantageous strategy. Of interest here are the means by which the dichotomy in these traits is maintained.

The dichotomy in juvenile types is distinct, such that juveniles can be either benthic or planktonic. Conversely, egg sizes are not strictly divided and many species exist that have intermediate egg sizes. However, evidence is presented here that indicates the extreme ends of the egg size continuum are favoured depending on the environmental niche that a species occupies. The variability of egg sizes among species suggests this

trait responds to a range of selection pressures, including those associated with macro-environmental variation, which also influence juvenile types. Although major transitions in egg size and juvenile type are likely to be rare, it is known that major transitions in egg size have the potential to directly influence juvenile type and may be the product of selection pressures that favour juvenile types. Furthermore, as large shifts in egg size may precede shifts in juvenile type (Hart 1996; Levitan 2000) selection pressures operating on egg size are likely to result in large transitions in egg size that will ultimately influence transitions in juvenile type.

The findings of this study indicated two factors have contributed to the evolution of egg sizes in benthic shallow-water octopuses, latitudinal variation, and more weakly, body size. It is likely that these factors have also been involved in the evolution of a dichotomy in reproductive strategies among species. In section 3.4.2.2, it was suggested that the dichotomy in egg sizes among shallow-water octopuses is maintained by a fecundity-survival trade-off and that under difficult environmental conditions where food availability and/or temperatures are low, large eggs are selectively advantageous (Bagenal 1969; Ferguson and Fox 1984; Perron 1986; McEdward and Miner 2003). Further, selection on juvenile types may also be strong and in high-risk conditions, such as cold and/or food poor environments, the benthic juvenile type is also expected to be selectively advantageous. This is because benthic offspring are known to be stronger and more robust, having a higher chance of survival at hatching than smaller juveniles (Werner 1986). Alternatively, in environments where food and/or temperature are high, even if food levels fluctuate, a high fecundity strategy (i.e. small eggs and planktonic juveniles) is likely to be advantageous (McEdward and Miner 2003). One of the key advantages to having small progeny and a planktonic juvenile type is that the risk of juvenile mortality can be spread widely via planktonic dispersal. Movement away from the natal habitat, both temporarily or permanently, in the event of a disturbance or catastrophic event improves the chances of survival for progeny if they are able to enter new environments.

The weaker negative relationship between egg size and body size after accounting for phylogeny, where larger females tended to have smaller eggs compared to closely related taxa, is characteristic of selection pressures operating on species to maximise fecundity. This relationship may also contribute to maintenance of a dichotomy among

the benthic shallow-water octopuses because selection for maximum female fecundity can be in conflict with offspring fitness, defined here as survival of offspring to the end of the developmental juvenile stage. It is generally accepted that high female fecundity is always selectively advantageous (Lack 1968). The small-sized eggs produced by species with high fecundity usually produce relatively underdeveloped offspring at hatching that generally have lower survivorship than offspring from larger eggs (Fox 2000). In environments that are cold and/or where food levels are low selection pressures often favor larger egg sizes (i.e. high survivorship of progeny) (Roff 1992; Stearns 1992; Bernardo 1996). In these types of environments juvenile fitness can be in conflict with maternal fitness such that selection on juvenile fitness can counter selection favoring maximal maternal fecundity (Parker and Begon 1986; Sibly and Calow 1986; Fox 2000). Selection for high maternal fecundity (i.e. very small egg sizes relative to body size) may therefore depend on the magnitude of selection favouring larger sized eggs (Roff 1992; Stearns 1992; Bernardo 1996).

Use of a comparative phylogenetic approach has provided a means of examining covariation and patterns of evolution in egg size and juvenile type that incorporates historical relationships among taxa into the analyses. The evidence from this study suggests that the pattern of evolution in egg size and juvenile types in the benthic shallow-water octopuses conform to predictions made by benthic marine invertebrate life history theory. However, other observations in this study indicate that some species, including *Octopus graptus*, may be subjected to alternative selection pressures, or have responded to selection pressure disparately to those of many other species, indicating a high degree of complexity in the evolution of these traits.

3.4.4 Conclusions and future directions

By incorporating phylogeny into the analysis of trait correlation and evolution, it was possible to discriminate between three scenarios of evolution among species in these traits a) phylogenetic inertia, b) coevolution and c) independent evolution of traits (Clutton-Brock and Harvey 1984; Felsenstein 1985a). There were four main conclusions concerning octopus reproductive life history evolution in this study. Firstly, the evolution of egg size has been adaptive but also constrained by phylogenetic inertia. Secondly, egg size covaries with latitude and more weakly with body size in octopuses and therefore at least two separate selective pressures are likely to be

operating on egg size, and perhaps juvenile type, to influence their evolution. Weaker selection pressures were also observed that maximise female fecundity and have resulted in smaller egg sizes in larger species while selection pressures to maximise offspring survival in risky environments have resulted in larger egg sizes in species that occupy niches associated with higher latitudes. Thirdly, the pattern of evolution in juvenile types, inferred from egg size as a percentage of adult body size, indicated that three independent transitions from the planktonic to the benthic juvenile type have occurred among these taxa. Finally, based on the covariation between egg size and latitudinal variation, juvenile types may also be an adaptation to particularly high-risk environments for juveniles (i.e. environments that are cold and/or food poor), responding to natural selection imposed by macro-environmental factors. Furthermore, this scenario may also apply to overall reproductive strategy evolution.

There are difficulties inherent in reconstructing trait evolutionary history because reasonably complex patterns of evolution usually require large amounts of data to estimate the most likely path of evolution to present day character states. Here, a large number of taxa were used to examine whether a correlation between egg size and latitude and egg size and body size exists when the influence of phylogeny was not adjusted for. Use of this large data set was advantageous as it allowed investigation of trends over a broad group of species. Unfortunately, when these trends of covariation were examined by removing the influence of phylogeny, a large amount of information was lost because information on phylogenetic relationships was only known for a subset of the aforementioned taxa. In order to examine whether the observations made in the present study apply to benthic shallow-water octopuses in general, phylogenetic information for more species would help to establish a more complete representation of reproductive life history evolution in this subfamily (i.e. the Octopodinae).

Juvenile types were inferred from egg size relative to body size but this method was prohibitive because it was not possible to know whether the estimated juvenile types truly reflected life history strategies within individual species. By gaining records of the measured juvenile stage of development for all species, including information on both the size of juveniles at the time of hatching and time that is spent in the plankton, it would be possible to make a more rigorous inference regarding the selective forces acting on juvenile type and egg sizes independently. Furthermore, the trade-off

between clutch size and egg size was discussed in relation to fecundity. In order to further investigate fecundity, data on average clutch sizes for all species are required. Finally, other factors such as competition for resources among juveniles and the levels of predation suffered by juveniles, which were not examined here, are also important selective factors in the evolution of egg size and reproductive strategy (Menge 1975; Clarke and Goetzfried 1978) and should be considered in future studies.

CHAPTER 4 General Discussion.

This research investigated the evolutionary biology of the benthic shallow-water octopuses. The first experimental chapter described the use of a molecular phylogenetics approach to elucidate evolutionary relationships among species from the subfamily Octopodinae. This phylogeny was then used in a comparative analysis in the second experimental chapter, to investigate whether the evolution of reproductive strategies in benthic shallow-water octopuses had involved adaptation to macro-environmental gradients associated with latitude. Here, a general discussion of the major findings of this study is presented.

4.1.1 Phylogeny of the benthic shallow-water octopuses

The phylogenetic relationships among members of the subfamily Octopodinae established here indicate that revision of the genus *Octopus* is necessary. Firstly, the genus *Octopus* is not a monophyletic group and its taxonomy should reflect this. Furthermore, based on the divergent clades observed here, sub-division of the genus *Octopus* into distinct genera and sub-genera is required. The three monophyletic species groups, *Abdopus*, *O. australis* and *O. vulgaris* (i.e. the systematic groupings of Robson (1929) and Norman (1993)) delineate taxa from the larger group. It is likely that the *O. macropus* and *O. aegina* groups are also distinct lineages but based on the present data there is limited resolution of the true *O. macropus* species group.

The present results indicate there are at least three to five monophyletic groups within the genus *Octopus*. Within these major groups a number of underlying relationships are likely to exist. For instance, the larger *O. macropus* group + *O. australis* group clade probably comprises a number of distinct lineages including: the "true" *O. macropus* group, *O. maorum* and its relatives, the *O. australis* group, and *Grimpella thaumastocheir* whose relatives are unknown. These relationships are presently indiscernible but the distinctive morphologies of these taxa suggest these groups are distinct lineages.

The shallow-water octopuses (subfamily Octopodinae) have been described as a diversifying group (Robson 1929), particularly the genus *Octopus* (Packard 1972). The

O. aegina + *Hapalochlaena* clade, observed in this study, offers speculative evidence that there has indeed been a recent evolutionary divergence among species. The key indicators of a recent delineation among species are the very short branch lengths between *O. aegina* group members compared to lengths between members of other groups, suggesting that the Indo-West Pacific and Australian species of the *O. aegina* group may have undergone a recent radiation burst. Some evidence also exists that *O. aegina* and *Hapalochlaena* are sister groups, potentially originating from a single common ancestor. This new hypothesis is suggested to indicate that the members of the *O. aegina* group that have skin patterns on their bodies known as ocelli may be an ancestral form of the extensive blue-ring patterning that is diagnostic for the genus *Hapalochlaena* (discussed in Chapter 2). Other *Octopus* species groups treated in this study may represent older lineages.

Examination of the phylogenetic relationships among the species in this study has provided a clear understanding of historical relationships among some of the benthic shallow-water octopuses. Further investigation of phylogeny in the Octopodinae would help to resolve the deeper divergences among species and clarify the relationships among clades of taxa. This would help to determine the more ancient relationships among the groups and provide a clearer examination of the ancestors to the benthic shallow-water octopuses.

4.1.2 Evolution of reproductive strategies in shallow-water octopuses

The pattern of evolution in inferred juvenile types and the factors that influence variation in egg sizes among benthic shallow-water octopuses were also examined. Under the principle that egg size and juvenile type are tightly related traits (Emlet et al. 1987; Sinervo and McEdward 1988; Emlet 1995; Hart 1995) I propose a number of hypotheses regarding the evolution of reproductive strategies in octopuses.

Based on the results for 22 benthic shallow-water octopus species, a number of generalisations regarding reproductive strategy evolution in benthic shallow-water octopuses are suggested. Firstly, small eggs and planktonic juvenile types are likely to be the ancestral states for shallow-water octopuses in general; traits that have probably been retained from an ancient pelagic cephalopod ancestor (Young et al. 1998). Further, based on the covariation of egg size with latitudinal variation, inter-specific

evolution in both egg size and juvenile type is suggested to reflect adaptations to natural selection resulting from large-scale ecological factors: a finding that is consistent with benthic marine invertebrate life history theory (Thorson 1950; Vance 1973; Christiansen and Fenchel 1979; Levitan 1993; Havenhand 1995; McEdward 1997). Large eggs and benthic juveniles may be an adaptation to high-risk conditions such as deep-sea (Voss 1988) and/or cold environments. This is supported by the tendency for transitions in juvenile type to occur in the direction of small egg size and planktonic juvenile type to large egg and benthic juvenile type amongst taxa. Finally, evidence that egg sizes are constrained by phylogeny was observed, which may also indicate a constraint on reproductive strategies such that transitions in strategy are rare.

The dichotomous reproductive strategies that exist among species of the benthic shallow-water octopuses are an exceptional life history feature that is only observed in one other cephalopod family, the Idiosepiidae (Boletzky 1977). However, many other benthic marine invertebrates also maintain dual reproductive strategies between species (e.g. Perron 1986; Lessios 1990; Hart 1995; Wellington and Robertson 2001) and a large body of theory exists regarding the manner in which these traits have evolved and been maintained throughout evolutionary history. Using a comparative phylogenetic approach it was possible to investigate hypotheses generated by optimality models and experimental observations in an historical context and to examine the patterns of evolution in traits.

The dichotomy in egg size and juvenile type appears to be maintained by a fecundity-survival trade-off (Bagenal 1969; Vance 1973; Smith and Fretwell 1974; Christiansen and Fenchel 1979; Ferguson and Fox 1984; Sinervo 1990) that responds in one of two ways to natural selection. This is thought to be influenced by the environmental conditions that a species inhabits. In environments where selection pressures favour a strategy that increases fecundity (e.g. tropical environments) the small egg - planktonic juvenile strategy is favoured. Alternatively, in environmental conditions where selection pressures favour a strategy that improves juvenile survival (e.g. temperate environments) the large egg - benthic juvenile strategy is favoured. These conclusions are consistent with hypotheses of life history evolution as described by a number of optimality models (Thorson 1950; Vance 1973; Christiansen and Fenchel 1979; Havenhand 1995; McEdward 1997; McEdward and Miner 2003).

For traits to evolve and be adaptive they need to be genetically variable (Roff 1992). The adaptive nature of egg size and juvenile types described here is indicative of a phenotypic and genetic flexibility that can facilitate responses to natural selection if new pressures are encountered. The level of flexibility in these traits among present day species may have originated during a time of evolutionary instability in life styles and/or niche occupation during the evolution of octopods. For instance Young et al. (1998) have suggested that a number of transitions from benthic adult life style to pelagic adult life style have occurred during the evolution of octopod groups within the suborder to which the benthic shallow-water octopuses belong (i.e. Incirrata). Life in highly heterogeneous environments such as the coastal shallow-water realm may have `further facilitated the maintenance of this variation because the variability of selection pressures in these types of environments are likely to maintain genetic variation (Stearns 1992). Subsequently, the dichotomy in reproductive strategies among the shallow-water octopuses has probably been maintained because it improves species fitness and permits adaptation to new and/or different environment types.

Egg size is thought to respond to selection with continuous changes in size rather than in a dichotomous manner (Levitan 2000). The present results indicate that variation in egg sizes is somewhat continuous but major transitions from one end of the egg size continuum to the other have occurred historically in the presence of strong selection pressures. The latter observation supports hypotheses of a number of optimality models that have predicted that egg sizes are generally dichotomous in their evolution (Thorson 1950; Vance 1973; Christiansen and Fenchel 1979; Havenhand 1995; McEdward 1997; McEdward and Miner 2003). A possible scenario of egg size evolution in octopuses is that variation is generally continuous among taxa but the close relationship between egg size and juvenile type may mediate large transitions in egg size if selection on the juvenile type is strong. In this way, egg size has the potential to mediate change in juvenile types in response to natural selection when one juvenile type is favoured over another.

Based on the present findings, it is not possible to discern whether the evolution of egg size and juvenile type among species is instantaneous or gradual. It would be of interest to examine whether egg size is a more evolutionarily labile trait than juvenile

type or if juvenile type also evolves continuously. This could be achieved by comparing the pattern of evolution in both egg size and juvenile types. The juvenile types would require measurement for each species to include the size of juveniles at hatching and the time spent in the plankton. This would help to identify whether evolutionary transitions in egg size precede transitions in juvenile type and whether the evolution of these traits is gradual and continuous rather than instantaneous as many optimality models suggest. Furthermore, it would be of interest to examine species and their life history traits at intermediary latitudes to examine if they have more variable and/or intermediate life history strategies compared to species at either end of the environmental gradient continuum. This would require knowledge of the range of egg sizes individual species have, in addition to the duration that juveniles spend in the plankton. Few studies examine this type of fundamental juvenile biology in cephalopods, but such information would help to identify whether species have intermediate life history strategies and whether they experience transitions in size and form according to variation in environmental selection pressures. In this way, it would be possible to distinguish whether the evolution of these traits is continuous and gradual or if it is punctuated and instantaneous, evolving from one end of the dichotomy continuum to the other.

Reproductive strategies have the potential to dramatically influence the evolution of species. The adaptive nature of these life history traits in octopuses allows species to maintain fitness in new environments even if conditions are extreme. By examining phylogenetic relationships among the benthic shallow-water octopuses and using this information to investigate the evolution of life history strategies among taxa it was possible to identify selective forces that have influenced reproductive strategy evolution. The findings of this study suggest complex phylogenetic relationships exist among species of the benthic shallow-water octopuses. This historical complexity is also reflected in the patterns of evolution in their reproductive strategies. It is hoped that this study has improved our understanding of the evolution of these relatively unknown benthic marine invertebrates.

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APPENDIX 1: Commands used in Bayesian phylogenetic analysis (MrBayes)

```
begin mrbayes;
set nowarnings=yes;
set autoclose=yes;
charset nuc = 1-135;
charset mt = 136-464;
partition bygene = 2:nuc,mt;
set partition=bygene;
lset rates=gamma ngamma=8;
prset aamodelpr=fixed(equalin);
prset applyto=(1)
statefreqpr=fixed(0.059,0.050,0.058,0.049,0.004,0.022,0.071,0.097,0.016,0.043,0.066,0
.077,0.013,0.036,0.088,0.050,0.059,0.014,0.015,0.113);
prset applyto=(2)
statefreqpr=fixed(0.033,0.015,0.049,0.024,0.012,0.014,0.029,0.065,0.044,0.097,0.130,0
.017,0.051,0.113,0.046,0.084,0.055,0.031,0.036,0.055);
mcmcp ngen=60000 printfreq=100 samplefreq=100 nchains=4 savebrlens=yes;
end;
```

APPENDIX 2: Genbank accession numbers for each gene sequence

Species	*cox3*	*cob*	EF-1α
Ameloctopus litoralis	AJ628207	AJ628171	AY651854
Argonauta nodosa	AJ628206	AJ628170	AY651855
Cistopus indicus	AJ628208	AJ628172	-
Grimpella thaumastocheir	AJ628209	AJ628173	AY651856
Hapalochlaena fasciata	AJ628210	AJ628174	AY651857
Hapalochlaena maculosa	AJ628212	AJ628176	AY651859
Hapalochlaena sp. 1	AJ628211	AJ628175	AY651858
Octopus aculeatus	AJ628213	AJ628177	AY651860
Octopus aegina	AJ628214	AJ628178	AY651861
Octopus alpheus	AJ628215	AJ628179	AY651862
Octopus aspilosomatis	AJ628216	AJ628180	AY651863
Octopus australis	AJ628217	AJ628181	AY651864
Octopus berrima	AJ628218	AJ628182	AY651865
Octopus bunurong	AJ628219	AJ628183	AY651866
Octopus cf. *kagoshimensis*	AJ628226	AJ628190	AY651871
Octopus cf. *tetricus* (WA) [1]	AJ628238	AJ628201	AY651882
Octopus cf. *tetricus* (WA) [2]	AJ628239	AJ628202	-
Octopus cyanea[1]	AJ628220	AJ628184	AY651867
Octopus cyanea[2]	AJ628221	AJ628185	-
Octopus dierythraeus	AJ628222	AJ628186	AY651868
Octopus exannulatus	AJ628223	AJ628187	AY651869
Octopus graptus	AJ628224	AJ628188	AY651870
Octopus kaurna	AJ628227	-	AY651872
Octopus maorum	AJ628231	AJ628194	AY651873
Octopus marginatus	AJ628232	AJ628195	AY651874
Octopus mototi	AJ628233	AJ628196	-
Octopus ocellate sp. A	AJ628234	AJ628197	AY651875
Octopus oculifer	AJ628235	AJ628198	-
Octopus pallidus	AJ628236	AJ628199	AY651876
Octopus sp. 10	AJ628229	AJ628192	AY651879
Octopus sp. 5	AJ628225	AJ628189	AY651878
Octopus sp. 8	AJ628228	AJ628191	-
Octopus sp. x (Sth. Africa)	AJ628230	AJ628193	AY651880
Octopus tetricus (NSW) [1]	AJ628237	AJ628200	AY651881

Species	*cox3*	*cob*	EF-1α
Octopus tetricus (NSW) [2]	AJ628240	AJ628203	-
Octopus vulgaris	AJ628241	AJ628204	AY651883
Opisthoteuthis sp.	AJ628242	AJ628205	AY651877

Note: Gene abbreviations: Cytochrome oxidase subunit III = *cox3;* Cytochrome *b* apoenzyme = *cob*; Elongation Factor-1α = EF-1α. X indicates accession numbers are presently unavailable and – indicates the gene region was not sequenced for that species.

APPENDIX 3a: Estimates of egg size, body size, latitude and residual egg size adjusted for body size for each species.

Species	Latitude abs. Mean	Body size (mm)	Egg length (mm)	Egg size % ML	Egg type Binary	Residuals
Callistoctopus macropus	12.50	155.00	2.50	1.60	0.00	-0.84
Eledone cirrhosa	53.00	150.00	9.00	6.00	0.00	0.45
Eledone moschata	33.50	150.00	14.00	9.30	0.00	0.89
Hapalochlaena lunulata	4.50	31.00	3.00	9.60	0.00	-0.29
Hapalochlaena nierstraszi	12.50	17.00	2.40	14.10	1.00	-0.37
Macrotritopus defilippi	11.00	55.00	2.10	3.80	0.00	-0.78
Octopus abaculus	10.75	33.00	2.40	7.30	0.00	-0.53
Octopus alecto	27.50	50.00	2.50	5.00	0.00	-0.58
Octopus bimaculatus	32.50	200.00	4.00	2.00	0.00	-0.43
Octopus bimaculoides	26.00	120.00	17.00	14.20	1.00	1.13
Octopus briareus	10.00	120.00	14.00	11.70	1.00	0.94
Octopus bocki	1.50	25.00	2.00	8.00	0.00	-0.64
Octopus burryi	15.75	65.00	2.50	3.80	0.00	-0.64
Octopus chierchiae	18.75	18.00	3.80	21.10	1.00	0.08
Octopus conispadiceus	44.25	210.00	28.00	13.30	1.00	1.50
Octopus digueti	27.50	50.00	10.00	20.00	1.00	0.81
Octopus dofleini dofleini	50.00	190.00	7.00	3.70	0.00	0.14
Octopus fangsiao typicus	37.50	52.00	10.00	19.20	1.00	0.80
Octopus filosus	12.50	72.00	1.70	2.40	0.00	-1.05
Octopus fitchi	28.00	40.00	6.00	15.00	1.00	0.35
Octopus globosus	33.50	32.00	9.00	28.10	1.00	0.80
Octopus hawaiiensis	19.50	30.00	3.00	10.00	0.00	-0.28
Octopus joubini	23.50	45.00	4.80	10.60	1.00	0.10
Octopus kagoshimensis	18.50	40.00	2.00	5.00	0.00	-0.75
Octopus lobensis	35.00	37.00	9.00	24.30	1.00	0.77
Octopus luteus	17.00	130.00	3.40	2.60	0.00	-0.49
Octopus maya	20.25	119.00	17.00	8.50	0.00	1.14
Octopus microphthalmus	11.00	40.00	11.00	27.50	1.00	0.95
Octopus micropyrsus	37.00	30.00	10.00	33.30	1.00	0.92
Octopus minor typicus	38.50	100.00	8.00	8.00	0.00	0.42
Octopus neglectus	17.50	64.00	0.80	1.30	0.00	-1.78
Octopus ochotensis	52.50	42.00	9.00	21.40	1.00	0.74

Species	Latitude abs. Mean	Body size (mm)	Egg length (mm)	Egg size % ML	Egg type Binary	Residuals
Octopus ornatus	1.00	130.00	3.00	2.30	0.00	-0.62
Octopus ovulum	36.50	45.00	3.00	6.70	0.00	-0.37
Octopus parvus	37.00	40.00	1.80	4.50	0.00	-0.86
Octopus polyzenia	23.00	37.70	8.00	21.20	1.00	0.65
Octopus pumilus	11.50	31.00	4.20	13.50	1.00	0.05
Octopus rex	0.75	76.00	3.00	3.90	0.00	-0.50
Octopus rubescens	37.20	100.00	4.00	4.00	0.00	-0.27
Octopus sasakii	33.50	45.00	10.00	22.20	1.00	0.83
Octopus selene	7.50	60.00	1.00	1.70	0.00	-1.54
Octopus siamensis	11.75	64.00	1.70	2.70	0.00	-1.02
Octopus superciliosus	35.75	26.00	9.50	36.50	1.00	0.91
Octopus tenebricus	20.00	19.00	1.20	6.30	0.00	-1.09
Octopus tonganus	20.00	35.00	1.20	3.40	0.00	-1.23
Octopus varunae	14.00	62.00	2.00	3.20	0.00	-0.85
Octopus vitiensis	8.50	60.00	2.00	3.30	0.00	-0.85
Octopus warringa	39.25	20.00	2.50	12.50	1.00	-0.37
Octopus yendoi	39.00	43.00	17.00	39.50	1.00	1.37
Octopus zonatus	10.50	30.00	6.10	20.33	1.00	0.43
Ameloctopus litoralis	21.00	30.00	10.00	33.30	1.00	0.92
Argonauta nodosa	25.00	100.00	1.50	1.50	0.00	-1.25
Cistopus indicus	15.00	86.00	5.00	5.80	0.00	-0.01
Grimpella thaumastocheir	35.75	40.00	15.00	37.50	1.00	1.26
Hapalochlaena fasciata	32.00	45.00	9.00	20.00	1.00	0.72
Hapalochlaena maculosa	36.25	57.00	9.00	15.80	1.00	0.67
Octopus aculeatus	0.50	58.00	3.00	5.20	0.00	-0.43
Octopus aegina	4.00	62.00	2.00	3.20	0.00	-0.85
Octopus australis	32.00	72.00	12.00	16.70	1.00	0.90
Octopus berrima	37.75	105.00	14.00	13.30	1.00	0.97
Octopus bunurong	36.25	95.00	10.00	10.50	1.00	0.66
Octopus cyanea	2.00	172.00	3.00	1.70	0.00	-0.68
Octopus exannulatus	6.00	50.00	3.90	7.80	0.00	-0.14
Octopus graptus	14.50	190.00	28.00	14.70	1.00	1.53
Octopus kaurna	36.25	85.00	11.00	12.90	1.00	0.78
Octopus maorum	41.75	255.00	6.00	2.40	0.00	-0.08

Species	Latitude abs. Mean	Body size (mm)	Egg length (mm)	Egg size % ML	Egg type Binary	Residuals
Octopus marginatus	8.50	80.00	3.00	3.80	0.00	-0.51
Octopus mototi	0.00	100.00	3.20	3.20	0.00	-0.49
Octopus ocellate sp. A	1.00	60.00	3.00	5.00	0.00	-0.44
Octopus pallidus	37.75	73.50	11.00	15.00	1.00	0.81
Octopus tetricus nsw	29.75	140.00	3.00	2.10	0.00	-0.64
Octopus cf. tetricus wa	29.75	250.00	2.40	1.00	0.00	-0.99
Octopus vulgaris	7.50	200.00	2.70	1.40	0.00	-0.83

Note: Abbreviations: abs. = absolute; ML = Mantle Length.

APPENDIX 3b: Estimates of species distributions and source references

Species	Northernmost country		Southernmost country		Ref.
Callistoctopus macropus	Florida, US	30N	N Brazil	5S	1
Eledone cirrhosa	Norway, Iceland	70N	MS, St.s Gibraltar	36N	2
Eledone moschata	MS	36N	-	31N	2
Hapalochlaena lunulata	E Indies	25N	S GBR	16S	3
Hapalochlaena nierstraszi	TLO, Aves Is.s	12N	-	-	4
Macrotritopus defilippi	Bahamas	27N	N Brazil	5S	1
Octopus abaculus	Batangas, S Negros	13.5N	N W Mindanao	8N	3, 5
Octopus alecto	G. Calif., Mex.	32N	-	23N	6
Octopus bimaculatus	P. coast Calif., US	42N	G. Calif., Mex.	23N	6
Octopus bimaculoides	P. mid coast Calif., US	37N	Mex.	15N	6
Octopus briareus	S Florida, US	25N	N Brazil	5S	7
Octopus bocki	Batangas, S Negros	13.5N	Fiji	16S	3, 6
Octopus burryi	N Carolina, US	36.5N	N Brazil	5S	7
Octopus chierchiae	Mex.	30N	P. coast Panama	7.5N	6
Octopus conispadiceus	N E Japan	46N	-	42.5N	8
Octopus digueti	G. Calif., Mex.	32N	-	23N	6
Octopus dofleini dofleini	Sub-Arctic	65N	Korea	35N	8
Octopus fangsiao typicus	P. coast Japan	42N	S Sea Japan	33N	8
Octopus filosus	Florida, US	30N	N Brazil	5S	7
Octopus fitchi	G. Calif., Mex.	32N	-	24N	6
Octopus globosus	Kyushu, Japan	33.5N	-	-	8
Octopus hawaiiensis	P., Hawaii	19.5N	-	-	6
Octopus joubini	Georgia, US	32N	C. Caribbean Sea	15N	7
Octopus kagoshimensis	Japan	42N	Sumatra	5S	8
Octopus lobensis	S Brazil	30S	S C. Argentina	40S	7
Octopus luteus	P. coast Japan	42N	Indonesia	8S	6
Octopus maya	Yucatan, Mex.	21.5N	Vera Cruz	19N	7
Octopus microphthalmus	TLO, Port Blair	11N	-	-	4
Octopus micropyrsus	P. coast Calif., US	42N	-	32N	6
Octopus minor typicus	Japan	46N	-	31N	8
Octopus neglectus	Taiwan	25N	G. Thailand	10N	16
Octopus ochotensis	Sea Okhotsk	60N	-	45N	8
Octopus ornatus	Japan	28N	N NSW, Aus.	30S	3, 9
Octopus ovulum	Honshu, Japan	41N	Nagasaki, Japan	32N	8

Species	Northernmost country		Southernmost country		Ref.
Octopus parvus	P. coast Japan	42N	-	32N	6
Octopus polyzenia	Dampier Arch., Aus.	21S	C. QLD, Aus.	25S	10
Octopus pumilus	Batangas, S Negros	13.5N	Siquijor	9.5N	3
Octopus rex	Andaman Sea	13.5N	N Aus.	15S	16
Octopus rubescens	P. coast G. Alaska	60N	Mex.	15N	6
Octopus sasakii	S Honshu, Japan	34N	W Kyushu, Japan	31N	8
Octopus selene	P. coast Panama	7.5N	-	-	6
Octopus siamensis	Andaman Sea	13.5N	G. Thailand	10N	16
Octopus superciliosus	GAB, S NSW	31.5S	Bass St., Aus.	40S	11
Octopus tenebricus	TLO, Bowen, Aus.	20S	-	20S	11
Octopus tonganus	TLO , Tonga	20S	-	-	5
Octopus varunae	TLO , Arabian Sea	14N	-	-	4, 6
Octopus vitiensis	PNG	1S	S P., Fiji	16S	6
Octopus warringa	GAB, S NSW, Aus.	31.5S	Stewart Is., NZ	47S	13
Octopus yendoi	Sea Japan	45N	-	33N	8
Octopus zonatus	Venezuela	12N	G. Darien	9N	7
Ameloctopus litoralis	Dampier Arch., Aus.	21S	S GBR, Aus.	21S	14
Argonauta nodosa	Indo W P., Namibia	12S	S VIC, Aus.	38S	5
Cistopus indicus	Pakistan	25N	Philippines	5N	3
Grimpella thaumastocheir	GAB, Aus.	31.5S	Bass St., Aus.	40S	15
Hapalochlaena fasciata	S QLD, Aus.	27S	S NSW	37S	11
Hapalochlaena maculosa	GAB, S NSW, Aus.	31.5S	S WA, Bass St., Aus.	41S	11
Octopus aculeatus	Vietnam, Philippines	20N	QLD, Aus.	19S	3, 5
Octopus aegina	Madras, India	13N	S E Asia	5S	3, 17
Octopus australis	S QLD, Aus.	27S	S NSW, Aus.	37S	12
Octopus berrima	GAB, S NSW, Aus.	31.5S	TAS, Aus.	44S	12
Octopus bunurong	GAB, S NSW, Aus.	31.5S	N TAS, Aus.	41S	13
Octopus cyanea	Africa	30N	C. QLD, Aus.	26S	11
Octopus exannulatus	Manila Bay	15N	S QLD, Aus.	27S	10
Octopus graptus	N Aus., GBR, Aus.	10S	Townsville, Aus.	19S	9
Octopus kaurna	GAB, S NSW, Aus.	31.5S	N TAS, Aus.	41S	13
Octopus maorum	GAB, C. NSW, Aus.	31.5S	Campbell Is.	52S	11
Octopus marginatus	Red Sea	30N	E Australia	21.5S	20
Octopus mototi	Okinawa, Japan	27N	Rapa Is.	27S	10
Octopus ocellate sp. A	G. Thailand	13N	N Australia	14S	20

Species	Northernmost country		Southernmost country		Ref
Octopus pallidus	GAB, C. NSW, Aus.	31.5S	TAS	44S	19
Octopus tetricus (NSW)	S QLD, Aus.	27S	S NSW, Aus.	34S	20
Octopus cf. tetricus (WA)	Shark Bay, WA Aus.	25.5S	Esperance, WA Aus.	34S	6
Octopus vulgaris	S England	50N	S E Africa	35S	1

Note: References: 1, (Mangold 1998); 2, (Norman 2000); 3, (Norman and Sweeney 1997); 4, (Toll 1998); 5, (Norman and Finn 2001); 6, (Hochberg et al. 1992); 7, (Voss and Toll 1998); 8, (Toll and Voss 1998); 9, (Norman 1992a); 10, (Norman 1992b); 11, (Stranks 1998); 12, (Stranks and Norman 1992); 13, (Stranks 1990); 14, (Norman 1992c); 15, (Stranks 1988a); 17, (Nateewathana and Norman 1999); 18, (Nateewathana 1997); 19, (Stranks 1988b); 20, (Norman 1998). Abbreviations: C. = Central; E = East; N = North; S = South; W = West; MS = Mediterranean Sea; P. = Pacific Ocean; Arch. = Archipelago; G. = Gulf; Is. = Island; St. = Strait; Calif. = California; Aus. = Australia; Mex. = Mexico; PNG = Papua New Guinea; GBR = Great Barrier Reef; NSW = New South Wales; QLD = Queensland; TAS = Tasmania; VIC = Victoria; WA = Western Australia; TLO = Type Locality Only.