

Systematics, Phylogeny, Phylogeography and Reproduction of *Neotrigonia* (Bivalvia: Palaeoheterodonta)

Ana Glavinic, BSc (Hon.)

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School of Biology, Faculty of Science and Engineering
Flinders University
Adelaide, Australia



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Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

~ Ana Glavinic

June, 2010

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Preface

This is a resubmission of a thesis, which was examined by two anonymous examiners and subsequently read by two advisors chosen by the Faculty. The Faculty permitted limited time for this revision. I have now addressed all of the comments made by examiners, and also acknowledge the helpful advice and suggestions made by advisors. This version of a thesis is a significant improvement upon the original.

This thesis comprises a series of manuscripts dealing with separate parts of the research project. This format is not typical of a traditional thesis, so there are some differences that need to be recognised beforehand. Firstly the tables and figures are not embedded within the text but are found at the end of each manuscript. Secondly, separate reference lists are provided at the end of each chapter. Finally some sections of the thesis can be repetitive at times because each chapter is meant to stand alone and thus can be read independently of the rest of the thesis. I apologise in advance for the repetition.

I wrote all of the chapters, however, other authors have been included for the purpose of publication and to acknowledge here their contribution to each of the separate pieces of work. The following table indicates the contribution of these co-authors to the piece of work:

Contribution of authors to manuscripts in the thesis:

Chapter	Concept	Method	Data analysis	Manuscript preparation
I	AG	AG	AG	AG/KB/PF
II	AG	AG/GR	AG	AG/GR/KB
III	AG	AG/GR	AG/GR	AG/NW/KB
IV	AG	AG/NW/GR	AG/NW/GR	AG/NW/GR
V	AG	AG/GR/ KB	AG/GR/ KB	AG/GR/ KB
VI	AG	AG	AG	AG/KB/PF

Where AG=Ana Glavinic, KB= Kirsten Benkendorff*, GR= Greg Rouse*, NW= Nerida Wilson[@] and PF= Peter Fairweather*. * = co-supervisors, @ = a collaborator of Prof. Rouse

Glossary

This glossary contains less common biological terms, but also common terms, which are used throughout the thesis to interpret a particular function or a morphological character.

acinus – singular Acini- a small saclike dilatation in ovaries containing eggs

acrosome - A caplike structure at the anterior end of a spermatozoon that produces enzymes aiding in egg penetration.

ciliary tracts- the respiratory tract that sweep in unison and help to sweep away fluids and particles.

ctenidia - A gill like structure, a respiratory apparatus of a mollusc.

denticulated- Finely toothed or notched, its use in the thesis is to describe the shell margin.

dissoconch – juvenile bivalve shell

eucheton- a small area on the shell near the umbo in the shape of the shield or a key hole like eucheton.

median carinae - Median carinae is a prominent feature on *Trigonia* and *Eotrigonia* specimens and it separates flank with radial ribs away from an area with parallel costae. In case of *Neotrigonia* this line is not as obvious, but it is present separating area from the flank.

metamorphic line- a shell feature delimitating prodissoconch from dissoconch

micropyle - a very small opening in the vitelline layer of an oocyte

oogonia - A cell that arises from a primordial germ cell (protogonia) and differentiates into an oocyte in the ovary.

palps- an elongated, often segmented appendage usually found near the mouth in invertebrate organisms such as molluscs.

prodissoconch – prejuvenile bivalve shell.

protogonia- a primordial germ cell of an oocyte.

spinous- pertaining to or like a spine, in the thesis it is used to describe shell rib ornamentation.

synonymisation- the act of identifying two known species to be identical and therefore synonyms.

vitelogenesis- process of yolk formation in an developing egg.

Abstract

This research investigates the evolution of *Neotrigonia* species (Bivalvia: Palaeoheterodonta), the remaining extant genus of the Trigonioida, a group of bivalves endemic to Australian waters. The intent of this research was to review the current systematics, investigate phylogeny and phylogeography of the genus, and advance scientific knowledge in regard to the presence of doubly uniparental inheritance in *Neotrigonia*, as well as to address some aspects of reproductive strategy and outline the process of oogenesis. The research has resulted in a thesis in manuscript format, where Chapter 1 is a general introduction to the thesis as a whole, Chapters 2-5 inclusive are research manuscripts, and Chapter 6 is a general discussion of the completed research.

In chapter 2, the type material of all of the seven extant, nominal species of *Neotrigonia* Cossman 1912 are reviewed and illustrated, based on available museum specimens and fresh collections. The type localities and currently-known distributions for each extant species are included. A cladistic analysis was performed using morphological characters of *Neotrigonia* species living and fossil, using *Eotrigonia subundulata* and *Trigonia miriana* as an outgroup. Results from parsimony analysis show that all *Neotrigonia* form a monophyletic clade, in which living and fossil *Neotrigonia* form reciprocally monophyletic sub-groups. The species status of *Neotrigonia bednalli*, Verco 1907, is revised based on examination of all available types, museum specimens and a relatively large number of newly-collected specimens from southern Australian waters. This assessment suggests that *N. bednalli* is a junior synonym of *N. margaritacea*. Species status is accepted for *N. gemma*, *N. lamarckii*, *N. uniophora*, *N. strangei* and *N. kaiyomaruae*. However, reclassification of *N. strangei* specimens from Western Australia to *N. margaritacea* would revise the previously disjunct distribution of this species to a narrower range in NSW. This chapter demonstrates the limitations in relying on shell morphology only for species classification in the *Neotrigonia*.

The contemporary knowledge of ocean currents, temperatures, and geological and climatic history across southern Australian waters represents a useful framework for phylogeographical analyses. There are already a number of studies that show coincident distribution patterns within some marine invertebrate groups across the Maugean, Flindersian and Peronian marine provinces. In Chapter 3, I examine the genetic structure of *Neotrigonia margaritacea* and *Neotrigonia lamarckii*.

Phylogenetic analyses based on COI and ITS gene sequence data reveals a split between southern Australian *Neotrigonia margaritacea* and eastern Queensland *Neotrigonia lamarckii*. The molecular analyses confirmed my synonymisation of *N. bednalli* to *N. margaritacea*. Population genetic analyses of the *Neotrigonia margaritacea* COI gene, in four different populations located hundreds of kilometres apart, revealed insight into genealogical pathways amongst haplotypes. These networks showed that there was no shared haplotypes among populations and most populations were significantly far from panmixia. The highest haplotype diversity was recorded from the Port Lincoln (South Australia) population. Haplotype variations across the range are discussed in terms of estimated population sizes and geographical barriers.

Several species of bivalves have been reported to have two mitochondrial DNA types, maternal and paternal. This system of mtDNA inheritance is known as doubly uniparental inheritance (DUI). In Chapter 4, the presence of the DUI phenomenon in *Neotrigonia margaritacea* is investigated within a phylogenetic framework for Paleoheterodonta, using COI and 16S rDNA molecular data. Results indicate the presence of DUI in *Neotrigonia margaritacea* and provide evidence for a masculinization event within this taxon. This phenomenon has so far been identified in six superfamilies of bivalves, so the new record of DUI in *N. margaritacea* was incorporated into a phylogenetic tree addressing the question of a single or multiple origins of DUI in Bivalvia. Parsimony transformations indicate that DUI is likely to be the ancestral state for all Bivalvia.

In Chapter 5, the ultrastructural stages of female gametogenesis are described for *Neotrigonia margaritacea*. The morphology of oocytes and gonad tissue are described for the first time using electron microscopy and histology techniques. Throughout the summer period, the ovary contains oocytes in various developmental stages. Oocytes develop from oogonia derived from protogonia and then undergo three distinct stages of oogenesis: previtellogenesis; vitellogenesis; and postvitellogenesis (or presence of mature oocytes). Based on gonad tissue and oocyte morphology, and as well as laboratory observations, it is inferred that *Neotrigonia margaritacea* is sequentially tachitictic, thus a trickle (continuous) spawner over an extended summer season.

In conclusion the museum collections of *Neotrigonia* and current systematics have provided valuable information on classification and distribution of this relic

bivalve genus. Morphological analysis has enabled preliminary synonymisation of species to establish species distributions. The results from molecular data confirmed aspects of phylogeny and revealed phylogeographic structure of *Neotrigonia margaritacea* in Southern Australian waters. The new molecular information regarding the presence of DUI and novel insight into reproductive strategies further our understanding of the evolutionary affinities of *Neotrigonia*. Based on the integration of these multidisciplinary results conservation assessment is suggested for *Neotrigonia margaritacea*.

General Introduction

Neotrigonia represents a relict lineage of the bivalve order Trigonioida. The marine Trigonioida is the sister taxon to the freshwater Unionoida, and together they form the monophyletic subclass Palaeoheterodonta (Giribet and Wheeler, 2002; Giribet, 2008) (Fig. 1.1). The evolutionary affinities of the Trigonioida have been a contentious subject for over a hundred years and are central to an understanding of bivalve mollusc character evolution (Cox, 1960; Newell, 1969; Morton, 1987; Healy, 1989). However this genus remains largely understudied, as *Neotrigonia* are rare organisms, endemic to Australian continental shelf waters (Stanley, 1984). This introduction provides a review of all literature on *Neotrigonia*. More specifically it provides information on the phylogenetic position of *Neotrigonia* within Bivalvia in the broader context of current methods used in phylogeny, phylogeography and systematics. The relevant background information is provided for upcoming research chapters addressing; number of extant *Neotrigonia* species, their phylogeography in Southern Australia; discovery of doubly uniparental inheritance within *Neotrigonia* and aspects of their reproduction, in particular oogenesis.

A systematic revision of the South Australian Cainozoic Trigoniidae by Darragh (1986) suggested that *Neotrigonia* evolved from *Eotrigonia* in the Oligocene or early Miocene, while *Eotrigonia* evolved from *Trigonia* spp. in the late Cretaceous or early Tertiary. This fossil record is consistent world-wide, as fossils have been found in America, France, Tanzania, Morocco, Papua New Guinea and Australia (Cox, 1969; Stanley, 1984). The history of Australian Trigoniidae during the last 60 million years is reasonably well documented in the Tertiary sediments of Victoria, South Australia and Tasmania (Fleming, 1964; Skwarko, 1966; Cox, 1969)

It appears, that *Eotrigonia* persisted with quite stable shell ornamentation for 40 million years, then gave rise to *Neotrigonia* in less than 5 million years; the descendent genus has remained remarkably stable for about 15 million years. Therefore, according to Simpson (1953), evolving *Eotrigonia* gave rise to *Neotrigonia*. Unfortunately this phase is poorly preserved in the fossil record and is therefore difficult to interpret (Fleming, 1964). However in South Australian fossil deposits, two trigoniid fossil genera *Eotrigonia* and *Neotrigonia* co-occur (Cox, 1952; Fleming, 1964; Darragh, 1986). Further to this the striking difference in surface ornamentation between the two supposedly successive groups has given rise to some

doubts (Saveliev, 1958) as to whether they are in fact as closely related as their distribution in time and space suggests. So rather than interpreting *Eotrigonia* as an ancestor to *Neotrigonia*, their relationship is better described as *Eotrigonia* being paraphyletic with respect to *Neotrigonia*. This explanation is more acceptable and less speculative, since there is no intermediate morphological link between the two genera (Darragh, 1986).

The morphology, functional biology and fossil history of Trigonioidea has been well documented in recent studies by Gould (1969), Gould and Jones (1974), Newell and Boyd (1975), Tevesz (1975), Stanley (1977), Darragh (1986) and Morton (1987). Newell (1965, 1969) established Trigoniidae as a family for the genus *Neotrigonia*, Cossman (1912). This genus contains six living and four fossil species according to Newell (1965, 1969), Newell and Boyd (1975), Tevesz (1975), Stanley (1977), Morton (1987) and Darragh (1986). However, Lamprell and Whitehead (1992) represented seven living species based on their morphological characters and distributions, which is restricted to Australian waters. The seven species are: *Neotrigonia margaritacea* (Lamarck, 1804); *Neotrigonia bednalli* (Verco, 1907); *Neotrigonia lamarckii* (Gray, 1838); *Neotrigonia gemma*, Iredale, 1924; *Neotrigonia uniophora* (Gray, 1847); *Neotrigonia strangei* (Adams, 1854); and *Neotrigonia kaiyomaruae* Habe and Nomoto, 1976. This publication provides the only plate where all seven species are illustrated together, unfortunately those shells are depicted in an inverted position (Fig. 1.2) (Lamprell and Whitehead, 1992). *Neotrigonia* fossil species were reviewed in Darragh (1986) and are: *Neotrigonia acuticostata* (McCoy, 1866); *Neotrigonia howitti* (McCoy, 1875); *Neotrigonia novaguineana*, Skwarko 1967 and *Neotrigonia medipontea* Darragh, 1986. Some of extant forms, i.e. *N. margaritacea*, *N. uniophora*, and *N. strangei*, have been positively identified in the fossil record as well as extant collections (Darragh, 1986).

Bivalve systematics and taxonomy have been predominantly based on morphological characters until recently when molecular data have been included. Morphological studies on neotrigonids have been based on an array of different anatomical, embryological and structural characters. The only neotrigonid that has been subject to extensive morphological study is *N. margaritacea* (Tevesz, 1975; Morton, 1987; Healy, 1996) and to a lesser extent of *N. gemma* (Healy, 1996). Hence it is critical to investigate other species because this will lead to more information on general morphology, function and the importance that morphology plays in

evolutionary relationships within this genus. The present study will further investigate evidence for species status based on museum collections, morphological characters and molecular evidence, where possible for both fossil and living species of *Neotrigonia*.

Phylogeny

Two contrasting phylogenies based on morphology of the higher-level relationships within the Bivalvia have been proposed by Salvini-Plawen and Steiner (1996) and Waller (1990). The major point of disagreement between the two phylogenies is the placement of the Trigonioidea. The Salvini-Plawen and Steiner (1996) hypothesis indicates that trigonioids are most closely related to pterimorph bivalves, with the Veneroidea being the sister group to the Unionoidea. Their proposed sister-taxon relationship for trigonioid and pterimorph bivalves was supported by the shared presence of byssate larvae and abdominal sense organs in these taxa (Salvini-Plawen and Steiner, 1996). This information was sourced from studies by Purchon (1957, 1960, 1968), who singled out three important facts from the anatomy of *Neotrigonia*: 1) the style sac and the mid gut are conjoined; 2) the rectum traverses the ventricle of the heart; and 3) the stomach is characterized by the concentration of ducts to the digestive diverticula into three large embayments, and has a reduced caecum, with a poorly developed tongue, but well-developed dorsal hood for food sorting. On the basis of these structural features, Purchon (1957) considered the stomach of *Neotrigonia* to most resemble those of representatives of the Limidae, Pectinidae and Anomiidae (Pteriomorphia). Likewise Pelseneer (1906, 1911) and White (1942) considered *Neotrigonia* to closely relate to Arcidae (Pteriomorphia) based on the kidney structure. This hypothesis was later supported by phylogeny based on molecular data (Adamkewicz *et al.*, 1997).

Alternatively, Waller (1990) hypothesized that trigonioids are most closely related to the Unionoidea, and these together form the sub-class Palaeoheterodonta. This was earlier proposed by Newell (1965, 1969) and Cox (1969) who established the order Trigonioidea as belonging to the subclass Palaeoheterodonta, and is represented by the single superfamily Trigonioidea. The consistent phylogenetic closeness in morphology within the Palaeoheterodonta, established by Waller (1990), is based upon similarities in shell structure (Taylor *et al.*, 1969, 1973; Tevesz and Carter 1980), gill speculation (Taylor *et al.*, 1969, 1973), sperm morphology (Popham,

1979; Healy, 1989) and gill cilia patterns (Ridewood, 1903; Pleseneer, 1911; Atkins, 1938; Tevesz, 1975; Morton, 1987). Representatives of the single extant genus *Neotrigonia* have a mixture of seemingly primitive features, such as filibranchtenidia (Fig. 1.3), lack of posterior mantle fusion and nacreous shells (Cox, 1960; Morton, 1987; Allen, 1985), with other derived features, such as a multi-vesicular sperm acrosome (Healy, 1989). This classification is not universally accepted (Morton, 1987), but is supported by shell (Newell and Boyd, 1975) and sperm ultrastructural characters (Healy, 1996). The hypothesis of Waller (1990) based on morphological data was later supported by the molecular sequence data by Hoeh *et al.*, (1998). Hoeh *et al.*, (1998) was the first study to include molecular data for Trigonioidea showing that this marine group is the sister taxon to the freshwater unionids, together comprising Palaeoheterodonta. The study by Hoeh *et al.*, (1998), based on sequences of the mitochondrial cytochrome *c* oxidase subunit I (COI) from 14 species of bivalves, monophyly of Autolamellibranchiata, Mytiloidea, Veneroidea, Unionoidea and Palaeoheterodonta was supported, although monophyly of bivalves was not supported.

The classification proposed by Waller (1990, 1998) was also largely supported by Giribet and Wheeler (2002) with a few notable changes. The research by Giribet and Wheeler (2002) combined molecular and morphological data for the first time for many bivalves, including *Neotrigonia margaritacea*. It is appropriate to combine molecular and morphological datasets if they are homogeneous with respect to phyletic indications, but not to combine data when it is heterogeneous (Bull *et al.*, 1993). Importantly, the decision to combine or not combine data sets relies heavily on the statistical procedure selected to evaluate heterogeneity (Bull *et al.*, 1993; Huelsenbeck *et al.*, 1996) and furthermore on availability of multiple data sets across specific groups of organisms. An approach known as total evidence (Kluge, 1989) combines a series of different kinds of data sets so that they can be analysed simultaneously. In contrast to consensus (taxonomic congruence), where mutual confirmation of the independent lines of evidence is taken as giving the strongest possible support, total evidence (character congruence) relies on the principle that all available evidence is considered concurrently (Lapointe *et al.*, 1999). Therefore under the taxonomic-congruence approach the data are analysed separately before trees are combined to form phylogenies. For all these reasons, it is best to employ both total evidence and consensus approaches in obtaining optimal phylogenies. In the

phylogenetic analysis based on the total evidence approach by Giribet and Wheeler (2002), monophyly of Palaeoheterodonta was confirmed; this finding is consistent with that of Graf and O'Foighil (2000) based on morphology. The same study by Giribet and Wheeler (2002) confirms the monophyly of Bivalvia (Fig. 1.1). Therefore the current research will be performed within this phylogenetic framework.

In this research data will be combined under parsimony analysis only when separate datasets do not show highly-supported topological differences and as long as inappropriate data (e.g. saturated transitions in the third codon position) are removed from the data set (Hoeh *et al.*, 1998). Combined datasets will be analyzed under Bayesian analysis where data are partitioned and different models of sequence evolution can be applied to each partition (Hall, 2004).

Phylogeography

Phylogeography is a field of study concerned with the principles and processes governing the geographical distributions of genealogical lineages, especially those at the intraspecific level (Avice *et al.*, 1987). The term was formulated by Avice *et al.*, (1987) and its use in the evolutionary genetics literature has grown exponentially since then. Well-resolved phylogenies form an excellent framework for investigating phylogeography, by incorporating information on the past environmental conditions that might have influenced character evolution. This is possible by investigating the historical biogeography of the group of interest and mapping the present-day distributions of the taxa or characters on a phylogenetic tree and tracing the change. Data from the geological literature, such as continental drift, climatic circumstances and/or changes in sea level (Veevers, 1984; De Queiroz, 2005) may provide approximate times of divergence. This enables scientists to formulate hypotheses in regard to divergence observed in a phylogenetic tree, and to postulate whether sympatric or allopatric speciation processes may have taken place. Present-day distributions of several marine invertebrate taxa in South Australia show distinct patterns correlated with historical environmental changes (Waters and Roy, 2004; Waters *et al.*, 2004). Therefore, investigating the phylogeography of *Neotrigonia* is of interest considering the rich history of this taxon.

The genetic structuring of a population of organisms, and ultimately the establishment of independent evolutionary lineages, is strongly influenced by the pattern of genetic exchange (gene flow) within and between populations (Avice *et al.*,

1987). Genetic variation is structured, not only by the contemporary forces of genetic exchange, but also by historical patterns of relationship (Avice *et al.*, 1987). For a given level of current genetic exchange, populations having recent common ancestry will be genetically more similar than those having more distant common ancestry (Avice *et al.*, 1987; Avice, 2000). Ultimately, if genetic exchange between two populations or species ceases altogether, then shared common ancestry will be the sole determinant of any genetic similarity between them. Therefore, historical relationships will contribute in some measure to the genetic structure of all species.

Within species, genetic exchange rather than historical relationship has traditionally been emphasized as the determinant of genetic structure. Classical models for describing this structure (e.g. *F* statistics, Wright, 1951) do not distinguish historical effects from recurrent processes. Estimates of gene flow (Nm) derived from these models assume that current population structure reflects an equilibrium between genetic drift and gene flow (Templeton *et al.*, 1995; Templeton, 1998). In many groups genetic exchange across the species range is severely restricted, either by the wide geographical distribution of populations or by limited ability to disperse (Avice, 2000). In these cases historical events such as climatic and habitat changes and population bottlenecks will be strong determinants of population genetic structure (De Queiroz, 2005). The observed genetic similarity between such populations owes more to recent common ancestry than to any ongoing process of genetic exchange (Avice, 1994).

In this study haplotype networks will be used to observe genetic differences and determine relationships within and between populations of *Neotrigonia*. With haplotype networks, genes are grouped by their similarities and haplotype classes. Whereas traditional methods often lack the power to resolve intraspecific relationships, such network approaches offer an appropriate representation of the haplotype relationships, including extinct or unsampled haplotype variants (Posada and Crandal, 2001). The advantage of haplotype networks over strictly bifurcating trees for estimating within-species relationships is that, networks can account effectively for processes acting at the species level and they might be able to incorporate predictions from population genetics theory (Posada and Crandal, 2001; Knowles and Maddison, 2002). Most network methods are distance methods, with the common idea of minimizing the distances or number of mutations among haplotypes (Posada and Crandal, 2001). Therefore in this research two parsimony-based software

packages will be implemented: TCS (Clement *et al.*, 2000), which is used to construct statistical parsimony networks to provide representations of gene genealogies at the population level (Templeton *et al.*, 1992); and ARLEQUIN 2.0 (Schneider *et al.*, 2000) program, which implements the maximum likelihood method, to estimate haplotype frequency (Nei, 1987). This result will be a novel contribution to phylogeography research in Southern Australia, as *Neotrigonia* have not previously been sampled and analysed at the population level.

Doubly Uniparental Inheritance

A further complication that must be considered when investigating *Neotrigonia* phylogeny and phylogeography using mitochondrial genes is the phenomenon of doubly uniparental inheritance (DUI) of mtDNA. DUI is a unique mode of inheritance where two types of mitochondrial DNA exist; one is transmitted via the egg to males and females and the other via sperm to males only (Zouros *et al.*, 1994a). This phenomenon has been reported in six bivalve taxa to date: Unionoida, Veneroida, Mytiloida, Margaritiferidae, Hyriidae, and Donacidae (Skibinski *et al.*, 1994a, 1994b; Zouros *et al.*, 1994a, 1994b; Liu *et al.*, 1996; Hoeh *et al.*, 1996; Passamonti and Scali, 2001; Curole and Kocher, 2002; Hoeh *et al.*, 2002; Serb and Lydeard, 2003; Passamonti, 2007; Theologidis *et al.*, 2008) and it is suspected to occur in *Neotrigonia* (Hoeh *et al.*, 1996).

In species with DUI, two types of mtDNA exist. One type of mtDNA is transmitted via the eggs to both female and male offspring. This mtDNA is known as maternal or type F genome. Due to uniparental inheritance, the other type of mtDNA is transmitted via the sperm only to the male offspring, and is known as the paternal or type M genome (Zouros, 2000). The males are thus heteroplasmic, where the type F genome predominates in the somatic tissue and the M type is restricted to the gonads (Stewart *et al.*, 1995; Sutherland *et al.*, 1998).

Phylogenetic analyses have revealed another unusual aspect of marine mussel genetics, where fidelity of DUI is sometimes compromised. Some males seem to lack a typical M genome (Hoeh *et al.*, 1997; Quesada *et al.*, 1999), and F genomes seem to invade the male route of inheritance such that they become transmitted from generation to generation only through sperm (Zouros, 2000, Saavedra *et al.*, 1997). This mode of inheritance has been referred to as ‘masculinisation’ or ‘role reversal

event'. The F genomes that have replaced the M genome are referred to as recently masculinized M types (Hoeh *et al.*, 1997; Quesada *et al.*, 1999).

Hoeh *et al.*, (2002) reported that the DUI characteristics observed in unionoid bivalves could resemble the DUI ancestral condition, and, given the basal position of unionoid bivalves and their sister relationship to *Neotrigonia*, then its existence or not within *Neotrigonia* may have an impact on interpretation of the origin of DUI. It is essential to work within stable phylogeny, such as the one recovered by Giribet and Wheeler (2002), when exploring the origin of doubly uniparental phenomenon within the Palaeoheterodonta and the whole of the Bivalvia. Consequently, the phylogeny recovered by Giribet and Wheeler (2002) (Fig. 1.1) will be used here to trace the origin of DUI within the Bivalvia.

Aspects of Reproduction

Neotrigonia are dioecious (Morton, 1987) but the Trigoniidae reproductive cycle is otherwise uncertain, although a number of authors (Tevesz, 1975; Healy, 1996; Prezant, 1998; O'Foighil and Graf, 2000) have published information on pre-juvenile ontogeny, veliger larvae development, sperm structure, egg size and external fertilization. Unfortunately only two species have been studied so far, *N. margaritacea* and *N. gemma* (Healy, 1996; O'Foighil and Graf, 2000) and the process of oogenesis is not detailed for either of these species.

According to Ockelmann (1965) and O'Foighil and Graf (2000), the prejuvenile shell (prodissoconch) morphology of *Neotrigonia margaritacea* is suggestive of early development. The prodissoconch of species in which obligate planktotrophy fuels larval development is characterized by the presence of an umbonate hinge line with a well-developed prodissoconch (Ockelmann, 1965; O'Foighil and Graf 2000). Absence of these larval shell characters excludes the possibility of larval planktotrophy, although some taxa with a non-umbonate prodissoconch may be facultative planktotrophs (Gros *et al.*, 1997). The prodissoconch morphology of *N. margaritacea* shows a distinct metamorphic line between the prodissoconch and the juvenile shell dissoconch (O'Foighil and Graf, 2000) (Fig. 1.4). According to O'Foighil and Graf (2000), prismatonacreous fine sculpture is evident in the dissoconch, while very fine radial and comarginal striae can be distinguished in the prodissoconch. Consequently, this information suggests that *N. margaritacea* is highly unlikely to have an obligate planktotrophic larval development

(O’Foighil and Graf, 2000). Furthermore, the ability of *N. margaritacea* to form a calcified bivalve prejuvenile shell suggests that the non-calcified lasidium/haustorium larvae found in a subset of freshwater palaeoheterodonts (Parodiz and Bonetto, 1963) are likely to represent derived character states among the Unionoidea (O’Foighil and Graf, 2000).

Healy (1996) described the spermatozoa of *N. margaritacea*. It consists of a thin acrosomal complex, a short nucleolus, a midpiece composed of 4 or 5 mitochondria and two centrioles positioned at the base of the nucleus and the single flagellum (Fig. 1.5). In the same study it was observed that the proacrosomal vesicles of *N. margaritacea* are larger than those of *N. bednalli* (Healy, 1996), although age or sample size were not reported, making this feature uncertain as a reliable taxonomic character (Healy, 1996). Nevertheless the study outlines the presence of multiple, unfused proacrosomal vesicles in mature spermatozoa of Trigonioida and Unionoidea as a valuable synapomorphy and thus a derived feature for the Palaeoheterodonta (Healy, 1989,1996).

According to Tevesz (1975) based on findings on *N. gemma*, it is assumed that neotrigonids have large, white, irregularly- shaped oocytes, which are produced in the visceral mass gonad, near the umbo. Tevesz (1975) also described the process of external fertilization where oocytes are extruded into the mantle cavity through two oviducts opening posterior to the visceral mass, just above the gills. Prior to ejection they travel on ciliary tracts to the posterior ventral part of the mantle cavity. However, oocyte ultrastructure and oogenesis have not been studied. This research will address the ultrastructural stages of female gametogenesis in *N. margaritacea* and endeavour to interpret timing of reproductive activity and mode of spawning.

Aims and Scope of the Thesis

The aim of this research was to enhance general knowledge regarding the evolutionary affinities of *Neotrigonia* species. The first objective was to establish the number of species belonging to the genus. A second objective was to develop a phylogeny, which would inform species delimitation and phylogeography. The third objective was to investigate the presence of Doubly Uniparental Inheritance within *Neotrigonia*. The fourth objective was to determine the process of oogenesis.

Each results chapter is presented in manuscript format as would be suitable for submission to biological journals. The consequence of this approach is that whilst ideas are communicated in a more concise manner, there is some repetition between chapters.

Chapter 2 is a taxonomic revision of *Neotrigonia*. In this chapter I investigate the species status for the seven extant species recognised by Lamprell and Whitehead (1992), based on live collected specimens, specimens (extant and fossil) deposited in museum collections and all available types. I also attempt to establish the current number of species using morphological character states, and thus verify species status and the geographic distribution of this genus.

Chapter 3 presents a molecular phylogeny of the southern Australian species complex. Species delimitation is examined using molecular characters. Further, I examine the population genetic structure based on high haplotypic diversity of the widespread *Neotrigonia margaritacea*. Multiple phylogenetic inference methods are contrasted (i.e. maximum parsimony and Bayesian) and a new means for assessing haplotype diversity is used.

In Chapter 4, the evidence for Doubly Uniparental Inheritance is presented for *Neotrigonia margaritacea*. The new findings are combined with previously published COI and 16s datasets from six bivalve taxa that have DUI (Skibinski *et al.*, 1994a, 1994b; Zouros *et al.*, 1994a, 1994b; Liu *et al.*, 1996; Hoeh *et al.*, 1996; Passamonti and Scali, 2001; Curole and Kocher, 2002; Hoeh *et al.*, 2002; Serb and Lydeard, 2003; Passamonti 2007; Theologidis *et al.*, 2008) and critically-important data on the very few species which have been found to lack DUI in the following groups,: Arcidae: *Arcanoae* (Theologidis *et al.*, 2008), Ostreidae: *Crassostrea virginica* and *C. gigas* (Obata *et al.*, 2008), Veneridae: *Venus verrucosa* and *Callistachione* (Theologidis *et al.*, 2008), and in Unionoida: Etheriidae (Walker *et al.*, 2006). The

phylogenies are constructed and used to generate an hypothesis with regard to the origin of DUI within the Bivalvia.

Chapter 5 describes the ultrastructural stages of female gametogenesis in *Neotrigonia margaritacea*. The morphology of the oocytes and gonad tissue are described for the first time using electron microscopy and histology techniques. The spawning mode is inferred based on gonad tissue and oocyte morphology, and using laboratory observations.

Chapter 6 is the general discussion, which synthesizes the major outcomes of this research and identifies the broader implications of our new understanding of *Neotrigonia*.

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Figure 1.1

The tree topology of bivalve relationships according to Giribet and Wheeler (2002) and Giribet (2008).

Figure 1.2

Neotrigonia images taken directly from Lamprell and Whitehead (1992) (with permission from Crawford House Press) where shells have been illustrated upside down: A) *Neotrigonia bednalli*; B) *Neotrigonia margaritacea*; C) *Neotrigonia uniophora*; D) *Neotrigonia kaiyomaruae*; E) *Neotrigonia lamarckii*; F) *Neotrigonia strangei*; and G) *Neotrigonia gemma*. These species are depicted in the right orientation in Chapter 2 figure 2.6.

Figure 1.3

Dissection showing soft morphology of *Neotrigonia margaritacea* showing A) lastrous nacre shell inside; B) schizodont hinge SH; and C) a diagrammatic presentation of internal features of *Neotrigonia gemma*. Image in C) taken directly from Tavesz (1975) where: A, foot with distinct heel and toe, B, adductor muscle, C, D, anterior pedal muscles, E, posterior pedal muscle, F, ctenidia with arrows indicating ciliary tracts, G, palps, and H, pseudofaeces.

Figure 1.4

Image of a juvenile *Neotrigonia margaritacea* shell. Interpretation of shell structure following O'Foighil and Graf (2000): left valve, showing prodissoconch (p) and dissoconch (d); arrow indicates metamorphic line.

Figure 1.5

Image of *Neotrigonia margaritacea* spermatid. Interpretation of characters following Healy (1998). A) ventral view of the spermatid showing; mitochondria (M) and flagellum (F), and B) dorsal view of spermatid showing, nucleus (N) and acrosome (a).

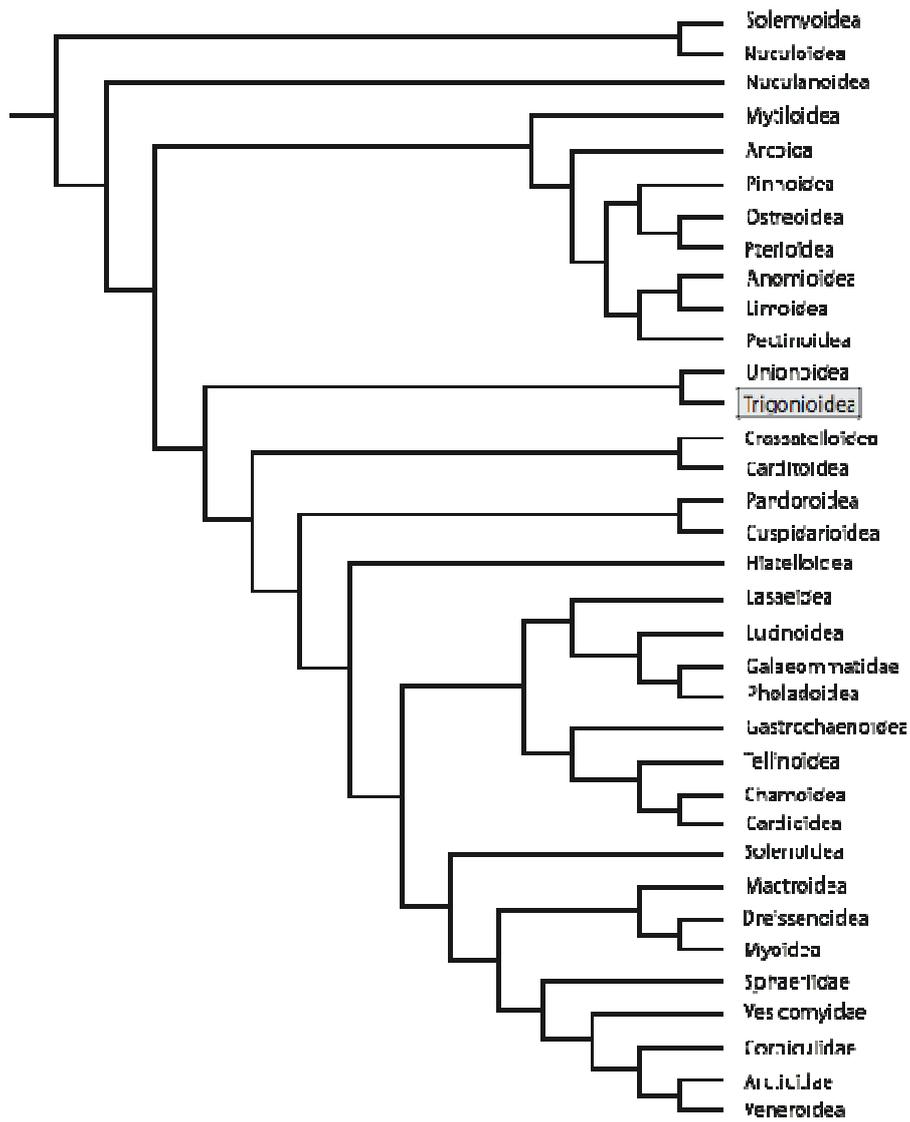


Figure 1.1

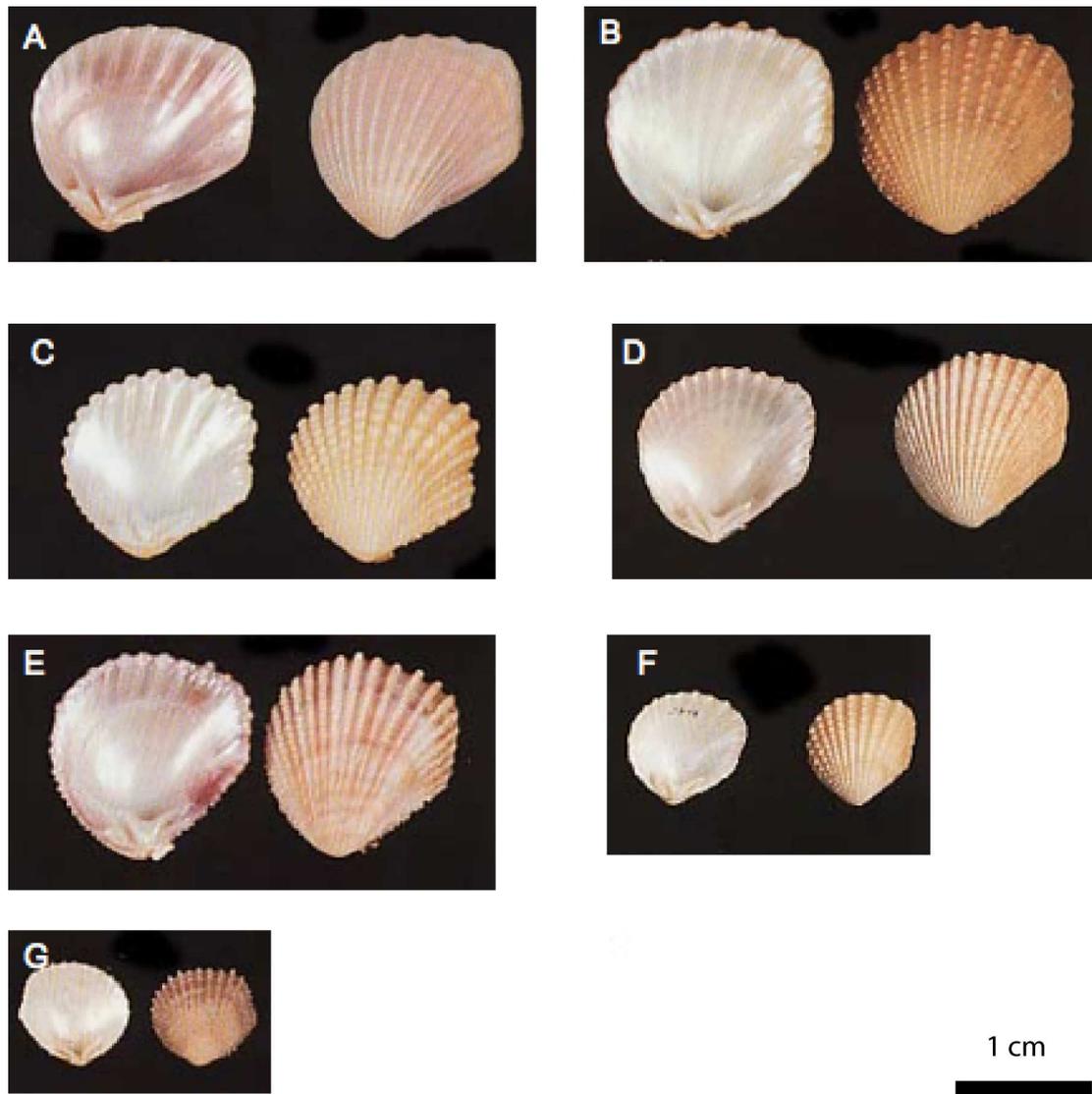


Figure 1.2

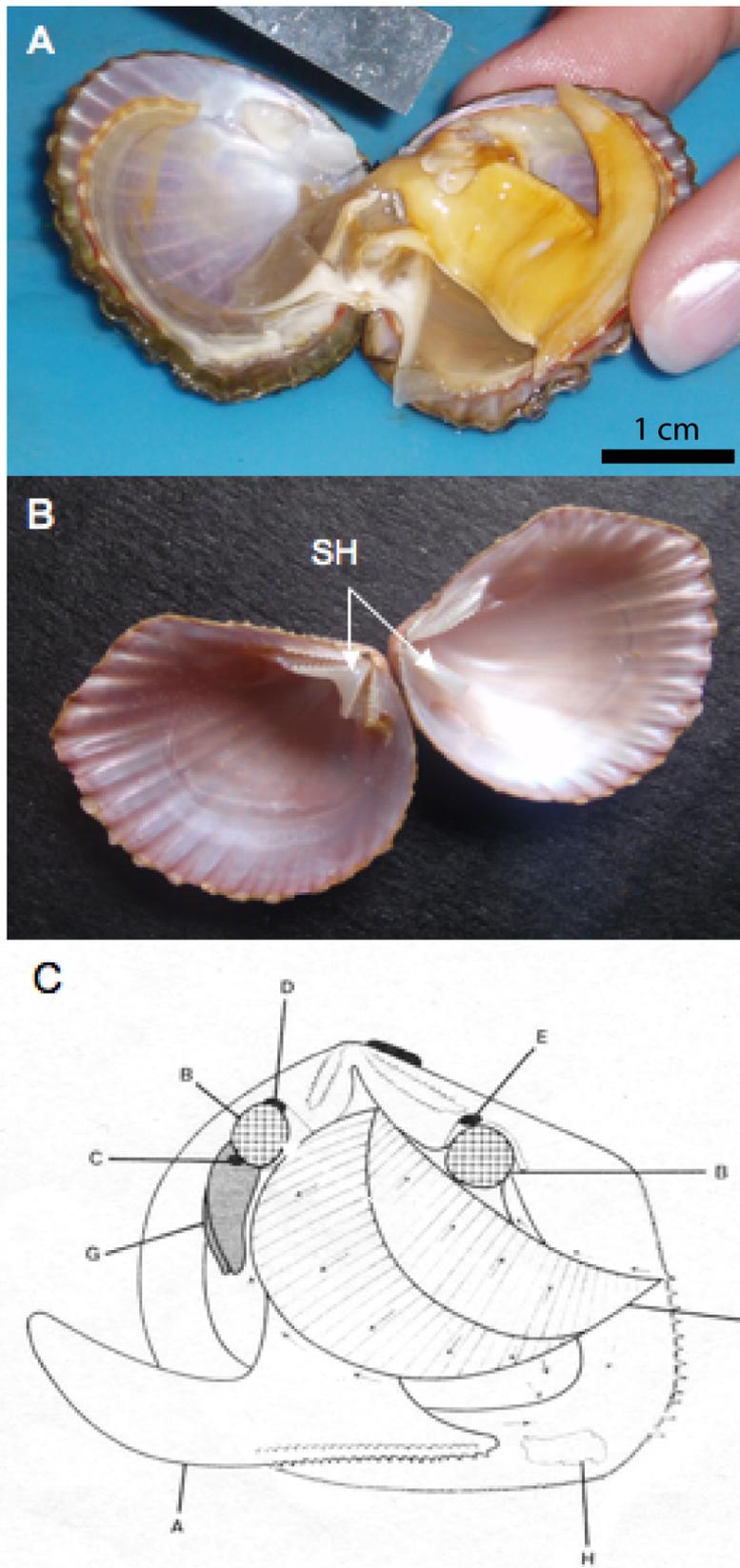


Figure 1.3



Figure 1.4

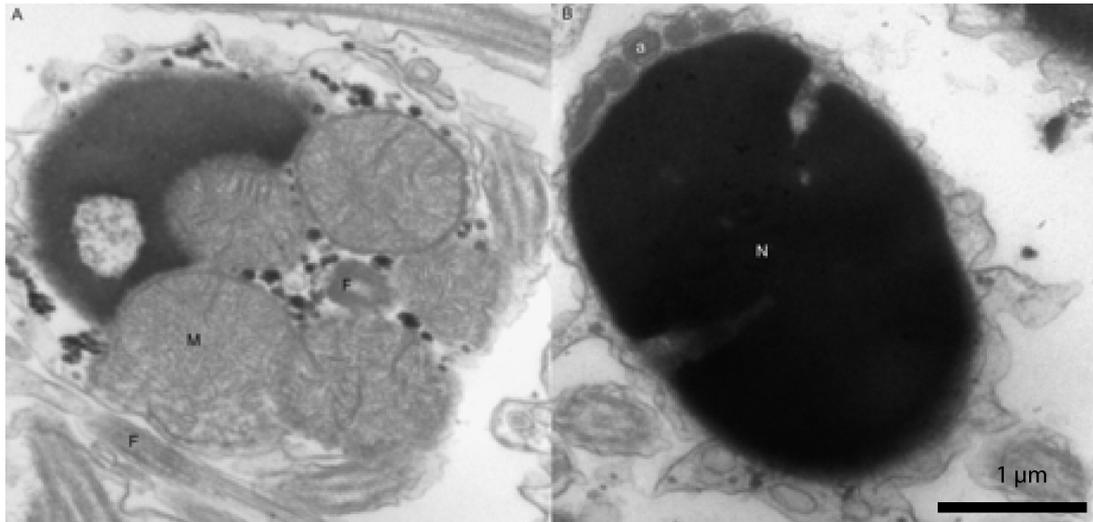


Figure 1.5

Systematics of *Neotrigonia* (Mollusca: Bivalvia: Palaeoheterodonta)

Ana Glavinic

Flinders University, Adelaide, PO Box 2100, Adelaide, SA 5001, Australia.

Author for correspondence: ana.glavinic@flinders.edu.au, telephone +61 8 82012267

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Abstract

The type material, localities and distributions of the seven living, usually-accepted species of *Neotrigonia* Cossman 1912, based on all available museum specimens and some fresh collections, are reviewed and illustrated. All species occur in Australian continental waters. A cladistic analysis based on shell morphological characters of all known fossil and living *Neotrigonia* species was performed using *Eotrigonia subundulata* (Jenkins, 1865) and *Trigonia miriana* (Skwarko, 1963) as outgroups. Results from parsimony analysis showed that the living and fossil *Neotrigonia* formed reciprocally monophyletic groups. The species status of *Neotrigonia bednalli*, Verco 1907, is revised based on examination of all available types, museum specimens and a large number of newly collected specimens from South Australia, Victoria and Tasmania. Parsimony analysis suggests that *N. bednalli* is a junior synonym of *N. margaritacea* (Lamarck, 1804). Species status is accepted for *N. gemma* Iredale, 1924, *N. lamarckii* (Gray, 1847), *N. uniophora* (Gray, 1847), *N. strangei* (Adams, 1854) and *N. kaiyomaruae* Habe and Nomoto, 1976, but with a range contraction to NSW for *N. strangei* due to previous misidentification of Western Australian *N. uniophora*. The validity of species status is accepted for *N. gemma* where a single character, increased width between nodules on radiating ribs, normally associated with juvenile shells, is the only feature that consistently distinguishes these specimens from other *Neotrigonia* species found along the eastern Australian coast. This study highlights the limitations in using shell morphology for *Neotrigonia* taxonomy, thus requiring molecular evidence to support any further revision in this genus.

Introduction

A major discovery by the French naturalist Francois Peron in 1802, on a beach at Adventure Bay in southern Tasmania, were some shells that appeared to be extant forms of the Trigonioidea, a once-diverse bivalve group thought to have gone extinct in the late Cretaceous (see Lamarck, 1804; Fleming, 1964; Skwarko, 1966; Gould, 1968; Stanley, 1984). Trigonoids were globally prominent in Mesozoic marine faunas and the extant Australian trigoniids have since been regarded as relictual, in the sense that *Neotrigonia* represents the sole surviving genus of a once-flourishing, diverse and widely-distributed taxon. The originally discovered living trigoniid specimens from Tasmania were described as *Trigonia margaritacea* Lamarck, 1804, with a series of species described over the subsequent years from Australian waters (McMichael, 1956; Habe, 1985). Initially placed in *Trigonia* Bruguière, 1789 by Lamarck (1804), the extant species were later moved to *Neotrigonia* Cossmann 1912.

Trigonioidea comprises eight families, with only Trigoniidae having extant forms (Cox, 1952, 1960). Trigonoid morphology, functional biology and fossil history has been well documented in studies by Gould (1969), Gould and Jones (1974), Newell and Boyd (1975), Tevesz (1975), Stanley (1977), Darragh (1986), and Morton (1987). The history of Australian Trigoniidae during the last 60 million years is reasonably well documented in the Tertiary sediments of Victoria, South Australia and Tasmania (Cox, 1952, 1960; Skwarko, 1966; Fleming, 1964). Species of *Neotrigonia* range back to the upper Miocene replacing the genus *Eotrigonia*, which ranges back to the lower Eocene or Pliocene (Cox, 1952; Fleming, 1964). A systematic revision of Trigoniidae by Darragh (1986) suggested that *Neotrigonia* evolved from *Eotrigonia* in the Oligocene or early Miocene, while *Eotrigonia* evolved from *Trigonia* spp. in the late Cretaceous or early Tertiary. However, four taxa, *Eotrigonia subundulata* (Jenkins, 1865), *Neotrigonia strangei* (Adams, 1854), *Neotrigonia howitti* (McCoy, 1875) and *Neotrigonia uniophora* (Gray, 1847), are found together in the Middle to Late Miocene outcrops, after which *E. subundulata* becomes extinct (Darragh, 1986). The striking difference in surface ornamentation between the *Eotrigonia* and *Neotrigonia* has given rise to some doubts (Saveliev, 1958) as to whether they are in fact as closely related as their distribution in time and space suggests. There are no known taxa that resemble a morphological link between *Eotrigonia* and *Neotrigonia* (Darragh, 1986), therefore their relationship is probably

better interpreted as *Eotrigonia* being paraphyletic with respect to *Neotrigonia*, rather than ancestral.

Newell (1965, 1969) established Trigoniidae as a family for the *Neotrigonia*, which contains six extant species according to Newell (1965, 1969), Newell and Boyd (1975), Tevesz (1975), Stanley (1977) and Morton (1987). However, Lamprell and Whitehead (1992) accepted seven valid species names for extant forms and four valid names for fossil species (Darragh, 1986). In this revision, specimens for all seven extant nominal species and four fossil species are examined through means of cladistic analysis. Extant forms are represented by: *Neotrigonia margaritacea* (Lamarck, 1804); *Neotrigonia bednalli* (Verco, 1907); *Neotrigonia lamarckii* (Gray, 1838); *Neotrigonia gemma* (Iredale, 1924); *Neotrigonia uniophora* (Gray, 1847); *Neotrigonia strangei* (Adams, 1854); and *Neotrigonia kaiyomaruae* Habe and Nomoto, 1976. Fossil species are: *Neotrigonia acuticostata* (McCoy, 1866), *Neotrigonia howitti* (McCoy, 1875), *Neotrigonia medipontea*, Darragh, 1986 and *Neotrigonia novaguineana*, Skwarko, 1967. Some of extant forms, *N. margaritacea*, *N. uniophora*, and *N. strangei*, have been positively identified in the fossil record, as well as living collections (Darragh, 1986). A contentious issue is the status of *Neotrigonia bednalli*; the initial description of this species was not detailed, and what could be regarded as the type locality stretches over 15 km of coastline (Verco, 1907).

Presented is a taxonomic revision of *Neotrigonia* based on examination of newly-collected specimens of *N. margaritacea* and *N. bednalli* from south east Australian subtidal benthos and other specimens deposited in museum collections and all available types. A cladistic analysis based on shell features is also presented. I also document all the available *Neotrigonia* types and specimens from museum collections worldwide.

Methods

Except for *Neotrigonia kaiyomaruae*, the types of all extant *Neotrigonia* species, and other specimens (hypotypes, incl *N. kaiyomaruae*) were loaned from collections of the South Australian Museum (SAM), National Museum of Victoria (NMVP), Australian Museum (AM), Western Australian Museum (WAM), Natural History Museum, UK (NHM) and Museum National d'Historie Naturelle, Paris (MNHN) and Bureau of Mineral Resources, Geology and Geophysics, Canberra (CPC) (Table 2.1).

Fresh *Neotrigonia* material was obtained from Noarlunga, Port Stanvac, Port Lincoln (S.A.), Stradbroke Island (Qld), Bruny Island, Hobart Harbour (Tas.) and Westernport Bay (Vic.) (Table 2.1) from subtidal habitats ranging from 12 to 40 m depth either via SCUBA or dredging (Table 2.2). Additional collections were attempted, but no live specimens were found (Appendix I). The dredge was constructed for manual use from small private vessels. It is 1200 cm long, 800 cm wide and 400 cm deep, with a mesh size of 1cm² (Appendix I). It was towed at a speed of 1 knot and for the period of 15 min per tow. Collection of fresh material by SCUBA allowed for assessment of the natural habitat of the specimens (Table 2.2). This material was preserved in 70% ethanol. Recently collected, 10% formalin-preserved material was also obtained from Abrolhos Archipelago, Esperance, Dampier Archipelago (WA) and Western Port (Vic) (Table 2.1). All other material kept in Museum collections was preserved in formalin or dry and often were beach-washed shells (Table 2.2). Therefore such material carries little information on natural habitat. Where possible, however, distributional data for each species was collated from all collection trips, collection records for all museum specimens and the available literature (Lamarck 1804; Verco 1907; Habe and Nomoto 1976).

The holotype specimens of *Eotrigonia subundulata* NMVP12250 and *Trigonia miriana* CPC4643 (Table 2.1) were used as outgroups following Fleming (1964) and Darragh (1986), because of their shell morphology and recent history documented in the fossil record. Character states were observed and scored (Tables 2.3, 2.4) according to the key list provided below. Maximum parsimony analyses were performed on the matrices using heuristic searches with 100 random additions of terminals using PAUP 4.0b10 (Swofford, 2003) with all other settings as defaults. Strict consensus trees were computed for an analysis based on a matrix from type specimens only, while strict and Adam's consensus trees were computed for an analysis of all available specimens. Most parsimonious reconstructions for characters were traced in MacClade version 4.08 (Maddison and Maddison, 2000).

Morphological features used to develop characters

To date *Neotrigonia* species diagnoses have been entirely based on shell morphological characters. Hence, the shell morphology of *Neotrigonia* and trigonioids is reviewed here with reference to those features that provide characters for the cladistic analysis. Relevant general papers that have reviewed the shells of

Neotrigonia include Newell (1965, 1969), Cox (1969), Gould (1969), Gould and Jones (1974), Newell and Boyd (1975), Stanley (1977), Darragh (1986) and Lamprell and Whitehead (1992). Descriptions of *Neotrigonia* species were also consulted (Lamarck, 1804; Gray, 1838, 1847; Adams, 1854; Verco, 1907; Cossman, 1912; Iredale, 1924; Habe and Nomoto, 1976, Graf and Cummings, 2006). The main diagnostic characters are the schizodont hinge and the shape of the shell (Fig. 2.1). The hinge is strong with large schizodont teeth interlocked by ridges and grooves. The shells of *Neotrigonia* are often described as ovate to subcircular, rarely subquadrate, equivalve and strongly inequilateral. The flank, posterior area and escutcheon are not prominently differentiated and all bear radial ribs (Fig. 2.1). Escutcheon was reported as absent by Cossman (1912), however, it is a present character for *Neotrigonia* according to Darragh (1986). The exclusively radial ribs, one of the most characteristic features, are generally sculptured with strong or weak scales, spines or tubercles, which may become obsolete towards the margin of the shell. The number of ribs is counted and reported in some of the species diagnosis (Lamprell and Whitehead, 1992) and it is always expressed as a range. The umbones are small to large, orthogyral, not projecting, and bear a discrepant juvenile sculpture of about eight comarginal ribs on the anterior and middle portions of the flank extending from the prodissoconch (Fig. 2.1). The margin, which is strongly denticulate, permits the valves to be interlocked (Cossman, 1912). The following list of shell characters 1-25 were developed to distinguish species of *Neotrigonia* following Darragh (1986). All scored characters are indicated on the *Neotrigonia margaritacea* shell in Figure 2.2:

1. Shell shape: 0, angular; 1, ovate. *Trigonia* and *Eotrigonia* specimens are often referred to as angular or strongly trigonal, while *Neotrigonia* can be described as sub-trigonal or ovate (Darragh, 1986) (Fig. 2.2). All margins, anterior, posterior, dorsal and ventral are considered to ascertain the shape.
2. Shell breadth; 0, compressed; 1, inflated. The shell was assessed from the anterior, posterior and ventral view (Fig. 2.2).
3. Median carinae: 0, strong; 1, weak. Median carinae is a prominent feature on *Trigonia* and *Eotrigonia* specimens and it separates flank with radial ribs away from an area with parallel costae. In the case of *Neotrigonia* this line is not as obvious, but it is present, and it separates the area from the flank (Fig. 2.2).
4. Umbone position on dorsal margin: 0, attenuated; 1, orthogyral (Fig. 2.2).

5. Size of the umbone: 0, small (< 2 mm), 1 large (>2 mm) (Fig. 2.2).
6. Shape of the umbone: 0, straight; 1, curved (Fig. 2.2).
7. Ventral margin denticulation: 0, absent; 1, present (Fig. 2.2).
8. Posterior margin denticulation: 0, absent; 1, present (Fig. 2.2).
9. Anterior margin denticulation: 0, absent; 1, present (Fig. 2.2).
10. Dorsal margin denticulation: 0, absent; 1, present (Fig. 2.2).
11. Ventral margin: 0, straight; 1 rounded (Fig. 2.2).
12. Posterior margin: 0, straight; 1 rounded (Fig. 2.2).
13. Anterior margin: 0, straight; 1 rounded (Fig. 2.2).
14. Dorsal margin: 0, straight; 1 rounded (Fig. 2.2).
15. Post larval shell sculpture: 0, simple concentric or absent; 1, radial (Fig. 2.2);
Based on electron micrographs of *Neotrigonia* postlarval sculpture (Ó Foighil and Graf, 2000).
16. Ornamentation on the post larval shell sculpture: 0, smooth; 1 subspinous (Fig. 2.2).
17. Adult shell sculpture: 0, shell bearing radial and concentric ribs; 1, exclusively radial ribs (Fig. 2.2).
18. Radial ribs width: 0, wide (> 1.5mm); 1 narrow (< 1.5 mm) (Fig. 2.2).
19. Radial ribs height: 0 high (> 1 mm), 1 low (< 1 mm) (Fig. 2.2).
20. Anterior interstitial grooves: 0 wide (> 2mm); 1 narrow (< 2mm) (Fig. 2.2).
21. Posterior interstitial grooves: 0 wide (> 1 mm); 1 narrow (< 1mm) (Fig. 2.2).
22. Nodules on radiating ribs: 0, rounded; 1, subspinose; 2, plated (Fig. 2.2).
23. Nodules spacing: 0 wide (> 1mm); 1 narrow (< 1 mm) (Fig. 2.2).
24. Escutcheon: 0, absent; 1, present. According to Darragh (1986), escutcheon is present in *Neotrigonia* species; however, it is not as well differentiated as in *Eotrigonia* members of the family (Fig. 2.2).
25. Escutcheon: 0, straight; 1, concave (Fig. 2.2).

Results

Neotrigonia spp. were normally found infaunally with up to 2 cm of sediment cover, in coarse sand exposed to moderate currents (Table 2.2). The museum records, where available, confirm that *Neotrigonia* species tend to occur in subtidal sand exceeding 18 metres depth. Apart from the climatic range to which the actual geographic sites belong (Fig. 2.3), natural habitats of where the different species were

collected varied little in physical character (Table 2.2). The distribution of each extant species is represented in Figure 2.3.

An analysis of the morphological data set for type specimen of *Neotrigonia* produced eight most parsimonious trees each with a length of 39 steps. There were 22 parsimony-informative and 3 parsimony-uninformative characters. The strict consensus tree based on type specimens presented in Figure 2.4 shows *Neotrigonia* as a clade is split in two reciprocally monophyletic groups, one representing all extant species and the other all fossil species. The monophyly of *Neotrigonia* is supported by the following character states: orthorygal umbone position (4), exclusively radial ribs (17) and narrow posterior interstitial grooves (21). Monophyly for extant *Neotrigonia* group is based on four apomorphies: ovate shell (1), large curved umbone (5,6) and posterior margin denticulation (8). Monophyly of fossil trigonioids is established based on angular compressed shell (1,2) and low radial ribs (19). Within the group representing extant species, *N. bednalli* and *N. margaritacea* form a polytomy. *N. margaritacea* and *N. bednalli* scored exactly the same state for each assessed shell character. Character (23) wide nodule spacing between, is shared between *N. uniophora* and *N. gemma*. *Neotrigonia strangei*, *N. uniophora*, *N. lamarckii* and *N. kayiomaruae* share a synapomorphy, straight escutcheon (25). Given the basal position of *N. uniophora*, which differs from the rest of the extant taxa by compressed shell breadth (2), strong median carinae (3) and smooth ornamentation on the post-larval shell structure (16), these characters are interpreted as plesiomorphic. Further plesiomorphies including rounded nodules (22) and wide radial ribs (18), were identified between *N. uniophora* and *N. strangei*.

A heuristic search performed on the subset of available specimens, where 22 characters were parsimony informative and 3 parsimony uninformative, produced 12 shortest trees. A result of this analysis is represented in an Adam's consensus tree. The results from this tree are congruent with the analysis on type specimens in that extant and fossil taxa form two reciprocally monophyletic groups, and that *N. uniophora* has a basal position within extant taxa. However, this tree did not reveal species-specific clades, nor did it indicate clade formation based on locality (Fig. 2.5).

The important result from this analysis is that contemporary identification of *Neotrigonia* specimens is not standardized. Although very few individual specimens score the same state for all characters when compared to the type specimen for each nominal species, most specimens for each species are in fact the closest match to the

type and share the distinguishing character. However, there are a few notable discrepancies from the type specimens. Based on this approach it is recommended that the specimens formerly identified as *N. strangei* from Pt Headland and New South Wales, C427815 AM and C427376, respectively, be renamed as *N. uniophora*. When *N. margaritacea* specimens (AM C010705, C048965) from Tasmania were analysed, the specimens scored exactly the same character states as the type specimen of *N. gemma*, and are recommended to be renamed as such. Likewise *N. bednalli* from Rottneest Island (BMNH: 20012697, 20050708 and 20050709) are to be renamed *N. kaiyomaruae* (Table 2.2).

Taxonomy

The known synonyms for all species are listed for each species below and the estimated distribution of each species based on all known collection localities is represented in Figure 2.3.

Neotrigonia margaritacea (Lamarck, 1804)

Synonyms: *Trigonia antarctica* Peron and Lesueur, 1807, *T. pectinata* Lamarck, 1819, Blainville, 1827, Crouch, 1827, Lesson, 1833, Deshayes, 1835, Chenu, 1846, Huxley, 1849; *T. margaritacea* Lamarck, 1804, Reeve, 1841, Adams, 1850, Adams and Adams, 1857, Sowerby, 1884, Hall, 1901, Hedley, 1902; *T. dubia* Sowerby, 1884; *T. nobilis* Adams, 1854; *T. acuticostata* McCoy, 1866; *T. reticulata* Tenison-Woods, 1878; *Neotrigonia pectinata* Lamarck, 1804, Cossman, 1912; and *Neotrigonia bednalli* Verco, 1907.

These synonyms proposed by Darragh (1986) are accepted because *Trigonia antarctica* Peron and Lesueur, 1807; *T. pectinata* Lamarck, 1819 and *Neotrigonia pectinata* Lamarck, 1804 are all based on the same specimen. *Trigonia nobilis* Adams, 1854 was described based on the specimen from an unknown locality, and it was not morphologically different. *Trigonia dubia* Sowerby, 1884 was stated to differ from *N. margaritacea* based on the colour, so species separation on these grounds is not warranted because specimens vary in colour within one locality and across the entire geographic range of the species.

Description: Shell moderately compressed, 20 to 24 narrow radiating ribs. Hinder ribs very compressed, frontal 7-8 ribs equally widely-spaced. Ribs decorated with repeating nodules. Nodules are subspinous, and are larger and more numerous at the anterior dorsal margin. Posterior dorsal margin nodules weak. Spacing between nodules larger at the posterior of flank than anterior. Interstitial grooves narrower than radial ribs, densely-scaled growth cords visible (Fig. 2.1). Umbo a paler colour than rest of shell, no nodules visible and ribbing slight. Interior nacreous; typical schizodont hinge (Fig. 2.1f). Size; height 37 mm, length 38 mm and breadth 28 mm (Fig. 5a).

Type specimens: Holotype held at Museum National d'Histoire Naturelle, no registration number (Fig. 2.6).

Type locality: The original description mentions a number of localities such as van Diemen's Land (Tasmania), King Island and Kangaroo Island, but the first specimen was collected from Adventure Bay, Bruny Island, south Tasmania. It was dredged from 15-20 fathoms depth (~28 metres). This locality was referred to by Lamarck (1819) but was later designated as type locality of the species by Iredale (1924). Other material examined: NMVP49500, NMVP49501 from National Museum of Victoria. Assessment of all museum collections returned in excess of 750 specimens labelled as *N. margaritacea*. Close to two hundred specimens (50 collected alive, ~100 preserved in formalin, and many dry), were examined in detail (Tables 2.1 and 2.2).

Species distribution: Temperate waters of southern Australia from Port Jackson (NSW) to Esperance (WA) including Tasmania (Fig. 2.3).

Remarks: The presence, appearance and number of radiating ribs is identical in all specimens of *N. margaritacea* and *N. bednalli* and all other synonyms across the entire range; 20-24 repeated ribs with subspinous nodules; hinder area ribs are narrower and nodules less protruding. Internally, shells are nacreous and pallial line is entire (Fig. 2.1). Previously shell colour was reported as the distinguishing character between *N. margaritacea* and *N. bednalli* (Lamprell and Whitehead, 1992). However, colour of the external shell varies greatly within single populations and therefore the representatives of this species complex range from pink, maroon, and orange to light cream in colour. Colour of the shell is further influenced by environment, with brown deposits on shells frequent in Victoria, and red algal deposits sometimes found on

specimens from South Australia. Therefore shell colour should not be used as a distinguishing character to split this species.

The initial description of *Neotrigonia bednalli* by Verco (1907) stated that this species is “a kind” of *N. margaritacea*, and it is referred to as a subspecies in many museum collections. However in subsequent literature (Stanley, 1984; Lamprell and Whitehead, 1992), the form has gained species status. There was no formal description by Verco (1907) of *N. bednalli* and hence the diagnostic characters are ambiguous. The type specimens for both *N. margaritacea* and *N. bednalli* are shells that were probably collected dead, as these specimens are missing a lot of the original ornamentation. Scoring of morphological characters of these two types revealed no difference, thus there was no justification for distinguishing them as two species (Fig. 2.4). It was important to assess synapomorphies between type specimens, but more significant to this investigation was the opportunity to collect fresh material from type localities. Fresh material assessment emphasizes the synapomorphies, such as the radiating ribs ornamented with subspinous nodules (Fig. 2.1). Close examination of juvenile shells reveals identical morphological characters from shells that were collected at the type locality and throughout the known range for *N. margaritacea* and *N. bednalli* (Fig. 2.1). This revision of the *N. margaritacea* – *N. bednalli* complex is further supported by recent molecular evidence (Chapter 3).

Neotrigonia lamarckii (Gray 1838)

Synonym: *Trigonia lamarckii* Gray, 1838.

This synonym is accepted as the name change occurred as a result of reclassification of the whole genus.

Original description: Shell rather ventricose, solid with 20 to 26 narrow, flat-topped, nodulose radiating ribs; the ribs of the hinder slope narrow, rather crowded; ribs convex, all close together and nodulose. Varies with the inside white, salmon-coloured, yellow or purple bronze (Gray, 1838).

In total 20 dry specimens, and 3 live specimens collected from Stradbroke Island (Qld) (Table 2.1 and 2.2) were examined. Based on these specimens the original description is confirmed with addition of following information. Average adult shell size: height 25 mm, length 32 mm and breadth 15 mm.

Type specimens: Holotype is held in the Natural History Museum (UK) as a part of Cumingian collection (Fig. 2.6). Hypotype C089121 held in the Australian Museum and was figured by Lamprell and Whitehead (1992).

Type locality: Port Jackson Head, New South Wales, 45 fathoms (~ 81 metres).

Species distribution: Eastern Australia from Port Jackson (NSW) to Stradbroke Island, 46m depth (Qld) (Fig. 2.3).

Remarks: *N. lamarckii* does not have a single unique feature; however, it is a valid species due to a unique combination of morphological characters (Table 2.3) such as narrow radial ribs, escutcheon not impressed and plated nodules (Fig. 2.6b).

Neotrigonia gemma Iredale 1924

Original description: Shell small for the genus, triangularly ovate, scarcely inequilateral, obese, rather solid easily separable by its small size and shape. The radial number about twenty two, each with about twelve triangular projections, easily counted from the edge, diminishing rapidly in size after that and becoming less pointed: the interstices are finely lined. The ribs are finer on the posterior side, which is little produced and simply indicated by an angle, but medially a little depressed. The juvenile discrepant sculpture is well marked and the hinge is strong for the size. Length 14 mm, breadth 14 mm (Iredale, 1924).

Type specimen: Holotype C90220 is held in the Australian Museum.

Type locality: Green Cape, New South Wales, depth 50-70 fathoms (~ 90-126 metres).

Thirty specimens were examined; all are held in the Australian Museum collection (Table 2.2).

Species distribution: Eastern Australia from Stradbroke Island (Qld) to Western Port (Vic), including Tasmania (Fig. 2.3).

Remarks: Some specimens appear to be juvenile specimens of either *N. lamarckii* or *N. margaritacea* and in fact the only character that sets them apart is the generally small size of *N. gemma* throughout the whole collection (Fig. 2.6c). Plated nodules on narrow radiating ribs are synapomorphies shared between *N. lamarckii*, *N. gemma* and *N. kaiyomaruae*. An escutcheon that is convex or not impressed is another synapomorphies shared between *N. margaritacea*, *N. lamarckii* and *N. uniophora*.

In the case of *N. gemma*, it can be suggested that the type specimen may represent juveniles of *N. lamarckii* and/or *N. margaritacea*, based on a lack of distinguishing characters except its small size. Potential synonymy could be suggested for *N. lamarckii* and *N. gemma* based on the morphological characters analysis (Fig. 2.4), where they share all but two characters. However, one distinguishing feature from *N. lamarckii* is the nodule spacing, where the *N. gemma* type specimen has wide distances between nodules more similar to those observed on juvenile specimens of *N. margaritacea* than *N. lamarckii*. The juvenile structure of nodules of all *Neotrigonia* species has been argued by Darragh (1986) to be a poor indicator of species difference because it could divide all six extant species in two separate groups. Further, the type specimen for *N. gemma* was collected from a locality where we commonly find both *N. lamarckii* and *N. margaritacea*, such that the species range is overlapping (Fig. 2.3). The Adam's consensus tree (Fig. 2.5) places *N. gemma* and *N. margaritacea* in the same clade corresponding to a New South Wales and Tasmanian distribution. Overall, the Australian Museum bivalve collection holds 83 records for *N. gemma* and the specimen locations exactly match that of *N. lamarckii* and *N. margaritacea*. Consequently, fresh collections are required throughout this range to resolve this species complex using additional soft-tissue characters and/or molecular markers.

Neotrigonia uniophora (Gray 1847)

Synonym: *T. uniophora* (Gray, 1947), Reeve, 1860, Sowerby, 1884, Smith, 1885; *Trigonia jukesii* Adams, 1850.

These synonyms are accepted because *T. uniophora* (Gray, 1947) and *Trigonia jukesii* Adams, 1850 are objective synonyms both based on the same specimen.

Original description: Shell ovate trigonal posteriorly truncated, the margin sinuated, radiately ribbed, ribs about 20-24, elevated, tubercularly nodulous; tubercles rounded, obtuse, ventral margin strongly pectinated (Gray, 1847).

Eight specimens were examined (Table 2.2). Average adult shell size: height 18 mm, length 25 mm and breadth 12 mm.

Type specimens: Holotype is held in the Natural History Museum (UK) as a part of Cumingian collection (Fig. 2.6). Hypotype WAM77.1345A is held in the Western Australian Museum.

Type locality: Cape York, North Queensland, 6 fathoms (~ 11 metres).

Species distribution: Tropical waters of Northern Australia, from Port Jackson (NSW) to Port Headland Western Australia (Fig. 2.3).

Remarks: This is accepted as a valid species based on its unique feature of marginal carinae running from umbo to posteroventral angle of shell delimitating posterior area (Table 2.3). *N. uniophora* shares similarities with *N. strangei*, *N. gemma* and *N. lamarckii*. The posterior side is slightly projected similar to *N. lamarckii*, but on average shell size is smaller in *N. uniophora*. The shell of *N. uniophora* is ornamented with blunt nodules, a primitive feature shared with *N. strangei* (Fig. 2.6d). Marginal carinae are strongly represented on the area of the shell, a character shared with *Eotrigoia subundulata*.

The majority of the specimens identified share a distinguishing character of low rounded ribs ornamented with blunt nodules. However, the Australian Museum collection contains a number of specimens that deviate from type specimen characteristics, and look more so like worn-out *N. lamarckii* shells. This again highlights limitations of distinguishing character plasticity from old shells, such as the current museum collections for this species.

Neotrigoia kaiyomaruae Habe and Nomoto 1976

Original description: Shell small for the genus, quadrately ovate in shape with the roundly curved anterior margin and widely truncated posterior margin, inequilateral with rather long straight posterior dorsal and short anterior dorsal margin, thin, solid, ventral margin weakly arched. Umbo slightly prominent, rosy yellow paler to light yellowish brown marginal portion. Surface with 29 strong radial ribs, with deep interstitial grooves which are narrower than radial ribs, crossing strongly and densely scaled growth cords. Hinge schizodonta as in other species. Interior pearly, pale purple, distinctly grooved corresponding with radial ribs on the surface and forming scalloped margin as the endings of ribs. Height 13.5 mm, length 14.0 mm and breadth 4.8 mm (Habe and Nomoto, 1976).

Including the hypotype, I examined 4 specimens in total (Table 2.2). Only an additional 3 dead shell specimens are available for this species in worldwide collections.

Type specimen: Holotype is reported to be held in the National Science Museum of Tokyo but was not located. Hypotype C303516 (Fig. 2.6) held at Australian Museum and was pictured by Lamprell and Whitehead (1992).

Type locality: Cheney Bay, Western Australia, latitude -35.101, longitude 115.525, collected from 73 m depth.

Species distribution: Great Australian Bight to Cheney Bay, WA (Fig. 2.3).

Remarks: The characteristic of this species are the narrow anterior interstitial grooves, like those of *N. lamarckii*, but are more densely placed. This is a single unique feature, but they do also have a unique combination of distinguishing morphological characters, such as low radial ribs, and straight escutcheon, not impressed. In shell surface area and size they are very similar to *N. margaritacea* (Fig. 2.6e).

Habe and Nomoto (1976) surveyed an extensive area of the continental shelf off Western Australia and collected various shells, of which one was a live specimen of *Neotrigonia*. This single specimen was described as *N. kaiyomaruae* species (Habe and Nomoto, 1976). These authors describe the single holotype specimen as an interesting shell with characteristic numerous radial ribs and densely-set nodules. However, the holotype was not available for further examination. Thus, it is difficult to confirm this species status, which is further complicated by the enormous cost of replicating surveys off the expansive WA continental shelf. Notably, the species range for *N. kaiyomarue* overlaps with that of *N. margaritacea* and it can only be distinguished from *N. margaritacea* by narrow anterior interstitial grooves and straight escutcheon, suggesting that further investigation into status of this species is needed.

Neotrigonia strangei (Adams 1854)

Synonyms: *Trigonia strangei* (Adams, 1854) Reeve, 1860, Sowerby, 1884, *Neotrigonia bednalli* (Verco, 1907) Chapman 1922, *Neotrigonia acuticostata* (McCoy, 1866) Deschet, 1966.

Trigonia strangei (Adams, 1854) synonym is accepted as the name change is a result of the reclassification of the whole genus. Fossil specimens of this species were previously identified as *Neotrigonia bednalli* (Verco, 1907) and *Neotrigonia acuticostata* (McCoy, 1866) but this is not warranted as shells were abraded and did not have characteristic large nodules.

Original description: Shell equivalve, inequilateral and subtrigonal. Intense longitudinal oostae. Costae ornamented with nodules. Anterior nodules more prominent than posterior. Posterior nodules widely spaced. Transverse ribs prominent and ornamented. Interstices transversally striated. Lateral anterior area of the shell rounded and posterior oblique and subtruncated.

Four specimens were examined (Table 2.2). Average adult shell size: height 14 mm, length 20 mm and breadth 12 mm.

Type specimens: Holotype held at Natural History Museum (UK), as part of Cumingian collection, was used to confirm original description (Fig. 2.6). Also examined was a hypotype NMVP13232 from National Museum of Victoria.

Type locality: Sydney Harbour.

Species distribution: Temperate waters of Port Jackson (NSW) (Fig. 2.3).

Remarks: This species is larger than *N. margaritacea*, and somewhat resembles the style and shell structure of *N. uniophora*. The form of the scales on the ribs distinguishes it (Table 2.3). However, the shape of the shell, especially the outline of the hind slope, is also very different (Adams, 1854). The distinguishing unique characters of this species are strong rounded nodules and wide interstices (Table 2.3). Nodules are blunt, thick and rounded. Ornamentation is often not continuous over the area of the shell and down the flank, but mostly covers the ventral part of the flank (Fig. 2.6f). As mentioned by Adams (1854) this species shares similarities with *N. uniophora*, in particular blunt nodules (Fig. 2.6f).

Neotrigonia strangei stays true to its name as this unique species is distinguished by marginal carinae and large round nodules, a primitive character, evident in fossil record for this species from late Miocene (Darragh, 1986). Further, this species shares similarities with *N. uniophora* (Lamprell and Whitehead, 1992), differing mainly in posterior ornamentation. Previous misidentification of WA specimens as *N. strangei* had resulted in an unexplained disjunct distribution for this species. Reclassification of these specimens as *N. uniophora* based on shell characters is therefore also more consistent with the known core distributions of these two species.

Discussion

Museum collections are primarily considered as large repositories of specimens, but they also provide data regarding species distribution (Hansen and Richardson, 1999) and species habitat (e.g. Hansen and Richardson, 1999; Stanistic, 1999) and Ponder (1999) has highlighted the importance of utilizing this information further. In this study, data gained from *Neotrigonia* holdings in museum collections has been utilized here to assess the taxonomy and biogeography of the group and generate distribution maps, after confirming the classification of all available specimens (Fig. 2.3, Table 2.1). Examination of available and revision of hard-shell morphology for all extant available specimens in *Neotrigonia* revealed here that the species status for *N. margaritacea*, *N. lamarckii*, *N. gemma*, *N. uniophora*, *N. strangei* and *N. kaiyomaruae* should be maintained. However, according to the recently-collected specimens, museum collections and type material, the species status of *Neotrigonia bednalli* is reduced to junior synonym of *Neotrigonia margaritacea*. Thus I can report a maximum of six extant species in *Neotrigonia* from Australian waters.

Based on the currently-available museum collections, the species range and distribution of *Neotrigonia* species around Australian continental waters show several overlapping and some clearly disjunct distributions (Fig. 2.3): *Neotrigonia kaiyomaruae* has been collected off Western Australia; *N. gemma* and *N. strangei* off the coast of New South Wales; and *N. uniophora* occurs in New South Wales, Queensland, Northern Territory and Western Australia. *Neotrigonia uniophora* is the most widely distributed species amongst the *Neotrigonia*, with a range that stretches over different climatic zones, from cooler subtropical to warm tropical waters (Fig.

2.3). This wide distribution may be explained by the East Australian Current, which brings warm water down the coast of New South Wales during the Australian summer (Ridgway and Godfrey, 1997). The extensive range of *N. uniophora* overlaps with that of *N. lamarckii*, *N. gemma*, *N. strangei* and *N. margaritacea* on the east coast of Australia (Fig. 2.3). In more temperate waters, the new range for *N. margaritacea*, established based on combining distributional ranges of former specimens identified as *N. bednalli* and current specimens of *N. margaritacea*, stretches from New South Wales, Victoria, Tasmania and South Australia to Western Australia (Fig. 2.3). Although identification of *Neotrigonia bednalli* in Western Australia is based on erroneous description, the suggestions by Lamprell and Whitehead (1992) that the range of this species extends to northern WA can not be confirmed or excluded at present due to a lack of fresh material. The Leeuwin and Great Australian Bight Currents can aid dispersal down the western coast and across the southern coast of Australia (O'Hara and Poore, 2003), potentially explaining the large distribution range of *Neotrigonia margaritacea*.

Neotrigonia appears to have a biogeographic structure similar to other Australian marine invertebrates (e.g. Helgen and Rouse, 2006; Kassahn *et al.*, 2003) in that there is generally a division of species between the far western and eastern coast of Australia. Apart from the climatic conditions characterising the actual geographic range for *Neotrigonia* spp., the natural habitat of specimens collected varied little and is characterized by subtidal (>18m deep) coarse sand, exposed to moderate currents. Shell morphology, in particular high radial ribs, interstitial grooves and nodules, enables this burrowing animal to remain *in situ* despite strong currents. *Neotrigonia* are able to burrow due to the foot, which Morton (1987) described as a complex compilation of oblique, circular and transverse muscles that give it extreme strength. This verifies Tevesz's (1975) speculation that *Neotrigonia* has an active burrowing lifestyle, with the added capacity of leaping. The muscular foot is protruded beyond the shell, it then contracts pulling along the whole animal (personal observation).

Ascertaining morphological characters that would be informative in delimiting species within the genus *Neotrigonia* was challenging. It was further complicated by assimilation of traditional and modern terminology applicable to both extant and fossil taxa. Although application of terminology describing shell shape and orientation is unlikely to be an issue among extant bivalve taxa, identifying anterior

and posterior ends of juvenile shells or from poorly preserved fossil specimens can be a speculative process (Hoggarth, 1987; Bailey, 2009). Likewise, traditional descriptions for most of *Neotrigonia* species contain some information on the number of ribs, either across the whole area of the shell or just the flank. Initially this comes across as a valid morphological character; however, this feature did not translate into a cladistically-valid character, since the number of ribs vary between 18-29 for extant species and for the fossil the range encompasses that of all extant taxa, ranging from 18-32.

Although morphological analysis has enabled six *Neotrigonia* species to be distinguished (Figs. 2.4, 2.6), it has also raised concerns in regards to identification of specimens to species level and ultimately the validity of taxonomic names used in museum collections based on morphology alone. The Australian Museum has the biggest collection of *Neotrigonia* specimens; *N. kaiyomarue* 7 records, *N. gemma* 83 records, *N. strangei* 6 records, *N. uniophora* 54 records, *N. margaritacea* 663 records, *N. lamarckii* 47 records and *N. bednalli* 23 records. However, for these specimens, and specimens in other museums, identifications are at times dubious, as illustrated by Table 2.1, supporting the reclassification of the purported WA *N. strangei* to *N. uniophora*, *N. bednalli* to *N. kaiyomaruae* and several Tasmanian *N. margaritacea* into *N. gemma*. Malacological specialists are able to adequately score morphological characters and identify specimens; however, not all collections are consistently curated to this level, hence misidentifications are possible. Shell morphology has been used in past to resolve taxonomy of *Neotrigonia* (e.g. Newell, 1969; Purchon, 1987; Waller, 1998), but more recently it has been shown that combining morphological and molecular data is a far superior approach for bivalve taxonomy (Giribet and Wheeler, 2002; Graf and Cummings, 2006). This reinforces a great need for fresh material to provide molecular data and further knowledge of the soft tissue anatomy, as well as the characterization of developing stages for all species within the genus.

This taxonomic revision of genus *Neotrigonia* was based on live collected specimens, specimens deposited in museum collections and all available types. The results were guided by museum identification of specimens and comparison to types, which further confirms the need for alive material to fully resolve all species in the genus. True species status can be confirmed only if specimens are collected fresh so that soft tissue anatomy, as well as molecular data can be obtained. This is not to say that current collections are not useful, rather the significance of these collections is

emphasized by their ability to aid in confirming species status and distribution range, as well as identifying gaps in knowledge for a number of species in this genus.

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Table 2.1. Subset of examined *Neotrigonia* extant and fossil specimens held at different museum collections, and registration information for neotype *Eotrigonia subundulata* and holotype *Trigonia miriana*, which were used as an outgroup taxa in cladistic analysis.

Species	State	Locality	Latitude/Longitude	Number collected (L/D),(E/F2), Fossil	Museum/Institution	Recommended name change
<i>N. margaritacea</i>	Victoria	Western Port	-38.48 145017	3 D	AM C 000663	
				1 LE	VM F 97351	
				2 E	VM F 97352	
				3 LE	VM F 97353	
				1D	AM C 011042	
	NSW	Port Philip Gabo Island Port Jackson Malabar Port Kembla	-37.850 144.933 -37.567 149.917 -33.855 151.247 -33.991 151.280 -34.465 151.075	1D	AM C 050283	
				4D	AM C 012038	
				1 L F	AM C 3636.9	
				10 D	AM C 151.075	
				2D	AM C 040705	<i>N. gemma</i>
	Tasmania	Port Arthur King Island Bruny Island	-43.150 147.850 -39.833 144.00 -43.383 147.283	8D	AM C 048965	<i>N. gemma</i>
1D				AM C 049023		
2D				AM C 079437		
1L E				SAM I.		
<i>N. bednalli</i>	SA	Kangaroo Island Great Aus. Bight St Francis Is	-37.000 138.550 -33.042 133.125	9 L F	AM C 065884	
				1 D	NMH	
				1 L F	AM C 426.923	
	WA	Wilson inlet Great Aus. Bight Rottneest Island	-35.200 117.000 -34.350 121.267 -32.020 115.500	10 L F	AM C 426924	
				1L F	BMNH	<i>N. kayiomaruae</i>

						20012697	
					1L F	BMNH20050	<i>N. kayiomaruae</i>
						708	
					1L F	BMNH20050	<i>N. kayiomaruae</i>
						709	
<i>N. lamarckii</i>	NSW	Sow and Pigs Reef	-33.838	151.268	20D	AM C 05436	
		Malabar	-33.991	151.280	20D	AM C 363318	
	Qld	Moreton Bay	-27.517	153.667	2 D	AM C	
						394.108	
		Mooloolaba	-26.667	153.600	1 L F	AM C 394144	
<i>N. gemma</i>	NSW	Cronulla	-34.067	151.133	8 L F	AM C 073050	
		Long Bay	-33.987	151.373	20 L F	AM C 158382	
		Cape Tree Points	-26.667	153.600	1 L F	AM C 016884	
	Qld	Jumpin Pin Bar	-27.733	153.525	2 D	AM C 393915	
	Victoria	Cape Everard	-38.177	149.283	2 D	AM C 393916	
		Churchill Island	-38.453	145.348	20 D	AM C 427483	
<i>N. uniophora</i>	NSW	Sow and Pigs Reef	-33.838	151.268	4 D	AM C 010709	
		Montague Island	-36.250	150.217	3 D	AM C 040704	
		Whale Point	-38.005	150.829	1 L F	AM C 361185	
		Wollongong			1 D	AM C 090777	
	Qld	Albany Passage	-10.750	142.617	20D	AM C 036125	
		Shelburne Bay			2 D	AM C 303167	
		South coast			1 D	AM C 303165	
	NT	Talc Head	-12.483	130.775	2 D	AM C 061433	
	WA	Roebuck Bay	-18.000	122.250	1 D	AM C 090774	
		Port Hedland	-19.515	118.820	20 D	AM C 148709	

<i>N. kaiyomaruae</i>	WA	Hood Point	-34.417	121.333	1 D	AM C 065910	
		Cheyne Bay	-34.917	119.000	3 D	AM C 065911	
<i>N. strangei</i>	WA	Port Hedland	-19.920	117.933	1 D	AM C 427815	<i>N. uniophora</i>
	NSW				1D	AM C 427376	<i>N. uniophora</i>
		Bass Point	-34.600	150.917	1 D	AM C 302837	
		Bass Point	-34.600	150.917	1 D	VM F 30042	
<i>N. acuticostata</i>	Vic	Beaumaris			1 Fossil	NMV P 12240	
<i>N. howitti</i>	Vic	Bairnsdale			1 Fossil	NMV P 12233	
<i>N. medipontea</i>	SA	Ranmark			1 Fossil	NMV P 47771	
<i>N. novaguineana</i>	New Guinea	Urapmin area			1 Fossil	CPC 6810	
<i>Eotrigonia subundulata</i>	Vic	Torquay			1 Fossil	NMV P 12250	
<i>Trigonia miriana</i>	WA	Giralia range			1 Fossil	CPC 4643	

Table 2.2. Listing of live collected *Neotrigonia* specimens and the habitat type they were collected from.

Species	State	Locality	Latitude/Longitude	Number collected (L/D ¹), Preservative (E/F ²)	Habitat type
<i>N. margaritacea</i>	Vic	Western Port	-38.48 145.017	34 L E	Silty, ammonia rich, high current
	Tas	Bruny Is	-43.383 147.283	4 L E	Well-sorted sand, moderate current
		Bass Straight	-39.20 145.30	2 L E	Well-sorted sand, high current
<i>N. bednalli</i>	SA	Port Lincoln	-34.44 135.52	54 L E	Sand, algae, high current
		Port Noarlunga	-35.167 138.500	8 L E	Coarse sand, moderate current
		Port Stanvac	-35.106 138.484	30 L E	Well-sorted sand, high current
	WA	Abrolhos Is ³	-28.184 113.355	1 L F	Well-sorted shell grit
		Rottneest Is ³	-31.595 115.539	3 L F	Well-sorted shell grit
		Dampier Is ³	-20.410 116.42	3 L F	Coral shell grit, high current
<i>N. lamarckii</i>	Qld	Jumpin Pin Bar	-27.733 153.525	3 L E	Well-sorted sand, high current

¹L, live; ²Specimens preserved in E = ethanol 70%, F = formalin 10%; and ³Specimens collected by J. Taylor and E. Glover, which were on loan from NHM.

Table 2.3. Morphological data matrix of characters 1 to 25 used to differentiate species of genus *Neotrigonia* based on type specimens. Scores are as follows: absence, 0, presence, 1, Missing or unknown, ?. Analysis based on this data matrix resulted in parsimony tree construction depicted in Figure 2.4.

Species name	Character scores		
	10	20	25
<i>Trigonia miriana</i>	0000000000	0000000000	00000
<i>Eotrigonia subundulata</i>	0000000000	1010100000	00010
<i>Neotrigonia acuticostata</i>	0001001000	00111?1110	11110
<i>Neotrigonia howitti</i>	0001001000	00111?1110	10110
<i>Neotrigonia medipontea</i>	0001001000	1100??1111	10110
<i>Neotrigonia novaguineana</i>	0011000011	1010??1111	10111
<i>Neotrigonia strangei</i>	1111111111	1010101001	10110
<i>Neotrigonia uniophora</i>	1001111111	1010101000	10010
<i>Neotrigonia gemma</i>	1111111111	1010111000	11011
<i>Neotrigonia lamarckii</i>	1111111111	1010111100	11110
<i>Neotrigonia kaiyomaruae</i>	1111111111	1010111111	11110
<i>Neotrigonia margaritacea</i>	1111111111	1010111100	11111
<i>Neotrigonia bednalli</i>	1111111111	1010111100	11111

Table 2.4. Morphological data matrix of characters 1 to 25 used to analyse the subset of specimens of genus *Neotrigonia* listed in Table 2.2. Scores are as follows: absence, 0, presence, 1, Missing or unknown, ? Analysis based on this data matrix resulted in parsimony tree construction depicted in Figure 2.5.

Species name	Character scores		
	10	20	25
<i>Trigonia miriana</i>	0000000000	0000000000	00000
<i>Eotrigonia subundulata</i>	0000000000	1010100000	00010
<i>Neotrigonia acuticostata</i>	0001001000	00111?1110	11110
<i>Neotrigonia howitti</i>	0001001000	00111?1110	10110
<i>Neotrigonia medipontea</i>	0001001000	1100??1111	10110
<i>Neotrigonia novaguineana</i>	0011000011	1010??1111	10111
<i>Neotrigonia strangei</i>	1111111111	1010101001	10110
<i>Neotrigonia uniophora</i>	1001111111	1010101000	10010
<i>Neotrigonia gemma</i>	1111111111	1010111000	11011
<i>Neotrigonia lamarckii</i>	1111111111	1010111100	11110
<i>Neotrigonia kaiyomaruae</i>	1111111111	1010111111	11110
<i>Neotrigonia margaritacea</i>	1111111111	1010111100	11111
<i>Neotrigonia bednalli</i>	1111111111	1010111100	11111
<i>N. bednalli</i> BMNH20012697	1111111111	1010111111	11110
<i>N. bednalli</i> BMNH20050708	1111111111	1010111111	11110
<i>N. bednalli</i> BMNH20050709	1111111111	1010111111	11110
<i>N. bednalli</i> C019242SA	1111111111	1010111100	11111
<i>N. bednalli</i> C073494SA	1111111111	1010111100	11111
<i>N. bednalli</i> BMNH20012696	1111111111	1010111100	11111
<i>N. bednalli</i> BMNH20050706	1111111111	1010111100	11111
<i>N. bednalli</i> BMNH20050707	1111111111	1010111100	11111
<i>N. bednalli</i> BMNH20050710	1111111111	1010111100	11111
<i>N. bednalli</i> F17399	1111111111	1010111100	11111
<i>N. bednalli</i> F18256	1111111111	1010111100	11111
<i>N. bednalli</i> S13732	1111111111	1010111100	11111
<i>N. bednalli</i> S13735	1111111111	1010111100	11111
<i>N. bednalli</i> S13736	1111111111	1010111100	11111
<i>N. margaritacea</i> C010705	1111111111	1010111000	11011
<i>N. margaritacea</i> C048965	1111111111	1010111000	11011
<i>N. margaritacea</i> C094195	1111111111	1010111100	11111
<i>N. margaritacea</i> C135613	1111111111	1010111100	11111
<i>N. margaritacea</i> C363287	1111111111	1010111100	11111
<i>N. margaritacea</i> C363546	1111111111	1010111100	11111
<i>N. margaritacea</i> F19065	1111111111	1010111100	11111
<i>N. margaritacea</i> C363547	1111111111	1010111100	11111
<i>N. margaritacea</i> F97355	1111111111	1010111100	11111
<i>N. margaritacea</i> F97354	1111111111	1010111100	11111
<i>N. gemma</i> C363358	1111111111	1010111000	11011
<i>N. gemma</i> C363580	1111111111	1010111000	11011
<i>N. gemma</i> C427308	1111111111	1010111000	11011
<i>N. gemma</i> F97357	1111111111	1010111000	11011

<i>N. kaiyomaruae</i> C303516	111111111	101011111	11110
<i>N. kaiyomaruae</i> C426919	111111111	101011111	11110
<i>N. lamarckii</i> C055419	111111111	101011100	11110
<i>N. lamarckii</i> C303168	111111111	101011100	11110
<i>N. lamarckii</i> C363316	111111111	101011100	11110
<i>N. lamarckii</i> C363318	111111111	101011100	11110
<i>N. lamarckii</i> C313607	111111111	101011100	11110
<i>N. lamarckii</i> F97358	111111111	101011100	11111
<i>N. strangei</i> C303166	111111111	101010100	10110
<i>N. strangei</i> C302837	111111111	101010100	10110
<i>N. strangei</i> F30042	111111111	101010100	10110
<i>N. strangei</i> C427815	100111111	101010100	10010
<i>N. strangei</i> C427376	100111111	101010100	10010
<i>N. uniophora</i> BMNH20012698	100111111	101010100	10010
<i>N. uniophora</i> BMNH20013145	100111111	101010100	10010
<i>N. uniophora</i> C090777NSW	100111111	101010100	10010
<i>N. uniophora</i> C303165Qld	100111111	101010100	10010
<i>N. uniophora</i> C303167Qld	100111111	101010100	10010
<i>N. uniophora</i> C361185NSW	100111111	101010100	10010
<i>N. uniophora</i> C363387NSW	100111111	101010100	10010
<i>N. uniophora</i> WAC14870	100111111	101010100	10010

Figure 2.1. Morphology of the *Neotrigonia margaritacea* species complex, showing synonymous shell shape and characters in specimens collected from A) South Australia (formerly called *N. bednalli*), B) Victoria and C) Tasmania. A single specimen of *N. margaritacea* from Victoria is used to illustrate a D) internal left valve, shizodont hinge Sh E) internal right valve showing the anterior adductor muscle scar AAdM, posterior adductor muscle scar PAdM, and shizodont hinge SH, and F) side view showing umbo, ligamental nymph and escutcheon.

Figure 2.2. *Neotrigonia margaritacea* is used to depict A) bivalve views and B) scored characters used in cladistic analysis, which are indicated on the *Neotrigonia margaritacea* shell. Character 1 is not shown on the figure, as the whole shell shape needs to be considered when scoring that character, and character 5 is indicated twice as each bivalve needs to be examined from both views to ascertain the size of the umbone.

Figure 2.3. Map of Australia illustrating distributions (shaded) of A) *N. margaritacea* with a stippled area indicating a former distribution of *N. bednalli*, B) *N. lamarckii*, C) *N. gemma*, D) *N. uniophora* E) *N. kaiyomarue*, and F) *N. strangei*. Type locality for each species is indicated with a white dot on the corresponding map.

Figure 2.4. Strict consensus tree topology obtained by parsimony analysis of morphological characters for type specimens (Table 2.3) of living and fossil *Neotrigonia* species, where *Trigonia miriana* and *Eotrigonia subundulata* were used as outgroup species.

Figure 2.5. Adam's consensus tree topology obtained by maximum parsimony heuristic search on morphological data based on a subset (Table 2.4) of specimens from museum collections including type specimens, which are indicated by an asterisk. When, data used to produce this figure, is filtered based on identical states for all characters so that each terminal taxon is different (in MacClade), Fig. 2.4 tree is achieved.

Figure 2.6. Holotypes or hypotypes for the six confirmed extant species of *Neotrigonia*: A) *Neotrigonia margaritacea*, holotype MNHN (no registration number); B) *Neotrigonia lamarckii*, holotype NHM Cummings collection; C) *Neotrigonia gemma*, holotype C90220; D) *Neotrigonia uniophora*, holotype NHM Cummings collection; E) *Neotrigonia kaiyomaruae* hypotype C303516; and F) *Neotrigonia strangei*, holotype NHM Cummings collection.

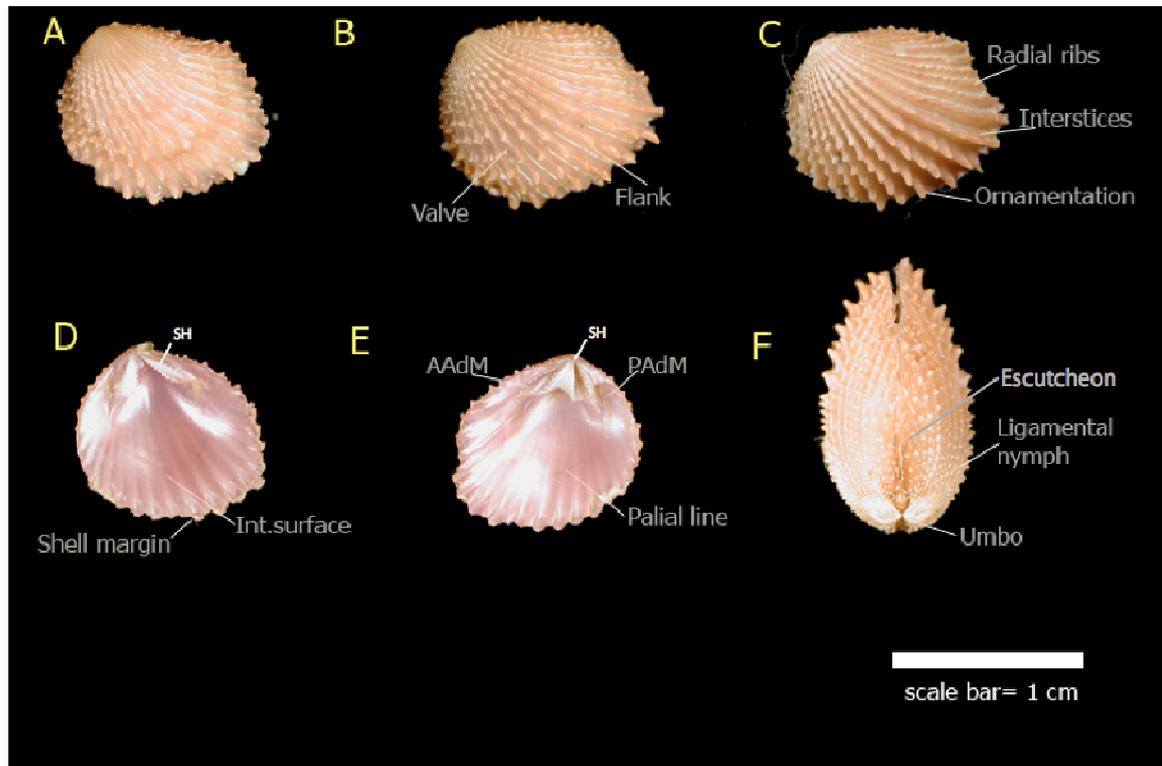


Figure 2.1

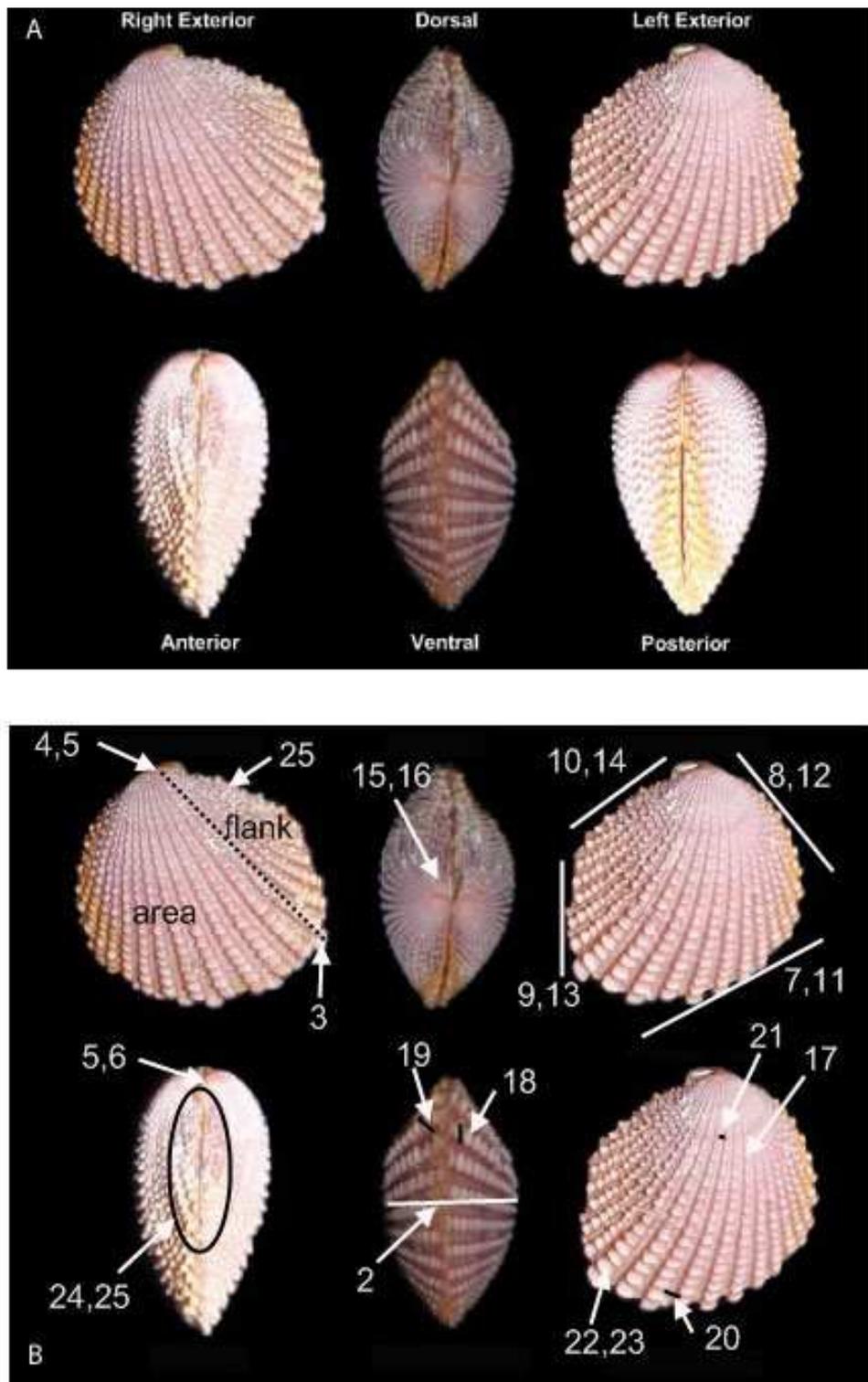


Figure 2.2

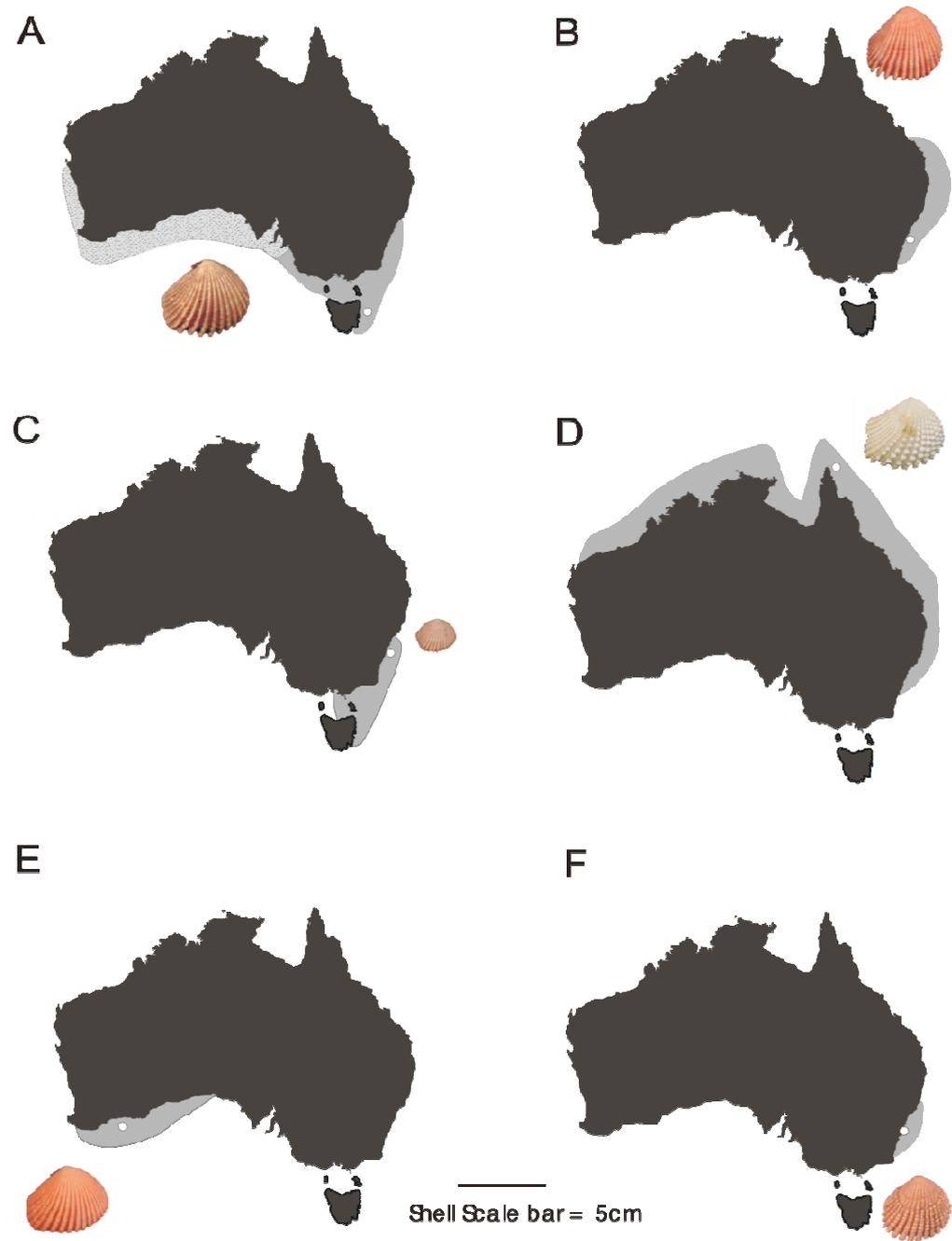


Figure 2.3

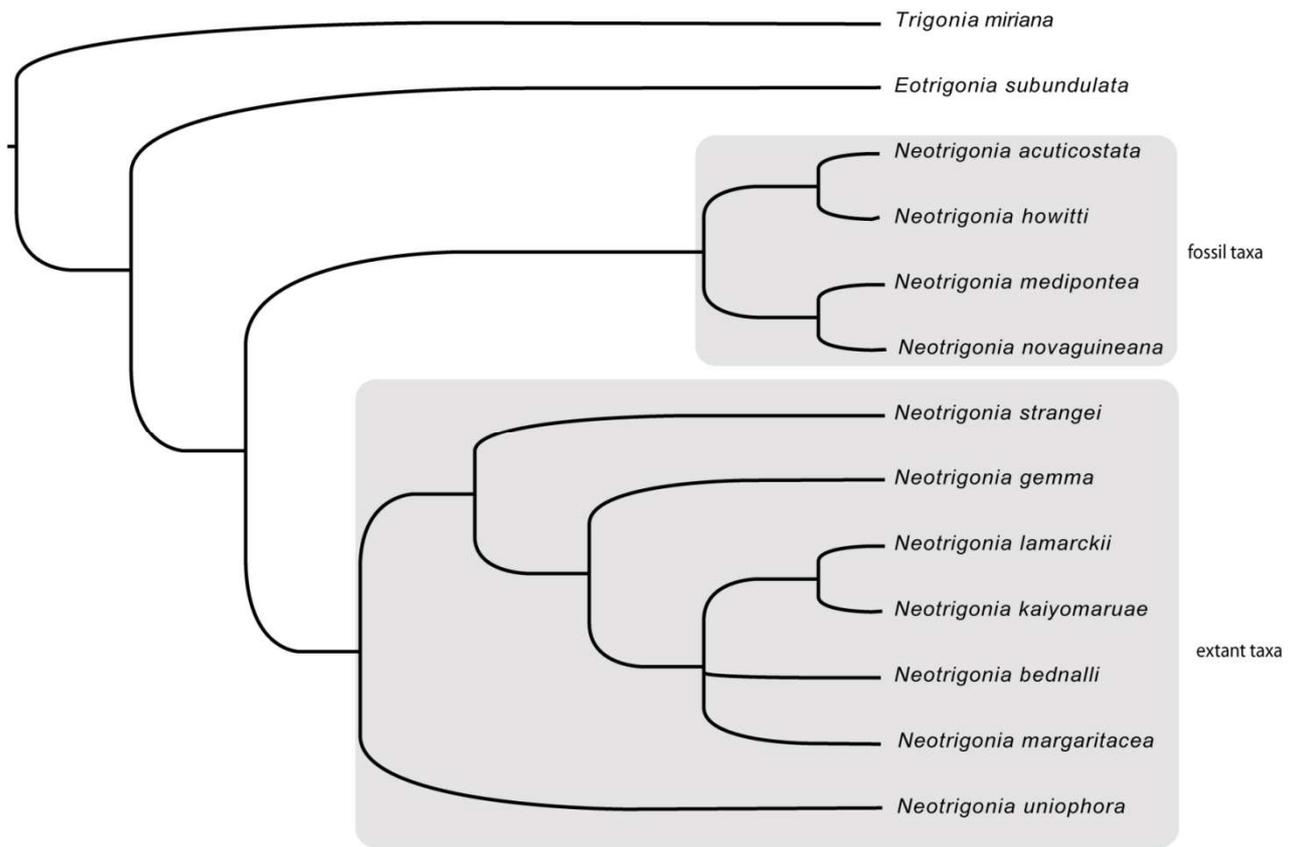


Figure 2.4

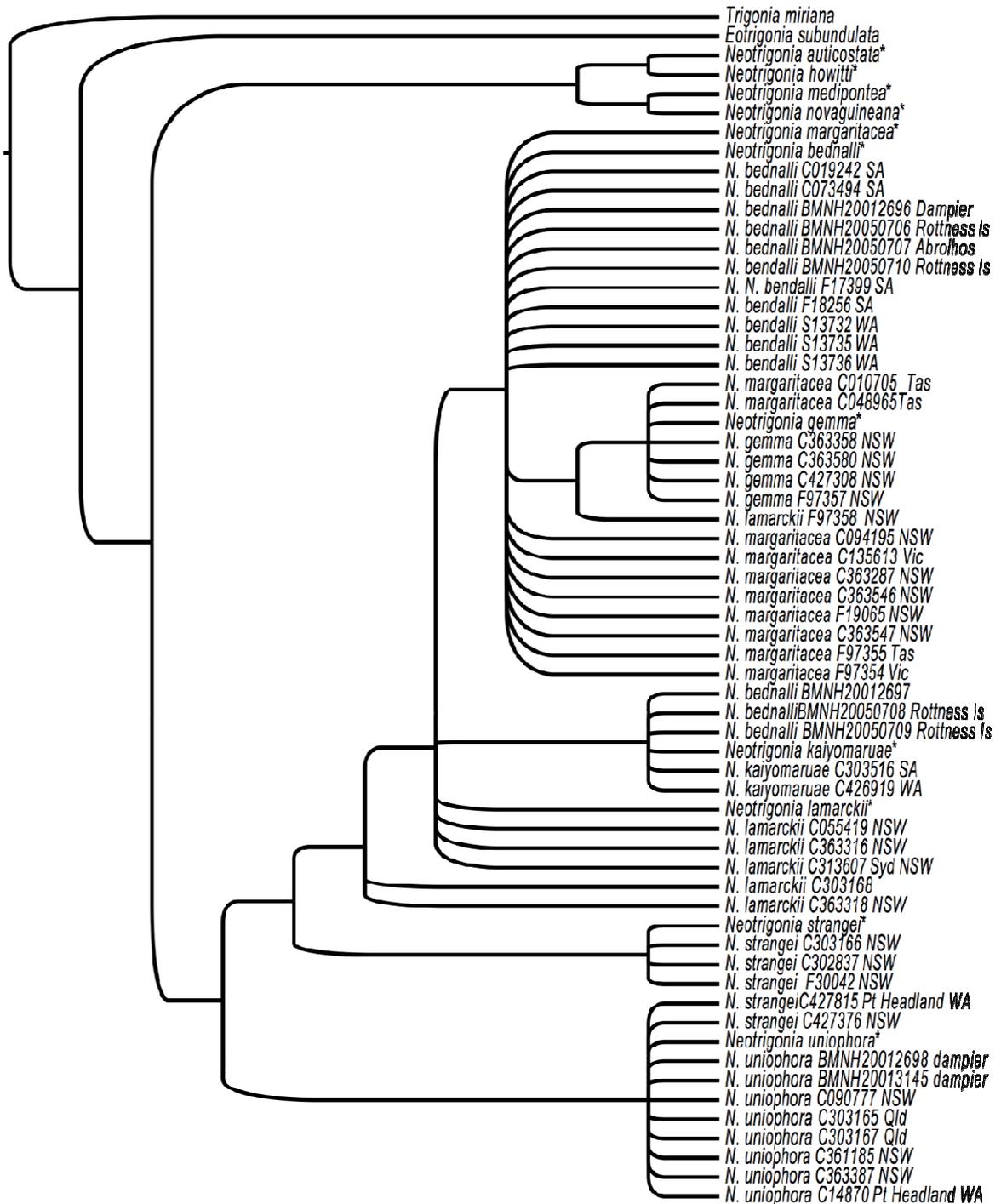


Figure 2.5

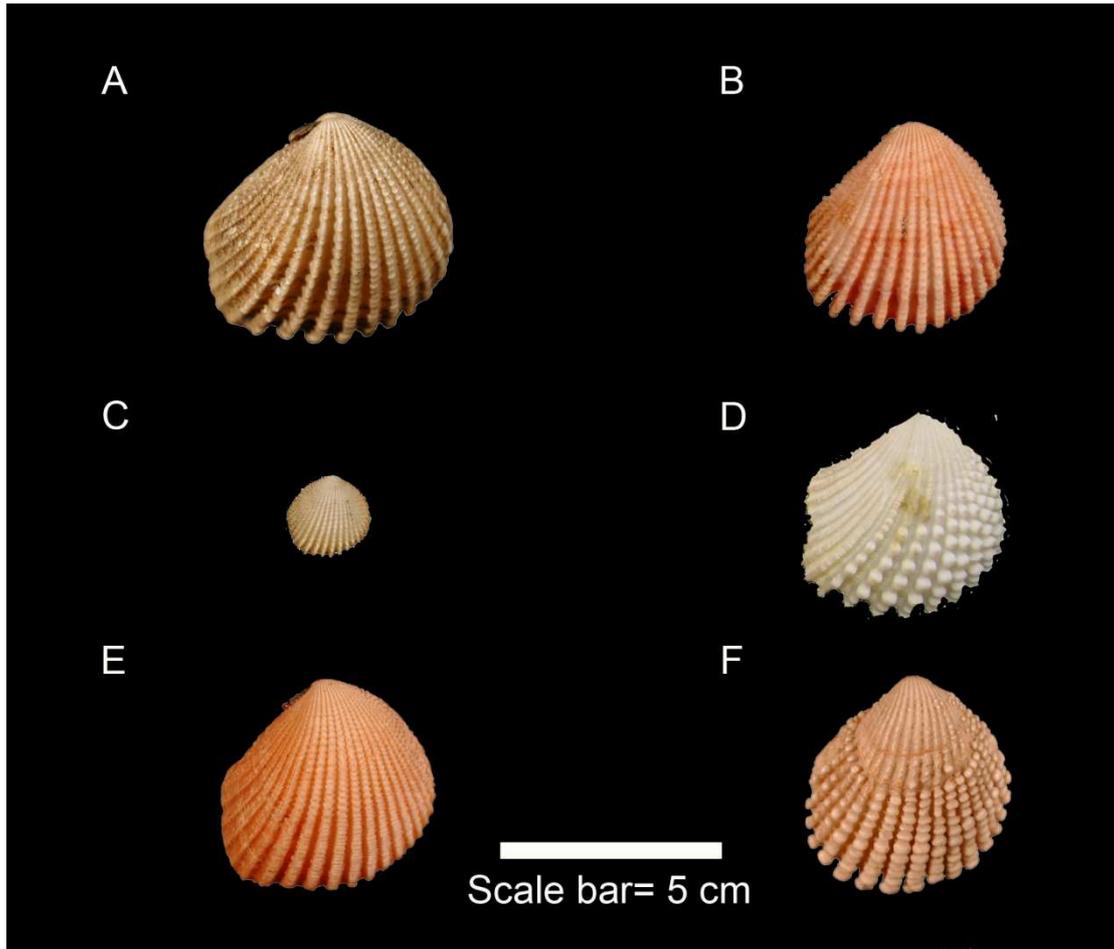


Figure 2.6

Phylogeography and gene flow in the living fossil *Neotrigonia margaritacea*

Ana Glavinic

Flinders University, Adelaide, PO Box 2100, Adelaide, SA 5001, Australia.

Author for correspondence: ana.glavinic@flinders.edu.au, telephone +61 8 82015747

Key words, phylogeography, haplotypes, gene flow, *Neotrigonia*, Maugean province, Flindersian, Bivalvia

Abstract

Knowledge of contemporary ocean currents, temperatures and geological history across southern Australian represents a useful framework for phylogeographical analyses. A number of studies show that some marine invertebrate distributions are coincident with biogeographic boundaries among Maugean, Flindersian and Peronian marine provinces. However, because of the differing histories of these taxa, it is hard to distinguish between causal and explanatory phylogeographic patterns. We focus on the marine 'living fossil' *Neotrigonia* (Bivalvia) that has been present in Australian waters for longer than 60 million years, and has therefore been exposed to most of the possible historical and contemporary factors invoked to explain phylogeographic patterns today. We first examined species delimitation with molecular data. Phylogenetic analyses based on COI and ITS sequence data confirmed that southern Australian *Neotrigonia margaritacea* and eastern Queensland *Neotrigonia lamarckii* are separate species. Molecular data also supported the recent synonymization of *N. bednalli* with *N. margaritacea*. We then examined the genetic structure within the widespread southern *Neotrigonia margaritacea*. Population genetic analyses based on COI data revealed high haplotypic diversity and strong genetic structure. Each sampled population contained only private haplotypes which suggests future sampling should be carried out on a smaller geographic scale. There was a significant lack of inferred gene flow between populations on the west side of the hypothesized Bass Strait land bridge barrier (Port Lincoln, Gulf St. Vincent). Populations near the east side of the historical Bass Strait land bridge barrier (Western Port, Bruny Island) were not significantly structured from each other and may represent recent recolonization events. The divergence between populations east and west of the Bass Strait land bridge was not markedly deeper than the divergence between the western populations. This is indicative of colonization from the west, and concurs with the known direction of flooding of the Bass Strait land bridge. This study highlights the benefits of incorporating paleontological inferences into *a priori* phylogeographic hypotheses.

Introduction

Australian temperate marine communities are highly structured despite occurring along a continuous, latitudinally similar coastline. Three main temperate provinces were first proposed by Bennett and Pope (1953), who identified a Peronian (south-east Australia), a Flindersian (south-west) and a Maugean (Tasmania and southern Victoria) province. A number of other marine biologists have recognised such marine biogeographical provinces on the basis of faunal distribution and physical parameters (Whitley, 1932; Bennett and Pope, 1953, 1960; Knox, 1963, 1980; Dartnall, 1974; Rowe and Vail, 1982). Many of these studies interpret biotic distributions in terms of contemporary environmental factors, whereas others emphasise the importance of geological, climatic and hydrographic history (Bennett and Pope, 1953; Wilson and Allen, 1987; O'Hara and Poore, 2000; O'Hara, 2001).

The strongest identifiable contemporary influences originate from the prevailing ocean currents (Fig. 3.1). The Leeuwin Current flows southwards along the west coast, then towards the east, along the south coast. Palaeontological evidence suggests that origin of Leeuwin current along the Western Australian coast predates the period between middle Eocene and mid to late Oligocene (Shafik, 1989; McGowran *et al.*, 1997). Since that time the current flow was prominent and intermittent, with strong pulses bringing warm waters into South Australia (Shafik, 1989). The East Australian Current moves southward along the east coast. By transporting tropical water masses these two ocean currents strongly influence water temperature, which plays a major ecological role in the establishment of marine invertebrates (Bennett and Pope, 1953, 1960; McGowran *et al.*, 1997, O'Hara and Poore, 2000).

In contrast, historical influences can be attributed to the Australian continent's geological past. During the late Cretaceous (90 Ma), the western side of the Australian landmass drifted north away from Antarctica (Veevers, 1984), forming the south Australian coastline. The western region of the coastline was then colonised by warm water species and cool-water fauna were concentrated to the east (O'Hara and Poore, 2000), where a connection still existed at the south Tasman Rise (Veevers, 1984). The final split between the Australian and Antarctic continents occurred 55-35 Mya ago (Veevers, 1984). The mixing of eastern and western faunas created a rich assemblage in southern Australia, further supplemented with tropical species as the Australian landmass drifted north (Darragh, 1989; Poore, 1994; O'Hara and Poore,

2000). There is also evidence of a smaller number of Southern Ocean species entering this ecosystem by the circumpolar subantarctic currents (Darragh, 1989; Edgar, 1986; Poore, 1994; O'Hara and Poore, 2000).

Allopatric speciation on the southern Australian coastline may also have been promoted by subsequent geological events (Bennett and Pope, 1953, 1960; Edgar, 1986; Waters and Roy, 2003; Waters *et al.*, 2004; Helgen and Rouse, 2006). One important period for allopatric speciation occurred during the Pliocene and Pleistocene eras, when glaciation repeatedly decreased sea levels by locking up large amounts of water as ice. During these periods, the area between Tasmania and mainland Australia, Bass Strait, formed a dry landbridge that separated populations on either side (Dartnall, 1974; Knox, 1980). The landbridge emerged and was subsequently flooded repeatedly during the late Pliocene to Pleistocene, disrupting gene flow for some 2 million years, providing a vicariance model for speciation between the Peronian and Flindersian provinces (Waters and Roy, 2003).

We explore the spatial patterns of genetic diversity in the marine bivalve *Neotrigonia* (Palaeoheterodonta: Trigonioida). The extensive fossil record shows *Neotrigonia* to be a "living fossil", a rare marine organism that remained present in southern Australian waters over many palaeontological eras (Darragh, 1986). The history of Australian Trigoniidae during the last 60 million years is reasonably well documented in the Tertiary sediments of Victoria, South Australia and Tasmania (Fleming, 1964; Skwarko, 1966; Cox, 1969). Species of *Neotrigonia* range back to the upper Miocene replacing the genus *Eotrigonia*, which spans back to the lower Eocene or Paleocene (Cox, 1952; Fleming, 1964).

Bivalves belonging to *Neotrigonia* display a broad distribution across the Australian coastline, occurring infaunally from the tropical sandy to temperate rocky substrate, but always in depths greater than 10 m (Tevesz, 1975; Stanley, 1977, 1984; pers. obs). A preliminary cladistic analysis using morphometric characteristics recognises six distinct extant species (Chapter 2). The majority of these extant species occupy warm to tropical habitats, including *N. lamarckii*. However, *N. margaritacea* is the dominant species in colder southern seas. The species range known for *N. margaritacea* (Victoria and Tasmania) now also encompasses the geographic distribution of the recently synonymized species *N. bednalli* from South Australia (Chapter 2).

Here, we investigate species delimitation within *Neotrigonia* and test the proposed synonymization of *N. bednalli* with *N. margaritacea* with molecular data. *Neotrigonia margaritacea* are benthic, burrowing animals whose larvae are thought to be lecithotropic (O’Foighil and Graf, 2000), so their reduced dispersal capacity may result in high levels of genetic structure. We examine gene flow and population structure in the widespread *N. margaritacea*, and use its prolonged existence in southern Australian waters as a model to improve sensitivity in understanding phylogeographic patterns around the southern Australian coastline.

Methods

Sample collection

Specimens of *Neotrigonia* spp. were collected subtidally and preserved in 70% ethanol (Table 3.1) (see Appendix I, for all collecting attempts). The holotypes of *N. margaritacea* and *N. lamarckii* were loaned from the Australian Museum, Sydney, and Muséum national d’Histoire naturelle, Paris, to establish identity, but could not be sequenced as these specimens comprise only the hard shells. We sampled newly-collected specimens from four populations of *Neotrigonia margaritacea*; Gulf St. Vincent, South Australia (n=21); Port Lincoln, South Australia (n=15); Western Port, Victoria (n=8); Bruny Is., Tasmania (n=4) and three specimens of *Neotrigonia lamarckii*, from North Stradbroke Is., Queensland (n=3). All populations of *N. margaritacea* were located in semi-closed gulfs or embayments except for Port Lincoln, South Australia, which represents an exposed coastline (Fig. 3.1). The populations from Western Port, Victoria and Bruny Is., Tasmania represents the Maugean province, while Gulf St. Vincent, and Port Lincoln populations from South Australia, represents the Flindersian marine province.

Markers and outgroup choice

We examined mitochondrial cytochrome *c* oxidase subunit I (COI) sequences in this study because: (i) universal invertebrate primers were available for a portion of this gene (Folmer *et al.*, 1994); and (ii) this gene region has provided useful information in phylogenetic studies of bivalves (Hoeh *et al.*, 1998; Giribet and Wheeler, 2002) and more generally in invertebrate phylogeographic studies. We also included data from the nuclear ribosomal Internal Transcribed Spacer region 1 (ITS1) to compare to the signal from the mitochondrial genome. Including data from both

mitochondrial and nuclear sources is preferred because differences in fixation times for the two genomes may reveal information from different timescales. A complicating factor in obtaining COI data was that doubly uniparental inheritance (DUI) of mitochondria is now also reported in *Neotrigonia* (Chapter 4). To prevent inadvertently including paternal or type M genome mtDNA data, only female specimens were included in this study.

The trees were rooted with *Anodonta anatina* (COI: EF571397, ITS1: DQ060181) and additional outgroups were *Unio tumidus* (COI: DQ060176, ITS1: DQ060191) and *Pyganodon grandis* (COI: EF418019, ITS1: EF488196). These are all freshwater bivalves in Unionoidea, the sister group of the marine Trigonioidea, which together form the diverse clade Palaeoheterodonta (Hoeh *et al.*, 1998; Giribet and Wheeler, 2002).

Extraction and amplification

All tissues were stored in ethanol and total genomic DNA was extracted according to the DNeasy blood and tissue kit (Qiagen, USA). The genomic DNA was then cleaned using the Cetyl Trimethyl Ammonium Bromide (CTAB) method (Scouras and Smith 2001) and the samples stored at -20°C. A 592-bp fragment of COI was amplified using the primers LCO1490 and HCO2198 (Folmer *et al.*, 1994), and 769-bp of ITS1 using primers G740 F (5'-TCCGTAGGTGAACCTGCGG-3') and G749 R (5'-GCTGCGTTCTTCATCGATGC-3') (White *et al.*, 1990). Polymerase chain reactions (PCR) were carried out using a Corbett FTS-320 thermal sequencer using 0.2 µl taqGold (5 units/µl), 2 µl per primer (5µM), 4 µl DNTP's (Deoxynucleotide Triphosphate) (10 µM), 8 µl MgCl₂ (25 µM), 5 µl TGold Buffer, 5 µl gDNA and 23.8 µl sterile water in a 50µl reaction. Denaturation was carried out at 94 °C for 45 seconds, and an annealing temperature of 48 °C (COI) or 56 °C (ITS1) was applied for 45 seconds, with an extension period at 72 °C for 60 seconds, repeated for 35 cycles.

Sequencing

PCR products were cleaned using UltraClean PCR clean-up spin columns (MoBio, USA). If multiple products were present, the desired product was isolated using agarose gel (1.5%) electrophoresis, and cleaned using the QIAquick gel extraction kit (Qiagen, USA). PCR products were labelled for sequencing using a

Hybaid Omn-E thermal cycler, with 5 μ l of PCR product, 1 μ l of primer (5 μ M) and 4 μ l Big Dye Version 3 combined in a 10 μ l reaction. The reaction was completed at a denaturation temperature of 96 °C for 30 seconds, an annealing temperature of 50 °C for 15 seconds, and an extension temperature of 60 °C for 4 minutes, repeated for 25 cycles. The product was cleaned using 70% isopropanol and sequenced at the Institute of Medical and Veterinary Science, Adelaide, SA, using an automated sequencer 3730 (Applied Biosystems). Bi-directionally sequenced data was reconciled and edited using seqEd v1.0.3, and aligned manually using SeAl v2.0a11 (Rambaut, 2002).

Phylogenetic analyses

We conducted maximum parsimony (MP) in PAUP* 4.0b10 (Swofford, 2003) on each gene, and then used combined COI and ITS data. MP analyses were performed with heuristic searches using 100 random sequence additions and tree bisection reconnection (TBR) branch swapping. Node support was assessed using 10,000 bootstrap replicates.

We also conducted Bayesian analysis on the combined data set using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). Data was organised in two unlinked partitions based on the general time reversible model (GTR+I+ Γ) recommended by Akaike Information Criterion (AIC) (see Posada and Buckeley, 2004) in MrModeltest 2.2 (Nylander, 2002). Bayesian analyses were run with default priors (rate matrix: 0-100, branch lengths: 0-10, Gamma shape: 0-1), a random starting tree and six Markov chains, where 3 chains were heated. One million (10^6) generations were run for two replicate analyses with a tree saved every 100 generations. The trees were used to construct a majority rule consensus tree providing the posterior probabilities for each clade. We used Tracer v1.3 (Rambaut and Drummond, 2005) to observe stationarity of obtained probabilities and discarded the first 500 trees that represented a pre-stationary phase.

Mitochondrial population structure

We used TCS 1.21 (Clement *et al.*, 2000) to construct statistical parsimony networks at the 95% connection limit based on the COI gene. This method is designed to provide representations of gene genealogies at the population level (Templeton *et al.*, 1992). The amount of population genetic structure was tested in ARLEQUIN 2.0 (Schneider *et al.*, 2000) by conducting a hierarchical analysis of molecular variance

AMOVA. Because molecular data are not normally distributed, significance was tested by permutating the data. ARLEQUIN 2.0 was used to estimate haplotype diversity (Nei, 1987) and to estimate Φ_{st} , the pairwise fixation index of genetic differentiation (Hudson *et al.*, 1992; Tamura and Nei, 1993). We also used ARLEQUIN 2.0 to calculate Tajima's D-test (Tajima, 1989) and Fu's F_s test (Fu, 1997) in a demographic context. Significance for these values was assessed in ARLEQUIN 2.0 with 1000 parametric bootstrapping replicates (Schneider *et al.*, 2000). Both of these statistical tests were used to assess the mtDNA haplotype distribution in the population, with the null hypothesis that all populations are expanding (Tajima, 1989; Fu, 1997). Negative values can be explained by selection, geographic barriers or recent mutation, while positive values are interpreted as recent secondary contact between previously differentiated lineages. To test for isolation by distance, we performed a Mantel test in ARLEQUIN 2.0 (Schneider *et al.*, 2000), which computes the linear correlation between two proximity matrices, in this study, Φ_{st} and geographical distance. Distances were measured using Google Earth 4.0.2694, where straight lines connected each population locality while avoiding coastlines.

Results

Topology-based species delimitation

Maximum parsimony (MP) analysis of 744-bp of aligned COI data (175 parsimony informative sites) produced four shortest trees of 351 steps (Consistency Index CI= 0.847, and Rescaled Consistency RC=0.874 for informative characters only) (Fig. 3.2). ITS1 data consisted of 799 aligned sites containing 352 informative sites, and MP analyses produced seven trees with a length of 835 steps (CI= 0.812 and RC= 0.671 for informative sites only) (Fig. 3.3). The topology recovered for each data set was largely congruent, with high support for basal nodes that defined species, but the topology within the species-level clades was either not supported or unresolved. Bayesian and MP analyses on combined data also produced congruent topologies (Fig. 3.4). All analyses strongly supported *N. lamarckii* and *N. margaritacea* as reciprocally monophyletic (bootstrap 100, posterior probabilities 1.00). Specimens that might be identified as *N. bednalli* by morphology nested inside the *N. margaritacea* clade and thus supports the synonymy implemented in Chapter 2.

Genetic distances

In total, 51 COI and ITS bi-directional sequences were obtained for four populations of *Neotrigonia margaritacea sensu lato* and one population of *N. lamarckii*. Interestingly, mitochondrial and nuclear genes contained similar amounts of variation. The mean uncorrected COI pairwise distance among *N. margaritacea* was 3.4 %, and the mean uncorrected *p*-distance among *N. lamarckii* was 3.2%. A mean COI *p*-distance of 11 % between *N. margaritacea* and *N. lamarckii* was recorded. The mean uncorrected *p*-distance for ITS1 within *N. margaritacea* was 2.8% and within *N. lamarckii* was 1.4%, while between *N. margaritacea* and *N. lamarckii* the mean uncorrected *p*-distance was 8.4%. The mean uncorrected *p*-distance among *N. margaritacea* populations was 3.8% based on COI, and 2.9% based on ITS1 data.

Phylogeography and demography

COI sequences from 48 individuals of *N. margaritacea* consisted of 33 unique haplotypes. Results from TCS resolve these haplotypes into a single star-like network, within the 95% limit of parsimony reconnection (Fig. 3.5). No haplotypes were shared among populations, and haplotypic diversity was high for all populations, ranging from 0.88–1.0. Mean nucleotide diversity was low, ranging from 0.01–0.07 (Table 3.2). An analysis of molecular variance found highly significant genetic variation among and within populations (Table 3.3). Pairwise Φ_{st} values between populations were high, and a significant lack of gene flow can be inferred among nearly all populations (Table 3.4). The exception was non-significant structuring between Western Port (Victoria) and Bruny Island (Tasmania) (Table 3.4). Within the four populations of *N. margaritacea*, no values recovered by Fu's F_s (Fu, 1997) or Tajima's D-test (Tajima, 1989) were significant (Table 3.2), rejecting an expanding population model and the null hypothesis of neutrality, respectively. A Mantel test based on Φ_{st} values and geographical distance indicated no evidence for an isolation-by-distance population model; the correlation coefficient R was equal to -0.173 and not significant ($p= 0.64$).

Discussion

Molecular phylogenetic analysis strongly supports *Neotrigonia margaritacea* and *Neotrigonia lamarckii* as distinct monophyletic group. These results are consistent with traditional views on the taxonomy of *Neotrigonia* that have largely relied on shell morphology (Darragh, 1986; Lamprell and Whitehead, 1992). However, traditional taxonomic approaches also recognised *N. bednalli* as a distinct species (Lamprell and Whitehead, 1992), whereas our results show that these specimens are highly supported within the *N. margaritacea* clade, confirming the recent morphological-based synonymisation with molecular data (Chapter 2). The smallest uncorrected COI divergence between two individuals of *N. margaritacea* and *N. lamarckii* is 6%. Although divergence percentages should not be used as an arbitrary threshold for species separation where 1.5% per lineage per million years rate is expected (Norgate *et al.*, 2009), our average uncorrected *p*-distance value for COI divergence of 11% between these two species corresponds to similar values within the sister group, Unionoida, where most interspecific uncorrected *p*-distance values ranged from 3.65% to 15.35% (Serb *et al.*, 2003). To date, the distribution of *Neotrigonia lamarckii* is confined to the eastern Australian Peronian marine province (Chapter 2). In contrast, *Neotrigonia margaritacea sensu lato* can now be regarded as widely distributed on the southern coast of Australia, ranging from South Australia to New South Wales (Stanley 1984), including Tasmania. That widespread distribution encompasses all three marine provinces proposed by Bennett and Pope (1953). We sampled populations of *N. margaritacea* from two of those provinces. Although the boundaries are not entirely discrete, populations from Port Lincoln and Gulf St. Vincent are both in the Flindersian Province that covers the majority of the southern coast of Australia. Port Lincoln is on the exposed coast, west of the major gulf system in South Australia, and is less likely to have been severely affected by sea-level change in the Pleistocene, whereas Gulf St. Vincent was likely to have been completely exposed during that period of lowered sea-level (Veevers, 1984). Populations from Western Port and Bruny Island are both in the shallow Bass Strait region of the Maugean province (see Fig. 3.1), which is likely to have been completely exposed during periods of lowered sea-level (Veevers, 1984).

An AMOVA showed significant genetic subdivision within and among all of the sampled populations of *Neotrigonia margaritacea*. Pairwise Φ_{st} comparisons were significant between all four populations along the southern Australian region,

except between Western Port and Bruny Island. These two Maugean province populations produced a pairwise Φ_{st} value within the range of the other pairwise populations comparisons, but were the least sampled of the *N. margaritacea* populations in this study, thus generating low power to detect a significant difference. Furthermore, the lack of any shared haplotypes between populations (Fig. 3.5) and the lack of corresponding phylogenetic structure (Figs. 3.2-3.5) supports the lack of gene flow between these Maugean populations. None of the optimality criteria applied to phylogenetic analyses here resulted in trees with a divergence between populations on either side of the hypothesized Bass Strait land bridge, possibly indicating it never presented a significant barrier for dispersal of this species. But in combination with the restricted dispersal, it seems more reasonable to infer that the populations in the Bass Strait region might represent a relatively recent colonization. These two Maugean province populations cannot be linked to each other by any clear current regime, and their lack of differentiation from each other may instead represent a lack of differentiation from a common source population. The level of divergence shown between eastern populations (Bruny Island and Western Port) and western populations (Port Lincoln and Gulf St. Vincent) is no greater than any other, indicating that recolonization may have originated from a westerly source population. This seems likely given that the Bass Strait land bridge was last flooded from a westerly direction after the last glacial maximum (Lambeck and Chappell, 2001). Further sampling of intermediate populations on either side of the Bass Strait land bridge will be necessary to resolve this. A similar phylogeographic pattern is observed in the barnacle *Catomerus polymerus* where the populations from South Australia are thought to source postglacial populations in Victoria and Tasmania (York *et al.*, 2008).

Although quite close geographically, Port Lincoln and Gulf St. Vincent populations are highly differentiated from each other, and each also contained high haplotypic diversity. Reasons for this may be two-fold, relating to both palaeoenvironments and contemporary local hydrodynamics. Firstly, although it is probable that the shallow Gulf St. Vincent was completely exposed during the last glacial maximum, there are Pleistocene and Pliocene fossil deposits that indicate contemporary gulfs could have served as possible refuges during glacial periods (Hocking *et al.*, 1988). Long periods of isolation and small population sizes would have increased the role of genetic drift in fixing the mitochondrial haplotypes

observed here. As each glacial event ended, subsequent contact among refuges might have resulted in the accumulation of haplotypic diversity generated in isolation.

Contemporary reinforcement of isolation may have also contributed to the genetic structure observed between Port Lincoln and Gulf St. Vincent populations of *Neotrigonia margaritacea*. Oceanographic modelling of the Gulf St. Vincent shows limited water exchange with the open ocean especially over summer months (Lennon *et al.*, 1987; Kaempf *et al.*, 2009). This would increase local retention of larvae, and preserve the incidence of private refugial haplotypes in the population (Appendix II). Based on the west to east path of the Leeuwin Current along the southern coast of Australia, the Port Lincoln population might be expected to serve as a source for the Gulf St. Vincent population due to its upstream position. However, the Leeuwin Current reverses direction during summer months (Baines *et al.*, 1983; Kaempf *et al.*, 2009). The reproductive period of *N. margaritacea* appears to be year round (Chapter 5), implying that any potential exchange between these two populations could occur in the winter months, but this is also the period when winter storms are frequent, originating from the Southern Ocean. How much this might prevent gamete exchange is difficult to estimate. Unfertilized oocytes are buoyant for a short period of time, exceeding no more than half an hour (pers. obs.) during which time they may be susceptible to onshore wave action. In any case, a similar pattern of population differentiation for Gulf St. Vincent is seen in the co-occurring giant Australian cuttlefish, *Sepia apama* (Kassahn *et al.*, 2003) and the bobtail squid, *Euprymna tasmanica* (Jones *et al.*, 2006). In those studies, South Australian populations are separated, but even more relevant to our results for *Neotrigonia*, there is connectivity between Melbourne and Tasmanian populations. These cephalopod species similarly have very limited dispersal post-hatching, and may not be mobile in the water column long enough to be influenced by major currents. This point is further corroborated by many species with dispersive larvae that do show a clear historical imprint of the Bass Strait Land Bridge on their population structure (Waters *et al.*, 2004, Waters *et al.*, 2005).

Neotrigonia margaritacea appears to have been present in its current range since the formation of the southern Australian coastline, approximately 1 Mya to 12 000 years ago (Darragh, 1986, McGowran *et al.*, 1997). For the most part, sampled populations appear to be very diverse and strongly genetically structured, perhaps reflecting a continued accumulation of mutations over a long period of time.

Understanding present day connectivity is complicated by the lack of any shared haplotypes and the rejection of an isolation-by-distance model, which may have been expected given the short larval dispersal for this genus. Although present divergence between geographically close populations can be explained in part by contemporary hydrodynamic patterns, historic explanations retain merit e.g. glacial gulf refuges and recent expansions in the Bass Strait region.

Interpreting these results was made more challenging by the large spatial scale between some of the sampled populations and a low number of individuals in others. Davis and Nixon (1992) point out that undersampling increases the chance of false recognition of differentiation (Nixon and Wheeler, 1992). We do not believe this is a major setback to the current study, as the haplotypic network easily resolves all individuals into one network. However, rare shared haplotypes may be uncovered through increased sampling within populations, and further sampling of any kind can only improve the inferences made. We anticipate that our work will renew interest in interpreting southern Australian phylogeographic patterns in light of the diverse origins of its resident taxa and highlight the utility of ‘living fossils’ in phylogeographic studies.

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Table 3.1. The location and number of *Neotrigonia* spp. collected for analyses.

Species	Location	Latitude	Longitude	N
<i>N. margaritacea</i>	SA, Gulf St. Vincent	35° 6'36.05"S	138°27'22.80"E	21
	SA, Port Lincoln	35° 9'42.79"S	135°50'41.58"E	15
	VIC, Western Port	38°21'10.58"S	145°14'26.81"E	8
	TAS, Bruny Island	43°11'33.34"S	148°2'37.81"E	4
<i>N. lamarckii</i>	QLD, North Stradbroke Is	27°23'25.12"S	153°38'4.11"E	3

Table 3.2. Mitochondrial COI diversity of *N. margaritacea* populations. N= number of sequences; K= number of haplotypes; h= mean haplotype diversity, π = mean nucleotide diversity.

Population	N	K	H	π	Tajima's D	Fu's <i>fs</i>
Gulf St. Vincent	21	9	0.88	0.06	1.54	2.88
Port Lincoln	15	9	1.0	0.02	-0.57	0.43
Western Port	8	4	0.83	0.05	0.95	0.73
Bruny Island	4	4	1.0	0.01	-0.79	2.59

Table 3.3. Analysis of molecular variance (AMOVA) among all four populations of *N. margaritacea* based on COI data.

Source of Variation	<i>df</i>	Sum of squares	Variance components	Percent of variation	<i>P</i> value
Among population	3	44.93	1.837	40.37	0.00
Within population	25	76.53	2.714	59.63	0.00

Table 3.4. Pairwise Φ_{st} among four populations of *Neotrigonia margaritacea*, based on COI data. Numbers in bold are significant values ($p < 0.05$).

Population	Gulf St. Vincent	Pt Lincoln	Western Port	Bruny Island
Gulf St. Vincent	0			
Port Lincoln	0.44	0		
Western Port	0.36	0.29	0	
Bruny Island	0.50	0.33	0.42	0

Figure 3.1

Map of Australia showing the locations of *Neotrigonia* collection, as well as the currents and marine provinces around southern Australia (inset) following Waters and Roy (2004), where abbreviations ZC represents the Zeehan Current, LC = Lewin Current and EAC = Eastern Australian Current. The single *Neotrigonia lamarcki* population from North Stradbroke Island is represented by a solid square, and populations of *Neotrigonia margaritacea* by solid circles for Port Lincoln, Gulf St. Vincent, Western Port and Bruny Island.

Figure 3.2

Strict consensus of four most parsimonious trees (length 351) from COI data for *Neotrigonia margaritacea* and *Neotrigonia lamarckii*. Tree terminals correspond to the two species from the following localities; Gulf St. Vincent, Bruny Island, Port Lincoln, Western Port and North Stradbroke Island. Maximum parsimony (MP) bootstrap values are shown above the line. Shaded terminals indicate specimens previously identified as *Neotrigonia bednalli*.

Figure 3.3

Strict consensus of seven most parsimonious trees (length 835) from ITS molecular data for *Neotrigonia margaritacea* and *Neotrigonia lamarckii*. Tree terminals correspond to the two species from the following localities; Gulf St. Vincent, Bruny Island, Port Lincoln, Western Port and North Stradbroke Island. Maximum parsimony (MP) bootstrap values are shown above the line. Shaded terminals indicate specimens previously identified as *Neotrigonia bednalli*.

Figure 3.4

Bayesian consensus tree using combined COI and ITS1 data for *Neotrigonia margaritacea* and *N. lamarckii* generated in MrBayes V3.1.2 (Huelsenbeck and Ronquist 2001). Tree terminals correspond to the two species from the following localities; Gulf St. Vincent, Bruny Island, Port Lincoln, Western Port and North Stradbroke Island. Values for posterior probabilities above the line and maximum parsimony (MP) bootstrap below the line. Shaded terminals indicate specimens previously identified as *Neotrigonia bednalli*.

Figure 3.5

Haplotype network of *N. margaritacea* COI data produced at the 95% confidence limit. Empty circles represent unsampled or possibly extinct haplotypes. The rectangle indicates a presumed ancestral haplotype, while large ovals represent two haplotypes and small ovals represent one haplotype. Haplotypes are grouped by a dashed line into their corresponding localities.

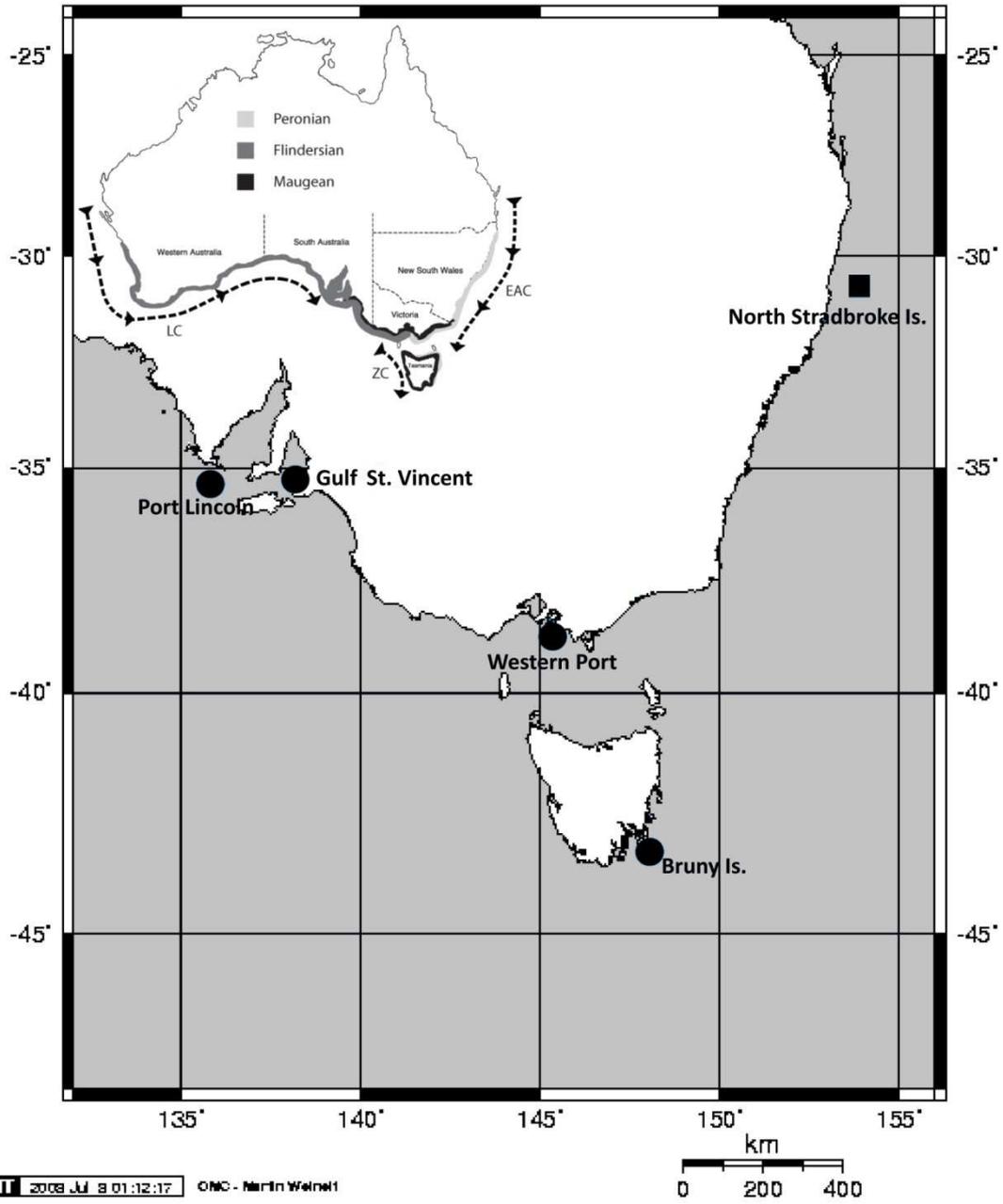


Fig. 3.1

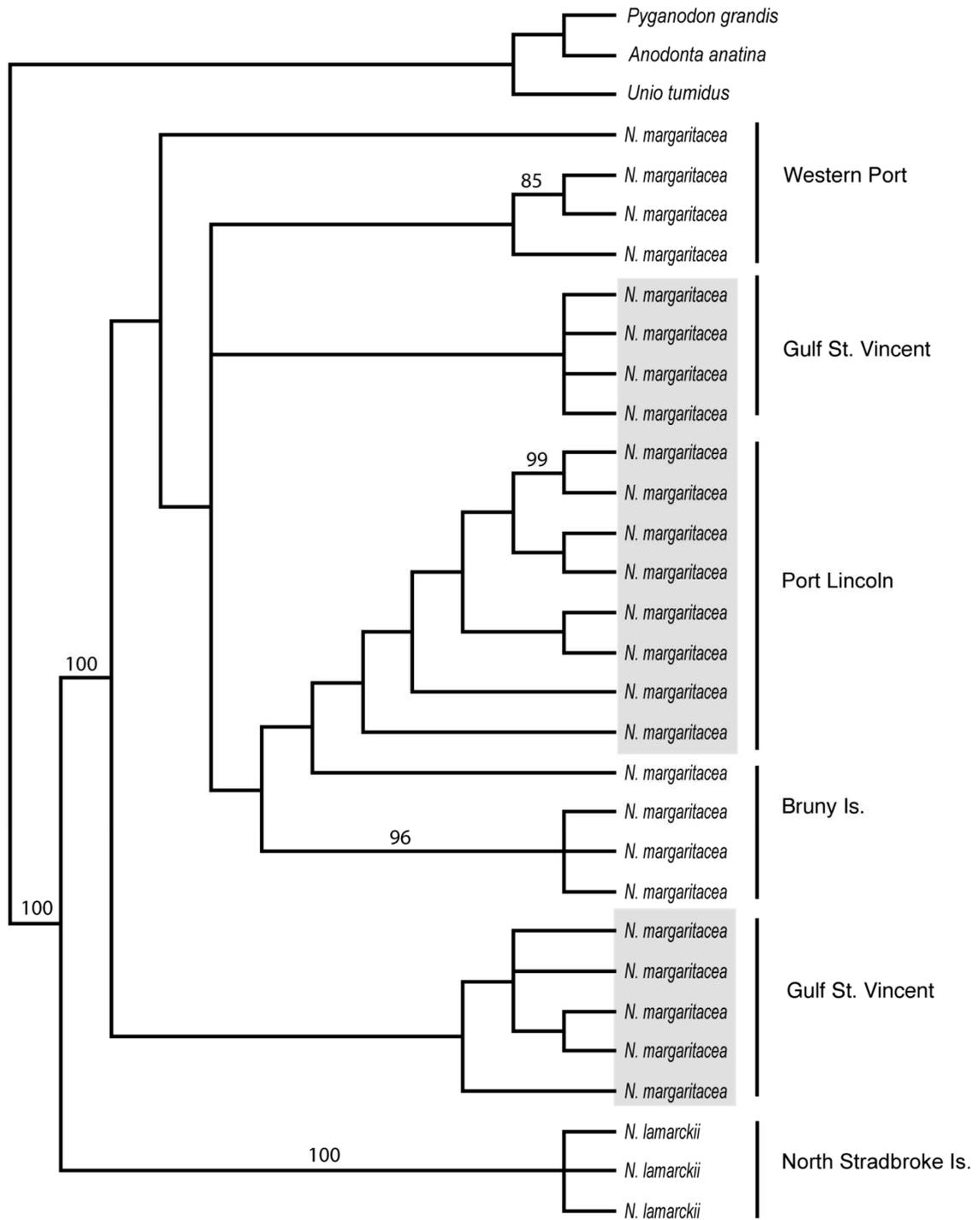


Fig. 3.2

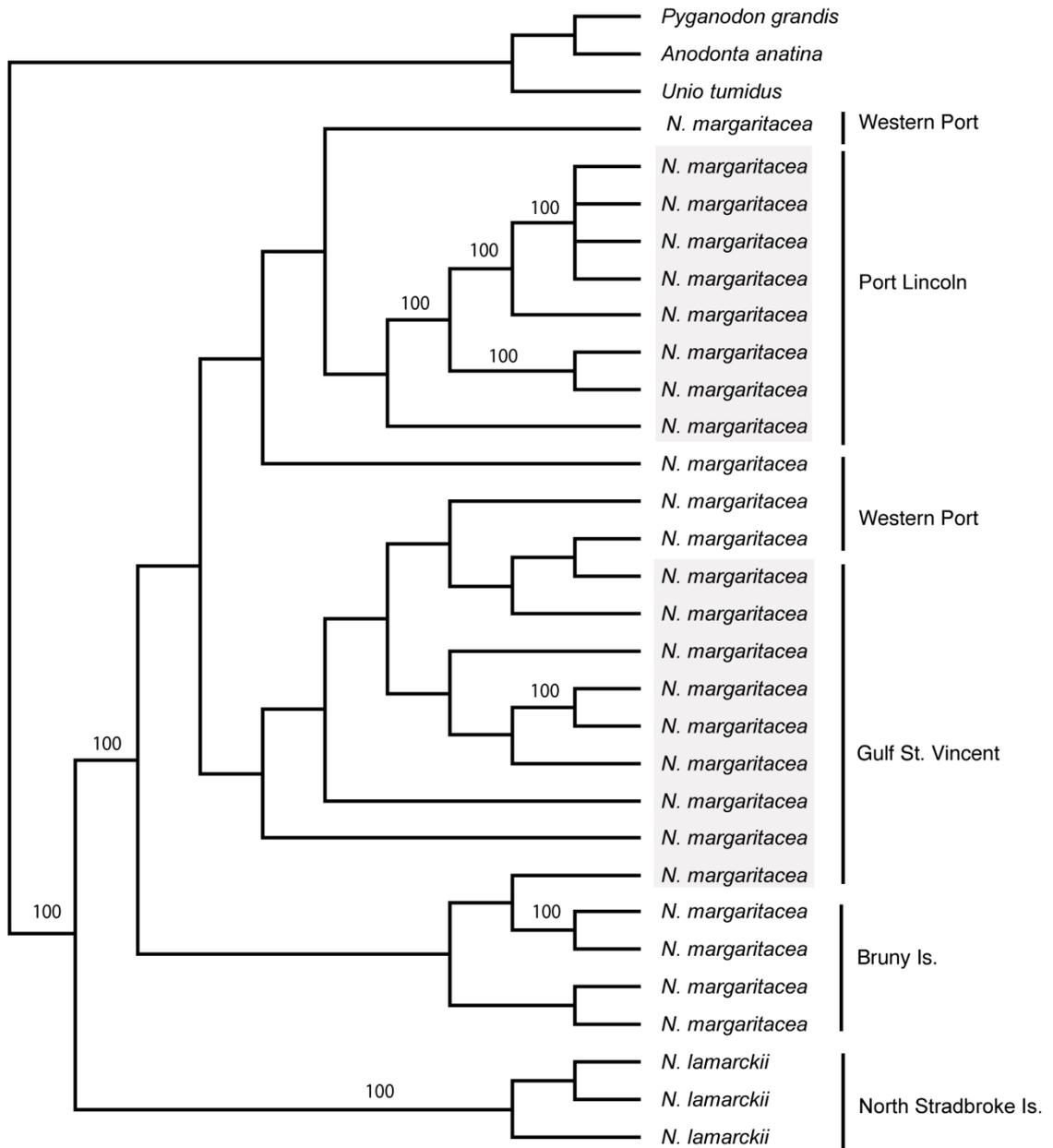


Fig. 3.3

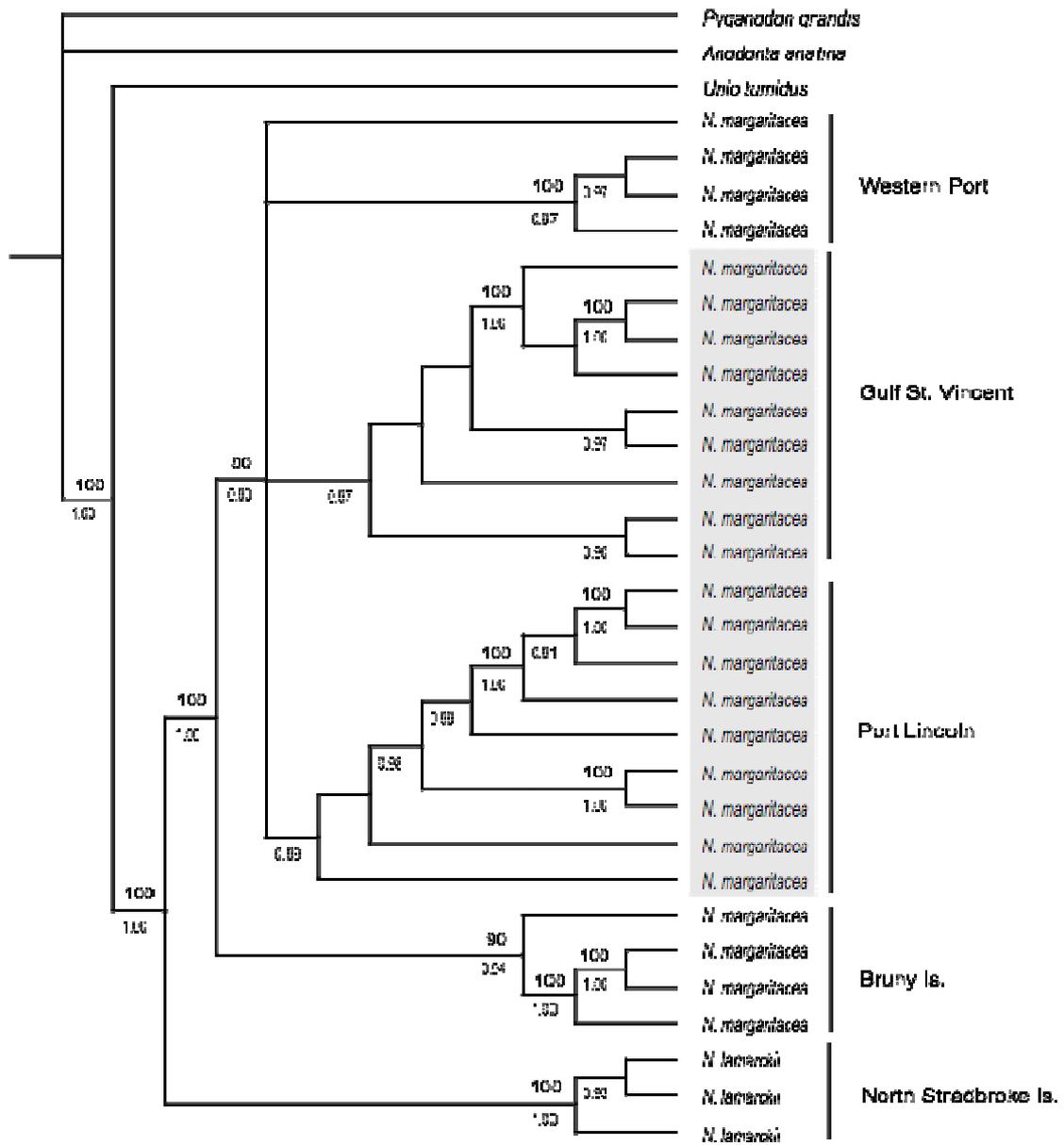


Fig. 3.4

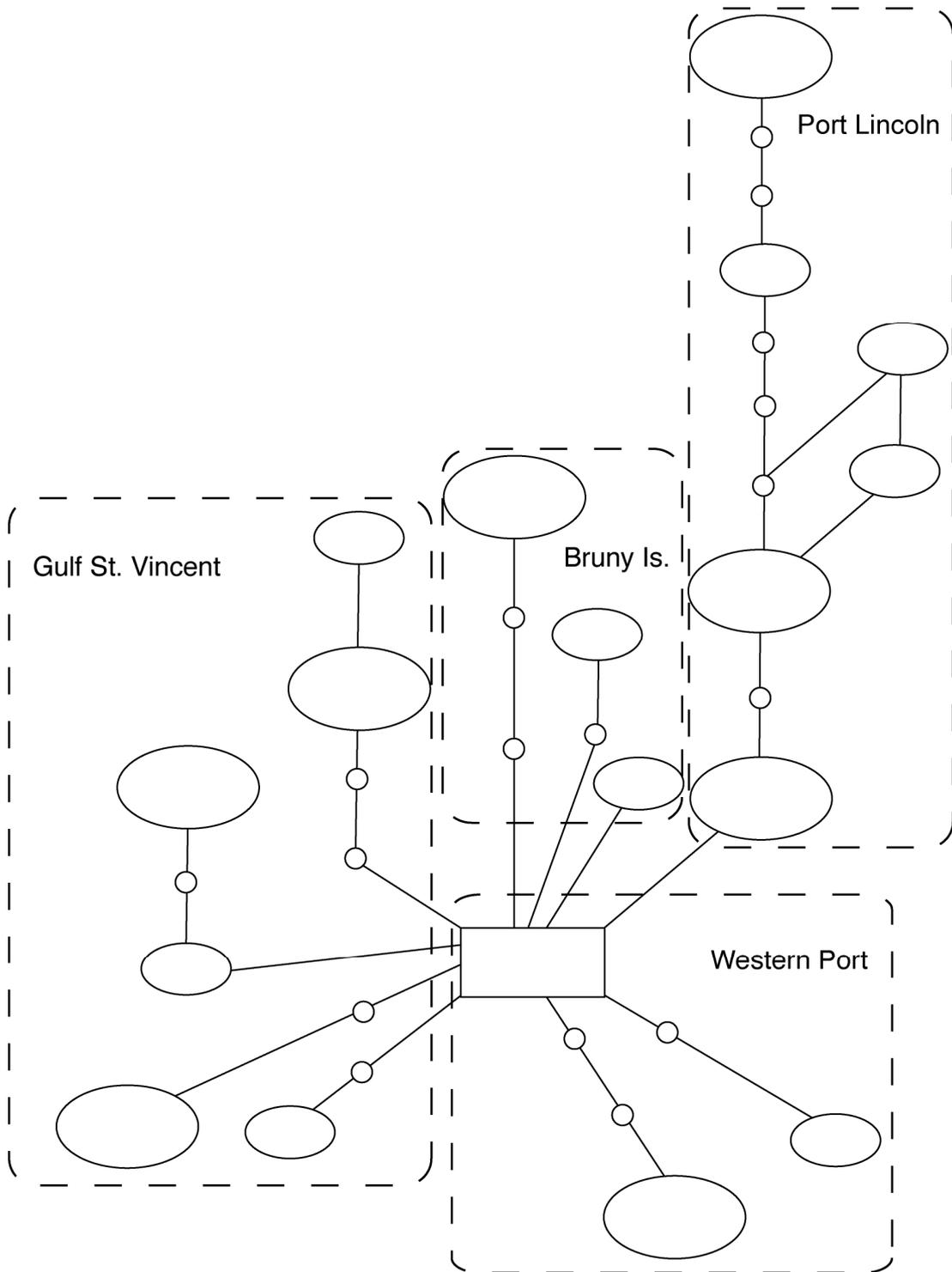


Fig. 3.5

**Doubly Uniparental Inheritance (DUI) of
mitochondrial DNA in *Neotrigonia margaritacea*
(Bivalvia: Palaeoheterodonta) and its evolution
in Bivalvia**

Ana Glavinic

Flinders University, Adelaide, PO Box 2100, Adelaide, SA 5001, Australia.

Author for correspondence: ana.glavinic@flinders.edu.au, telephone +61 8 82015747,
facsimile +61 8201 3015

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transmission route, mitochondrial DNA, Bivalvia, *Neotrigonia*, origin of DUI

Abstract

Several families of bivalves have been reported to contain two mitochondrial DNA types, maternal and paternal, which are contained in different body tissues. These two mitochondrial genomes are inherited separately, and this system of mtDNA inheritance is known as doubly uniparental inheritance (DUI). Here the presence of DUI is reported for *Neotrigonia margaritacea* (Lamarck, 1804), using mitochondrial DNA from Cytochrome C Oxidase I (COI) and 16S ribosomal DNA genes (16S rDNA). Male and female mitotypes, from gonad and somatic tissue, were identified for both COI and 16S rDNA within each of the males that were sequenced. The low divergences between the male and female mitotypes indicated either a recent acquisition of DUI in *Neotrigonia*, or a recent masculinization event. To elucidate this further, the data was analysed in a phylogenetic framework by adding terminals from its extant sister group, Unionoida, which also show DUI. Congruent with previous results, female *Neotrigonia* COI mitotypes formed the sister group to female unionid COI mitotypes, suggesting that no masculinization events (where the reversals in the route of mitotype transmission resulting in reset divergences of F and M mitotypes) have occurred in unionids since their divergence from *Neotrigonia*. The addition of male *Neotrigonia* COI mitotypes showed that they were nested among the female *Neotrigonia* mitotypes. Interestingly, analyses with a *N. lamarckii* (Gray, 1838) mitotype, derived from somatic tissue, showed that the two *Neotrigonia* species did not group according to gender. Rather, the *Neotrigonia lamarckii* mitotype was sister to all M and F mitotypes of *N. margaritacea*. If the homology of DUI between *Neotrigonia* and Unionoida is accepted then this lack of gender-affiliation within *Neotrigonia* suggests a recent masculinization event within *Neotrigonia margaritacea*. The analysis of 16S rDNA sequence data showed that male and female mitotypes of *Neotrigonia* were very similar sequences, though males did show two different mitotypes. The phylogenetic analysis of this data differed from the COI result in that Unionoida male and female mitotypes formed a clade that was sister group to the *Neotrigonia*. This result would suggest that DUI in Unionoida could have appeared after the split with *Neotrigonia* or that the rate of 16S rDNA evolution has been much slower than COI. When the presence or absence of DUI was mapped onto a phylogeny of all bivalves, the most parsimonious transformation suggests that DUI is the ancestral state for all Bivalvia but has been lost on several occasions, though the amount of missing data means that much further investigation is required.

Introduction

Animals transmit their mitochondrial genome predominantly through the maternal lineage (Hayashi *et al.*, 1978; Birky, 1995). However, various forms of paternal mtDNA transmission are known in several invertebrate and vertebrate species (Korpelainen, 2004). Within the Mollusca, biparental transmission of mtDNA, known as doubly uniparental inheritance (DUI) (Zouros *et al.*, 1994a), has been reported in numerous bivalves, mainly in Unionoida, including species in Unionoidae (Liu *et al.*, 1996; Hoeh *et al.*, 1996, 2002; Serb and Lydeard, 2003), Margaritiferidae and Hyriidae (Hoeh *et al.*, 1996; Curole and Kocher, 2002), but also within Veneroida (Passamonti and Scali, 2001), Mytiloida (Fisher and Skibinski, 1990; Skibinski *et al.*, 1994a,b; Zouros *et al.*, 1994a,b; Passamonti, 2007), and Solenidae and Donacidae (Theologidis *et al.*, 2008). In these species, two types of mtDNA exist. One type of mtDNA is transmitted via the eggs to both female and male offspring. This mtDNA is known as the maternal or type F mitotype. The other type of mtDNA is transmitted through the sperm only in male offspring, and is known as the paternal or type M mitotype (Zouros, 2000). The males are thus heteroplasmic, where the type F mitotype predominates in the somatic tissue and the M mitotype is restricted to the gonads (Stewart *et al.*, 1995; Sutherland *et al.*, 1998). There have been reports of traces of the paternal mitotype in the somatic female and male tissues (Stewart *et al.*, 1995; Garrido-Ramos *et al.*, 1998; Dalziel and Stewart, 2002), as well as in eggs (Obata *et al.*, 2006). However, the sperm has been reported to be free of the male's maternal mitotype (Venetis *et al.*, 2006). DUI is a phenomenon so far only detected in bivalves, although not all bivalves have DUI and many more remain to be assessed. So far, absence of DUI has been reported for species within the Arcidae: *Arca noae* (Theologidis *et al.*, 2008), Ostreidae: *Crassostrea virginica* and *C. gigas* (Obata *et al.*, 2008), Veneridae: *Venus verrucosa* and *Callista chione* (Theologidis *et al.*, 2008), and in Unionoida: Etheriidae (Walker *et al.*, 2006), although these latter two families predominantly have DUI (Hoeh *et al.*, 1996, 2002).

DUI should be easy to detect as it generates two distinct mtDNA lineages with different distributions in female and male tissues (Zouros *et al.*, 1994a; Theologidis *et al.*, 2008). Amongst the mytiloid bivalves, the average divergence between the paternal and maternal lineage is 20% for p-distances based on the partial sequence for Cytochrome C Oxidase subunit I gene (COI) (Mizi *et al.*, 2005), whereas for unionoid bivalves divergence values can be as high as 50% (Ladoukakis and Zouros, 2001;

Breton *et al.*, 2009). The sequences for the same genes in unionids were so different between sexes that DNA primers would recognise only sequences from the genomes of one sex and failed to amplify the other (Ladoukakis and Zouros, 2001). However, divergence values between the sexes do not necessarily have to be so high. *Mytilus galloprovincialis* (Ladoukakis and Zouros, 2001) and *Mytilus trossulus* (Burzynski *et al.*, 2003) have divergence ranges between 2 and 20%. A reason behind this lower divergence value may be homogenisation of the two genome lineages through recombination (Burzynski *et al.*, 2003). Recent investigation into DUI by Doucet-Beaupre *et al.*, (2010) revealed that gene content and gene order vary between the complete M and F genomes among the Unionoida bivalves.

Another reason why the amount of divergence can be so variable between the gender-associated mtDNA within a male is due to the occurrence of "masculinization" events (Hoeh *et al.*, 1996, 1997) or "role reversal" (Quesada *et al.*, 1999). These events occur when a gender-associated mitochondrial lineage is lost and replaced by the opposite gender lineage. Masculinization occurs when the original M-mitotype mtDNA lineage is replaced by F-mitotype mtDNA, but is subsequently only inherited through males (Hoeh *et al.*, 1997; Quesada *et al.*, 1999). This resets the 'divergence clock' between the two lineages, and the divergence is expected to increase over time with further accumulation of mutations. Unlike in *Mytilus* species, there is no evidence for recent DUI masculinization events occurring in unionoids because the amount of divergence between M and F type mtDNA is very high, ranging from 28% to 50% for COI (Hoeh *et al.*, 1996, Doucet-Beaupre *et al.*, 2010).

An interesting consequence of DUI is that the F and M genomes from different taxa may form distinct clades based on sex rather than taxa, if DUI pre-dated the speciation events of the examined taxa (Theologidis *et al.*, 2008). This has been shown in closely-related species such as *Mytilus edulis* and *Mytilus trossulus* (Rawson and Hilbish, 1995; Stewart *et al.*, 1995) and similar results have been reported within the Unionoida (Hoeh *et al.*, 2001; Curole and Kocher, 2005). In unionoid bivalves there is an exclusive M-mitotype extension in the Cytochrome C Oxidase subunit II gene (MCox2e), and a novel localisation of tRNA histidine (TrnH) in the M genome (Doucet-Beaupre *et al.*, 2010). This distinguishes the unionid M genome not only from the unionoid F genome, but from all other F genomes in bivalves that possess DUI (Doucet-Beaupre *et al.*, 2010). When different bivalve families, mytilids, venerids and unionids, were analysed together, the F and M mitotypes of each family

cluster together, away from genomes of other families, leading to suggestions that DUI emerged independently in each family (Hoeh *et al.*, 1996; Mizi *et al.*, 2005). However, masculinization events provide an alternative explanation for this result.

Given the relatively basal position of Unionoida within bivalve phylogeny (Giribet and Wheeler, 2002; Dryer and Steiner, 2006), Doucet-Beaupre *et al.*, (2010) reported that the DUI characteristics observed in unionoid bivalves could resemble the DUI ancestral condition. Although the origins of DUI are still not known, Hoeh *et al.*, (2002) predicted that this phenomenon has been operating in bivalves for over 200 million years. This was based on the inclusion of a single, presumably F mitotype COI sequence of *Neotrigonia margaritacea* in a phylogeny, which showed this sequence to be the sister to the F mitotype clade of the freshwater Unionoida. *Neotrigonia* is a marine “relic” genus in Trigoniodea, which forms the sister group to freshwater Unionoida together forming the monophyletic subclass Palaeoheterodonta (Hoeh *et al.*, 1998; Giribet and Wheeler, 2002; Dryer and Steiner, 2006). Despite inclusion of only somatic DNA as the probable F mitotype for *Neotrigonia*, Hoeh *et al.*, (2002) predicted that DUI has been present in Palaeoheterodonta prior to the origin of freshwater unionids. For this chapter, the possibility of DUI operating in *Neotrigonia margaritacea* was assessed by sequencing mtDNA (COI and 16S) extracted from gonad tissue of males and females. The results of these analyses are then combined with previously published information on the absence and presence of DUI within bivalves and used to examine the origin of DUI with respect to the Palaeoheterodonta clade and further within Bivalvia.

Methods

DNA extraction, amplification and sequencing

Twenty-two specimens of *Neotrigonia margaritacea* were collected from subtidal depths exceeding 12 metres from Gulf St. Vincent, South Australia, and preserved in 70% ethanol. Gender was determined by microscopic inspection of gonad tissue. Of the 22 specimens (labelled SW samples in Table 4.1), seven were male and fifteen were female. Seven male specimens were sequenced using separate DNA extractions on gonad and somatic tissue to obtain male and female mitotypes of Cytochrome C Oxidase I (COI) and two out of seven males were sequenced for 16S ribosomal DNA genes (16S rDNA). In addition, newly-obtained sequences were combined with all other available *Neotrigonia* somatic tissue-derived COI sequences,

in total 18 *Neotrigonia margaritacea* (including Genbank samples) and three *Neotrigonia lamarckii* specimens (Table 4.1). These genes were specifically chosen to allow the broadest possible comparison using existing datasets on species known to either have DUI or lack it.

Total genomic DNA was extracted according to the Qiagen DNeasy (Qiagen, USA) protocol for animal tissues. The DNA was isolated using spinfilter followed by ethanol precipitation. The genomic DNA was then cleaned using the Cetyl Trimethyl Ammonium Bromide (CTAB) method (Scouras and Smith, 2001) and the samples stored at -80°C. A 590 base pair (bp) fragment of COI and 425 bp of 16S rDNA were amplified using the primers CO1490-L and CO2198-H (Folmer *et al.*, 1994) for COI and 16Sar-L (Kocher *et al.*, 1989) and 16Sbr-H (Palumbi *et al.*, 1996) for 16S rDNA. Polymerase chain reaction (PCR) was carried out using a Corbett FTS-320 thermal sequencer using 0.2 µL taqGold (5 units/µl), 2 µL per primer (5µM), 4 µL DNTP's (Deoxynucleotide Triphosphate) (10 µM), 8 µL MgCl₂ (25 µM), 5 µL TGold® (Applied Biosystems), buffer, 5 µL gDNA and 23.8 µL sterile water in a 50µL reaction. Denaturation was carried out at 94 °C for 45 seconds, with annealing temperature of 48 °C for COI and 46.5 °C for 16S rDNA applied for 45 seconds and extension at 72 °C for 60 seconds. This was repeated for 35 cycles.

PCR products were cleaned using MoBio spin clean kit (MoBio, USA). If more than one product was present, the desired product was isolated using agarose gel (1.5%) electrophoresis and cleaned using the Qiagen QIAquick gel extraction kit (Qiagen, USA). PCR products were amplified for sequencing using a Hybaid Omn-E thermal cycler (Hybaid Limited, USA), 5µL of PCR product, 1 µL of primer (5µM) and 4 µL Big Dye Version 3 were combined in a 10µL reaction. The reaction was completed at a denaturation temperature of 96 °C for 30 seconds and annealing temperature of 50 °C for 15 seconds and an extension temperature of 60 °C for 4 minutes, repeated for 25 cycles. The product was cleaned using 70% isopropanol and sequenced at the Institute of Medical and Veterinary Science, Adelaide, SA, using an automated sequencer 3730 (Applied Biosystems). Sequenced data was edited using seqEd v1.0.3. Sequences were aligned using MacClade version 4 (Maddison and Maddison, 2000) and MAFFT (Kato *et al.*, 2009). Mitotypes were inferred using ARLEQUIN 2.0 (Schneider *et al.*, 2000) and TCS 1.21 (Clement *et al.*, 2000).

Analyses

Female and male mitotypes of *Neotrigonia margaritacea* were combined with all other additional published sequences of bivalve species known to possess DUI (Table 4.1). In addition we used outgroups as chosen by Hoeh *et al.*, (2002) and Theologidis *et al.*, (2008) due to their taxonomic appropriateness and low chance that they would have DUI (Table 4.2).

To evaluate the level of divergence between the datasets derived from the female and male sequences for COI and 16S rDNA in *Neotrigonia margaritacea*, the pairwise uncorrected p-distances were determined for each mitotype sequence. The two groups were compared with using analysis of molecular variance (AMOVA), to test whether variability within male mitotype and female mitotype sequence is significantly different. Because molecular data are not normally distributed, significance was tested by permutating the data. TCS 1.21 (Clement *et al.*, 2000) was also used to construct statistical parsimony networks at the 95% connection limit based on the COI gene. This method is designed to provide representations of gene genealogies at the population level (Templeton *et al.*, 1992) and here it is used to identify the ancestral mitotype for all male and female mitotypes.

As well as incorporating new data for *Neotrigonia*, the effects of outgroup choice and 3rd position saturation in COI on previous analyses of DUI in Paleoheterodonta (Unionoida + Trigonoida) were assessed. It has been suggested by Hoeh *et al.*, (1998) that third-position transversions are saturated for bivalves at this level of divergence. Saturation can exacerbate problems for older divergence and for analyses that cover a wide range of hierarchical levels (Whitfield and Cameron, 1998; Schwarz *et al.*, 2004). Also, heterogeneity in base composition among taxa can cause problems in phylogenetic analysis if they do not reflect shared ancestry, leading to spurious attraction between taxa with similar compositional bias (Lockhart *et al.*, 1994). The third position for COI was recoded (as RY) by Hoeh *et al.*, (1997,1998, 2002) and this was also followed here to compare the effect against the normally coded dataset. The outgroup choice of the insect *Drosophila* and other non-bivalve molluscs by Hoeh *et al.*, (2002) was also assessed here by using outgroups more closely related to Paleoheterodonta.

Analyses of dataset 1

Maximum Parsimony (MP) analyses were conducted using COI nucleotides, and using the same outgroups as in Hoeh *et al.*, (2002) (Table 4.2), excluding the snail *Albinaria turrita*, which had a very long branch. PAUP* 40b10 (Swofford, 2003) was used with default settings, except for stepwise addition using 100 random addition sequences. Jackknife support values (37% deletion) were assessed in PAUP* using 100 replicates of 100 random addition sequences. Bayesian analyses on the same data set was performed with MrBayes 3.1 (Ronquist and Huelsenbeck, 2003), with COI data organised in three unlinked partitions according to codon position, and applying the general time reversible model (GTR+I+ Γ) chosen based on the Akaike Information Criterion AIC (see Posada and Buckeley, 2004) in MrModeltest 2.2 (Nylander, 2002). All Bayesian analyses were run with default priors as follows: rate matrix: 0-100; branch lengths: 0-10; Gamma shape: 0-1. Six Markov chains were started from a random tree and all chains ran simultaneously with a tree saved every 1000 generations for 10,000,000 generations. The first million trees were discarded as burn-in, chosen after examination of the log likelihood plots to see when stationarity was reached using Tracer v1.4 (Rambaut and Drummond, 2007). The majority rule consensus tree of the last 9001 trees for each analysis gave the posterior probabilities for each clade.

Analysis of dataset 2

COI dataset saturation in the third position was assessed using the test by Xia (2003) implemented in DAMBE, and found it to be significantly saturated with respect to outgroups. Examination of a plot of transitions and transversions against a corrected genetic distance in DAMBE suggested that the saturation would be mostly with transitions. The dataset was then recoded using RY coding in Mesquite (Maddison and Maddison, 2009), so that only transversions were coded at the third position and so potentially eliminating a transition bias. A MP analysis were repeated as above on the RY data set.

Analyses of dataset 3

Further data exploration was performed by removing the distant outgroups: *Drosophila yakuba*, *Lepetodrilus elevatus*, *Dentalium sp.* and *Katharina sp.* (Table 4.2), while the bivalves *Solemya velum* and *Musculista senhousia* (male and female

sequences) were retained as outgroups (Table 4.2) based on the phylogeny of Giribet and Wheeler (2002). The MP and Bayesian analyses were again repeated, and the MP analyses were repeated with both the new outgroup strategy and RY coding.

Analyses of 16S rDNA data

MP and Bayesian analyses were also conducted for 16S rDNA data. The 16S rDNA dataset contained new sequences for *Neotrigonia margaritacea*, sequences used in Theologidis *et al.*, (2008) for all available bivalve with DUI and the outgroup sequences following Hoeh *et al.*, (2002). The Unionoida plus *Neotrigonia* 16S rDNA sequences were also analysed using *Solemya velum* and *Musculista senhousia* as outgroups.

Analysis of the origin of DUI

To trace the origin of DUI in Bivalvia, DUI was scored as either present (1), absent (0) or unknown (?), based on references in Theologidis *et al.*, (2008) and Doucet-Beaupre *et al.*, (2010) and the results found here for *Neotrigonia*. This character was traced in MacClade v4 (Maddison and Maddison, 2000) onto the phylogeny of Bivalvia generated by Giribet and Wheeler (2002). Known absences of DUI are critical to this analysis and so these are listed here: *Arca noae*, *Venus verrucosa*, *Callista chione* (Theologidis *et al.*, 2008), *Crassostrea virginica*, *C. gigas* (Obata *et al.*, 2008) and Etheriidae (Walker *et al.*, 2006).

Results

Sequences for two genes, COI, 590 nucleotides in length and 16S rDNA, 425 nucleotides in length, were obtained for 22 *N. margaritacea*. Out of 15 female specimens, there were four COI mitotypes (Table 4.1). Two different COI sequences were identified in each of the seven male specimens sequenced, one type from gonad and the other from somatic tissue (Table 4.1). The COI sequence from somatic tissue of the four male specimens corresponded with two of the female mitotypes. Three unique “male” mitotypes were obtained from the seven male gonad samples. A level of divergence, based on uncorrected p-distances, was found between mtDNA COI sequence data from somatic tissue and testis (F and M types), with divergence between male and female mitotypes at 2.1%. The variability of COI sequence was greater among the male gonad mitotypes than the somatic mitotypes of *Neotrigonia margaritacea*. Values of sequence divergence evaluated by uncorrected p-distance

were between 0.0013 and 0.032 in F mitotype and between 0.0061 and 0.0044 in M mitotype sequence. AMOVA confirmed that variability within male mitotype and female mitotype sequence was significantly different ($P=0$, where $\alpha=0.05$) (Table 4.3). Results from TCS resolved these mitotypes into a single star-like network, within the 95% limit of parsimony reconnection (Fig. 4.1). Mitotype B was inferred as the ancestral mitotype to all male and female mitotypes (Fig. 4.1). An uncorrected COI p -distance of 0.11 between *N. margaritacea* and *N. lamarckii* was recorded.

Two female 16S rDNA mitotypes were obtained from all female specimens, these corresponded to two sequences available on GenBank (DQ093489, DQ280034). Two male 16S rDNA mitotypes were obtained from male gonad samples. A low divergence between male and female mitotypes was evaluated at 0.072%, based on uncorrected p -distances (3 base differences). Non-significant variability within male type and female mitotype 16S rDNA sequence was confirmed by AMOVA ($P=0.13$, where significance was $\alpha=0.05$) (Table 4.3).

Analyses of dataset 1

MP analysis of 702 bp of aligned COI data, with all codon positions included, (387 parsimony informative sites) produced 2 shortest trees of 3418 steps (Consistency Index CI= 0.92, and Rescaled Consistency RC=0.81 for informative characters only). The tree in Figure 4.2 shows that both male and female sequences of *N. margaritacea* form a clade that is the sister group to the *N. lamarckii* sequence. The *Neotrigonia* clade was then sister group to the female-type Unionoidea, with the male Unionoidea clade as the sister group to this assemblage (Fig. 4.2). The Bayesian analysis of the same dataset gave congruent results (Fig. 4.2).

Analysis of dataset 2

MP analysis of the same COI data set, now RY coded (transitions excluded), contained 332 parsimony informative sites. The strict consensus tree was produced from 12984 trees of 1885 steps (Consistency Index CI= 0.89, and Rescaled Consistency RC=0.90 for informative characters only) (Appendix III). The tree topology indicated that male unionoid mitotypes form a well-supported clade, sister to a clade containing the *N. margaritacea* and *N. lamarckii* and female unionoid mitotypes. Within *Neotrigonia*, *N. lamarckii* is a sister to both M and F mitotypes of *N. margaritacea*. This result was congruent with both the maximum parsimony and

the Bayesian analyses of dataset 1 (Fig 4.2) where the third position transitions were not excluded.

Analyses of dataset 3

With *Solemya velum* and *Musculista senhousia* as outgroups for the COI dataset there were 528 parsimony informative sites and the MP analysis produced 6510 shortest trees of 2082 steps (Fig. 4.3). The additional informative sites when compared with the more inclusive outgroups appears to be due to the divergent *Musculista senhousia* sequences (Fig.4.3). As with the analysis 1 and 2 the results of both the MP and Bayesian analyses showed that male unionoid mitotypes form a well-supported clade that was sister to a clade containing *N. margaritacea* (male and female mitotypes) and *N. lamarckii* and the female unionoid mitotypes.

Analyses 16S rDNA data

Both the analyses with more distant outgroups and all bivalves with DUI as well as the more restricted analysis of Paleoheterodonta showed a similar result that differed from the COI result. In each case (Fig. 4.4) the male and female mitotypes of Unionoida formed a well supported clade that was sister group to *Neotrigonia*. The tree topology produced by MP analysis of 581bp of aligned 16S rDNA data, (287 parsimony informative sites) produced 15 shortest trees of 1066 steps (Consistency Index CI= 0.58, and Rescaled Consistency RC=0.70 for informative characters only) (Fig. 4.4). Bayesian analysis produced the same topology as the MP analysis (Fig.4 and Appendix III).

Analysis of the origin of DUI

The result showing that DUI occurs in *Neotrigonia* allowed for further assessment of the origin and evolution of DUI in Bivalvia. Mapping the presence or absence of DUI onto the phylogeny of Bivalvia generated by Giribet and Wheeler (2002) suggested that, under a maximum parsimony criterion, DUI is plesiomorphic for Bivalvia with several losses, although the large amount of missing data means that this result must be viewed with caution (Fig.4.5).

Discussion

The results here provide the first evidence for the presence of DUI in *Neotrigonia*. Based on their being different mitochondrial gene sequence in gonad and somatic tissue respectively, of male *Neotrigonia margaritacea*. The observed tissue specific distribution of male and female mitochondrial sequences parallels that predicted by a doubly uniparental mechanism of mitochondrial inheritance (Skibinski *et al.*, 1994a, Zouros *et al.*, 1994a).

The sequence divergence between male and female mitotype COI sequences in *Neotrigonia margaritacea* is only 2.1%, which is much lower than reported for unionoids, where divergence between M and F type mtDNA ranged from 28% to 33% (Hoeh *et al.*, 1996). However this low divergence between *Neotrigonia margaritacea* sexes is similar to that of *Mytilus galloprovincialis* (Ladoukakis and Zouros, 2001) and the European *M. trossellus* (Burzynski *et al.*, 2003). It has been shown that such close similarity between M and F mitotype sequences corresponds to a masculinization event, where male genomes that have been paternally inherited, but have recently arisen from female mitotypes (Quesada *et al.*, 1999). The strong evidence in support of a masculinisation event in *Neotrigonia margaritacea* is shown in the phylogenetic affinity of male mitotype to female mitotype (Fig. 4.2 and 4.3). This is also supported by the mitotype network for *N. margaritacea*, which shows that the male mitotypes are connected to a female mitotype B (Fig. 4.1), which can be inferred as the ancestral mitotype to all M mitotypes, therefore suggesting that M mitotypes arose relatively recently. However, since it is unknown if the replacement of a former male lineage by a newly-masculinized lineage is a random event, we are unsure if sequence divergence between the F and M mitotypes in COI of *N. margaritacea* could potentially increase as the masculinization event progresses through to the final stage. As suggested by Hoeh *et al.*, (1997), the old M lineage could be totally replaced by the new M mitotype.

If we now accept the occurrence of DUI in *Neotrigonia margaritacea* and recognize the complications associated with detecting masculinisation events, we can consider the variability of COI sequence between male and female mitotype. Several authors have made the observation that the M genome evolves faster than the F (Skibinski *et al.*, 1994b; Rawson and Hilbish, 1995; Stewart *et al.*, 1995; Hoeh *et al.*, 1996). Consistent with this, the variability of the COI sequence was found to be greater in male-type than female-type *Neotrigonia margaritacea*, suggesting an

overall higher turnover rate for the M genome. This result is comparable to the study on *Mytilus* COIII data (Stewart *et al.*, 1995), which established that the M lineage evolves faster than the F lineage due to relaxed selection against the M type.

However, a more noteworthy aspect of the COI data in regards to DUI origin is revealed through phylogenetic analyses. The position of the genus *Neotrigonia* as a sister clade to a grade of Unionoidea F mitotype sequences is shown in Figures 4.2 and 4.3. It was necessary to perform multiple phylogenetic analysis to establish an appropriate method, which in turn has accounted for underlying mutational mechanisms. For COI data MP trees were congruent with Bayesian trees (Appendix III) however, improved outgroup taxon sampling and RY coding lead to stronger support within the trees. The results from COI data set also show the Unionoidea male mitotype is more divergent than female. This result is expected considering a higher rate of nucleotide substitution in the unionoidean mitochondrial M genome (Hoeh *et al.*, 2002). This is consistent with the hypothesis of Hoeh *et al.*, (1998, 2002) and Walker *et al.*, (2006) that DUI has been operating in the Palaeoheterodonta prior to the trigonioid-unionoid divergence. Phylogenetic analysis shows that within *Neotrigonia*, the *N. lamarckii* mitotype derived from somatic tissue is a sister to all M and F mitotypes of *N. margaritacea*. The mitotypes from two *Neotrigonia* species thus do not affiliate according to gender, a pattern reported for *Mytilidae* genera (Hoeh *et al.*, 1997). This indicates that while it is arguable *Neotrigonia* species descended from an ancestral bivalve that had DUI, reversals in the route of mitotype transmission occur during masculinization events, so a recent origin of DUI (i.e., divergence of F and M mitotypes) can be inferred for *Neotrigonia margaritacea*. However, it is critical that both gonad and somatic DNA are analysed for *N. lamarckii* male and female specimens.

Conversely, the 16S rDNA analysis (Fig. 4.4) suggests that DUI in Unionoidea could have appeared after the split with *Neotrigonia*, or that the rate of 16S rDNA divergence has been much slower than for COI gene. The mitochondrial genome contains a number of highly conserved genes. One of the most conserved is that of the mitochondrial 16S rDNA gene (Neefs *et al.*, 1990; Wang *et al.*, 1999). Therefore low variation between the M and F type 16S rDNA sequences of *N. margaritacea* can be explained by the fact that this gene is inherited independently from the nuclear rRNA genes (Garey *et al.*, 1998). Both male and female mitochondrial genomes can be expressed in male gonads, making difficult the direct sequencing of PCR product.

This non-significant variation of a 3-bp difference between M and F mitotype sequence could also be attributed to low PCR success. It is not uncommon that universal primers fail to detect both male and female types. A related problem is contamination of the target tissue by one or the other genome (Theologidis *et al.*, 2008). However, DUI is a complex mechanism and, if we consider that mtDNA recombination occurs in bivalves (Ladoukakis and Zouros, 2001), then the possibility exists that the mtDNA molecule that dominates the gonad of the males is in fact the M type, as observed with COI sequence. The 16S rDNA region of the mtDNA may have incorporated an F-like sequence through recombination between an F and an M molecule. It is speculated that 16S rDNA of the two *N. margaritacea* males sampled here is of the F mitotype, yet in terms of function and mode of transmission, this sequence behaves like an M mitotype. Similar evidence was used by Hoeh *et al.*, (1996, 1997) to propose masculinisation, where a maternally-transmitted genome may revert into a paternally-transmitted one.

It is suspected that DUI has operated in unionoid genera for 100 my (Hoeh *et al.*, 1996) and Rawson and Hilbish (1995) estimated the time of the split between the most common M and F mitotypes in Mytilidae genera at 5.3 mya. If we consider the first occurrence of *Neotrigonia margaritacea* to have been in the Pliocene (~5mya) according to the fossil record (Darragh, 1986), then newly-masculinized *Neotrigonia margaritacea* M mitotypes could be even more recent. If newly-masculinized *Neotrigonia margaritacea* M mitotypes totally replaced the original M mitotype (Hoeh *et al.*, 1997), then a consequence of this process is that the detected origin of DUI within *Neotrigonia margaritacea* can be equated with the most recent masculinization event. The masculinization explanation would imply that there was enough time since the separation of venerids, solenids, donacids and mytilids for at least two masculinization events in the lineage leading to each of these groups (Theologidis *et al.*, 2008). Masculinization events have not occurred after the emergence of unionoids but, under the assumption that DUI arose once in an ancestral bivalve lineage, a masculinisation event must have occurred in Unionoidea prior to the split between M and F mitotypes (Hoeh *et al.*, 1996, 1997).

Including this study, there are now 37 bivalve species shown to have evidence of DUI. The 37 species belong to six superfamilies: Mytiloidea, Unionoidea, Tellinoidea, Solenoidea, Veneroidea (Skibinski *et al.*, 1994a,b; Zouros *et al.*, 1994a,b; Liu *et al.*, 1996; Hoeh *et al.*, 1996, 2002; Passamonti and Scali, 2001; Curole and

Kocher, 2002; Serb and Lydeard, 2003; Passamonti, 2007; Theologidis *et al.*, 2008) and now Trigonioidea. The results from the COI data confirm the prediction by Hoeh *et al.*, (2002) and Walker *et al.*, (2006) that DUI has been present in Palaeoheterodonta prior to the origin of freshwater unionids and that *Neotrigonia margaritacea* has DUI. The known distribution of DUI within bivalves (Fig. 4.5) suggests a single origin of DUI for the whole of Bivalvia. This is contrary to the hypothesis proposed by Theologidis *et al.*, (2008), who suggested that DUI arose multiple times, independently among distantly-related species. Independent evolution of DUI within the Bivalvia would require one origin for all of the Paleoheterodonta and one for each of the families Solenidae, Veneridae, Donacidae and Mytilidae, followed by the separate origin required for four mytilid genera (Rawson and Hilbish, 1995; Stewart *et al.*, 1995; Theologidis *et al.*, 2008). A single origin for DUI is more likely considering the complexity of the DUI phenomenon in regards to its linkage to sex inheritance and molecular intricacy (Zouros, 2000; Cogswell *et al.*, 2006). Therefore, if we were to assume that DUI has a single origin, then patchy occurrence of DUI within the class would imply loss of DUI in some lineages and retention in others, as is the case of Etheriidae (Walker *et al.*, 2006). It is a widespread assumption among biologists that a complex character, such as DUI, is more easily and frequently lost than gained (Budd and Jensen, 2000). Testing the single origin of DUI hypothesis will require further investigation of other species within Bivalvia to ascertain the frequency of DUI in more-recently evolved lineages and the consistency between species within a lineage.

In conclusion, the finding of gender associated mtDNA heteroplasmy in *Neotrigonia margaritacea* widens the distribution of DUI in the Bivalvia. Considering that a masculinization event is likely to have occurred in *Neotrigonia margaritacea*, the hypothesis of a single origin of DUI is favoured. However, it is acknowledged that this can only be confirmed by further work on additional taxa within Bivalvia. Recognition of DUI within *Neotrigonia* has implications for further phylogenetic and phylogeographic work, in particular haplotype-based studies will need to consider the sex of all specimens sampled. This study also revealed that not all genes corresponding to an M and an F type sequence diverge to the same degree, potentially masking the presence of DUI. Therefore the best approach is to analyse sequences using standard and specifically-designed primers for more than one gene.

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Table 4.1. Bivalve ingroup taxa with GeneBank (NCBI) accession numbers for COI and 16S rDNA sequences used. Sequences where sex is unknown and it is presumed that they were derived from somatic tissue DNA, are indicated by an asterisk (*).

Order	Family	Subfamily	Species	COI DNA F-mitotype	COI DNA M-mitotype	16s rDNA F-mitotype	16s rDNA M-mitotype	
Unionoida	Unionoidae	Hyriidae	<i>Hyridella menziesi</i>	AF231747	AF406802			
			<i>Hyridella depressa</i>	AF156496				
			<i>Alathyria jacksoni</i>	AY386977				
			<i>Castalia stevensi</i>	AF231736				
			<i>Diplodon deceptus</i>	AF231744				
			<i>Velesunio angasi</i>	AF231743				
			<i>Velesunio ambiguus</i>	AF305371				
			<i>Velesunio ambiguus</i>		AF231743			
			<i>Velesunio sp1</i>	AY387018				
			<i>Velesunio sp2</i>	AY386999				
			<i>Velesunio spA</i>	AY211550				
			<i>Velesunio spB</i>	AY211558				
			<i>Velesunio spD</i>	AY211587				
			Unioninae	<i>Inversidens japonensis</i>			AB055625	
				<i>Inversidens japonensis</i>				AB055624
			Ambleminae	<i>Fusconaia flava</i>	AF231733			
				<i>Fusconaia flava</i>		AF406799		
				<i>Fusconaia flava</i>			AY238481	
				<i>Fusconaia flava</i>				AY498702
				<i>Amblyma plicata</i>			APU72548	
		<i>Plectomerus dombeyanus</i>			AY655057			

Order	Family	Subfamily	Species	COI DNA F-mitotype	COI DNA M-mitotype	16s rDNA F-mitotype	16s rDNA M-mitotype
			<i>Quadrula quadrula</i>				FJ809751
			<i>Actinonaias ligamentina</i>	AF231730			
			<i>Actinonaias ligamentina</i>		AF406796		
			<i>Actinonaias ligamentina</i>			AY655027	
			<i>Cyrtonaias tampicoensis</i>	AF231749			
			<i>Cyrtonaias tampicoensis</i>		AF406798		
			<i>Cyrtonaias tampicoensis</i>			AY655032	
			<i>Lampsilis teres</i>	AF406803	AF406794		
			<i>Ligumia recta</i>	AF231748			
			<i>Ligumia recta</i>		AF406795		
			<i>Ligumia recta</i>			EF213054	
			<i>Pseudodon vondembuschianus</i>	DQ206793			
			<i>Pseudodon vondembuschianus</i>		DQ206795		
			<i>Potamilus purpuratus</i>	AF406804			
			<i>Potamilus purpuratus</i>		AF406797		
			<i>Potamilus purpuratus</i>			U72573	
		Anodontinae	<i>Gonidea angulata</i>	DQ206792			
			<i>Gonidea angulata</i>		DQ206794		
			<i>Pyganodon fragilis</i>	AF406805			
			<i>Pyganodon fragilis</i>		AF406800		
			<i>Pyganodon grandis</i>	AF231734			
			<i>Pyganodon grandis</i>		AF406801		
			<i>Pyganodon grandis</i>			AY238490	

Order	Family	Subfamily	Species	COI DNA F-mitotype	COI DNA M-mitotype	16s rDNA F-mitotype	16s rDNA M-mitotype
			<i>Pyganodon grandis</i>				AY498700
		Lampsilinae	<i>Glebula rotundata</i>			AY655044	
		unclassified Unionidae	<i>Ventustaconcha ellipsiformis</i>			AY655082	
		Margaritiferidae	<i>Dahurinaia dahurica</i>	AY579123			
			<i>Dahurinaia dahurica</i>		DQ241802		
			<i>Margaritifera margaritifera</i>	AF303339			
			<i>Margaritifera margaritifera</i>		AY785399		
			<i>Margaritifera falcata</i>	AY579126			
			<i>Margaritifera auricularia</i>	AY579125			
			<i>Margaritifera laevis</i>	AY579124			
			<i>Cumberlandia monodonta</i>	AY785393			
			<i>Cumberlandia monodonta</i>		AY785397		
	Muteloidae	Iridinidae	<i>Chambardia rubens</i>	DQ241807			
		Mutelidae	<i>Mutela dubia</i>	DQ241805			
		Mutelidae	<i>Mutela rostrata</i>	AY785387			
		Etheriidae	<i>Acostaea rivoli</i>	AF231739			
		Mycetopodidae	<i>Anodontites guanarensis</i>	AY785383			
			<i>Anodontites trigonus</i>	AF231738			
			<i>Tamsiella tamsiana</i>	AY785384			
			<i>Monocondylaea minuana</i>	AF231745			
		Etheriidae	<i>Etheria elliptica</i>	DQ241803			
Mytiloida	Mytilidae	Crenellinae	<i>Musculista senhousia</i>	AY570041			
			<i>Musculista senhousia</i>		AY570043		

Order	Family	Subfamily	Species	COI DNA F-mitotype	COI DNA M-mitotype	16s rDNA F-mitotype	16s rDNA M-mitotype
			<i>Musculista senhousia</i>			AY570065	
			<i>Musculista senhousia</i>				AT570055
Trigonioida	Trigoniidae	Trigonioidae	<i>Neotrigonia margaritacea</i>	NMU56850* Mitotype P			
			<i>Neotrigonia margaritacea</i>			DQ093489*	
			<i>Neotrigonia margaritacea</i>			DQ280034*	
			<i>Neotrigonia margaritacea</i> F (SW1)	Mitotype A			
			<i>Neotrigonia margaritacea</i> F (SW2)	Mitotype A			
			<i>Neotrigonia margaritacea</i> F (SW3)	Mitotype B			
			<i>Neotrigonia margaritacea</i> F (SW4)	Mitotype B			
			<i>Neotrigonia margaritacea</i> M (SW5)	Mitotype B	Mitotype C		
			<i>Neotrigonia margaritacea</i> F (SW6)	Mitotype A			
			<i>Neotrigonia margaritacea</i> M (SW7)	Mitotype B	Mitotype C		Mitotype 3
			<i>Neotrigonia margaritacea</i> F (SW8)	Mitotype B			
			<i>Neotrigonia margaritacea</i> F (SW9)	Mitotype D		Mitotype 1	
			<i>Neotrigonia margaritacea</i> F (SW10)	Mitotype E		Mitotype 2	
			<i>Neotrigonia margaritacea</i> M (SW11)		Mitotype Q		
			<i>Neotrigonia margaritacea</i> M (SW12)	Mitotype A	Mitotype Q		

Order	Family	Subfamily	Species	COI DNA F-mitotype	COI DNA M-mitotype	16s rDNA F-mitotype	16s rDNA M-mitotype
			<i>Neotrigonia margaritacea</i> F (SW13)	Mitotype B			
			<i>Neotrigonia margaritacea</i> M (SW14)		Mitotype R		
			<i>Neotrigonia margaritacea</i> F (SW15)	Mitotype D			
			<i>Neotrigonia margaritacea</i> F (SW16)	Mitotype D			
			<i>Neotrigonia margaritacea</i> M (SW17)		Mitotype R		
			<i>Neotrigonia margaritacea</i> F (SW18)	Mitotype A			
			<i>Neotrigonia margaritacea</i> F (SW19)	Mitotype A			
			<i>Neotrigonia margaritacea</i> F (SW20)	Mitotype A			
			<i>Neotrigonia margaritacea</i> M (SW21)	Mitotype B	Mitotype R		Mitotype 4
			<i>Neotrigonia margaritacea</i> F (SW22)	Mitotype A			
			<i>Neotrigonia margaritacea</i> (MW2)	Mitotype G			
			<i>Neotrigonia margaritacea</i> (MW3)	Mitotype H			
			<i>Neotrigonia margaritacea</i> (MW5)	Mitotype F			
			<i>Neotrigonia margaritacea</i> (MW6)	Mitotype H			
			<i>Neotrigonia margaritacea</i> (NB1)	Mitotype I			
			<i>Neotrigonia margaritacea</i> (NB2)	Mitotype I			

Order	Family	Subfamily	Species	COI DNA	COI DNA	16s rDNA	16s rDNA
				F-mitotype	M-mitotype	F-mitotype	M-mitotype
			<i>Neotrigonia margaritacea</i> (NB3)	Mitotype I			
			<i>Neotrigonia margaritacea</i> (PtL2)	Mitotype M			
			<i>Neotrigonia margaritacea</i> (PtL5)	Mitotype J			
			<i>Neotrigonia margaritacea</i> (PtL7)	Mitotype L			
			<i>Neotrigonia margaritacea</i> (PtL13)	Mitotype K			
			<i>Neotrigonia margaritacea</i> (NMT1)	Mitotype N			
			<i>Neotrigonia margaritacea</i> (NMT2)	Mitotype O			
			<i>Neotrigonia margaritacea</i> (NM3)	Mitotype O			
			<i>Neotrigonia margaritacea</i> (NMT4)	Mitotype O			
			<i>Neotrigonia lamarckii</i> (NL1)	Mitotype NL1			
			<i>Neotrigonia lamarckii</i> (NL2)	Mitotype NL1			
			<i>Neotrigonia lamarckii</i> (NL3)	Mitotype NL1			

Table 4.2. Outgroup taxa with GeneBank (NCBI) accession numbers for COI DNA and 16S rDNA sequences used. The reason for using these outgroups was to replicate the analysis conducted by Hoeh *et al.*, (2002).

Phylum	Class	Species	COI	16S
Mollusca	Bivalvia	<i>Solemya velum</i>	U56852	DQ280028
Mollusca	Scaphopoda	<i>Dentalium</i> sp.	U56843	DQ280026
Mollusca	Polyplacophora	<i>Katharina</i> sp.	U56845	EU407006
Mollusca	Gastropoda	<i>Lepetodrilus elevatus</i>	U56846	EF549688
Arthropoda	Insecta	<i>Drosophila yakuba</i>	X03240	

Table 4.3. Sequence variability and AMOVA results for mitochondrial COI and 16S rDNA ribosomal genes. AMOVA was performed to test whether variability within male mitotype and female mitotype sequence was significantly different, significance level ($\alpha=0.05$) with significant values in bold.

Comparisons	d.f.	Sum of squares	Variance components	Percentage of variation
<i>COI</i>				
Among groups: F-type vs. M-type	1	1.369	0.079	15.19
Within groups	41	18.119	0.441	84.81
<i>16S rDNA</i>				
Among groups: F-type vs. M-type	1	0.917	0.226	42.03
Within groups	4	1.250	0.312	57.97

Figure 4.1

Mitotype network of *N. margaritacea* COI data produced at the 95% confidence limit. Empty circles represent unsampled or possibly extinct haplotypes. The rectangle indicates a presumed ancestral haplotype, while large ovals represent two haplotypes and small ovals represent one haplotype. Mitotypes are grouped by a dashed line into their corresponding M or F types.

Figure 4.2

A Bayesian analysis tree based on Cytochrome C Oxidase subunit I using outgroup taxa as in Hoeh *et al.*, (2002). Posterior probabilities (pp) > 0.95 values are shown below the line and MP jackknife >70 values are shown above the line. Terminal names correspond to NCBI accession numbers listed in Table 4.1 and Table 4.2. Male, M, and female, F, types are marked according to available information, *Neotrigonia margaritacea* GenBank sequences are annotated by an asterisk*.

Figure 4.3

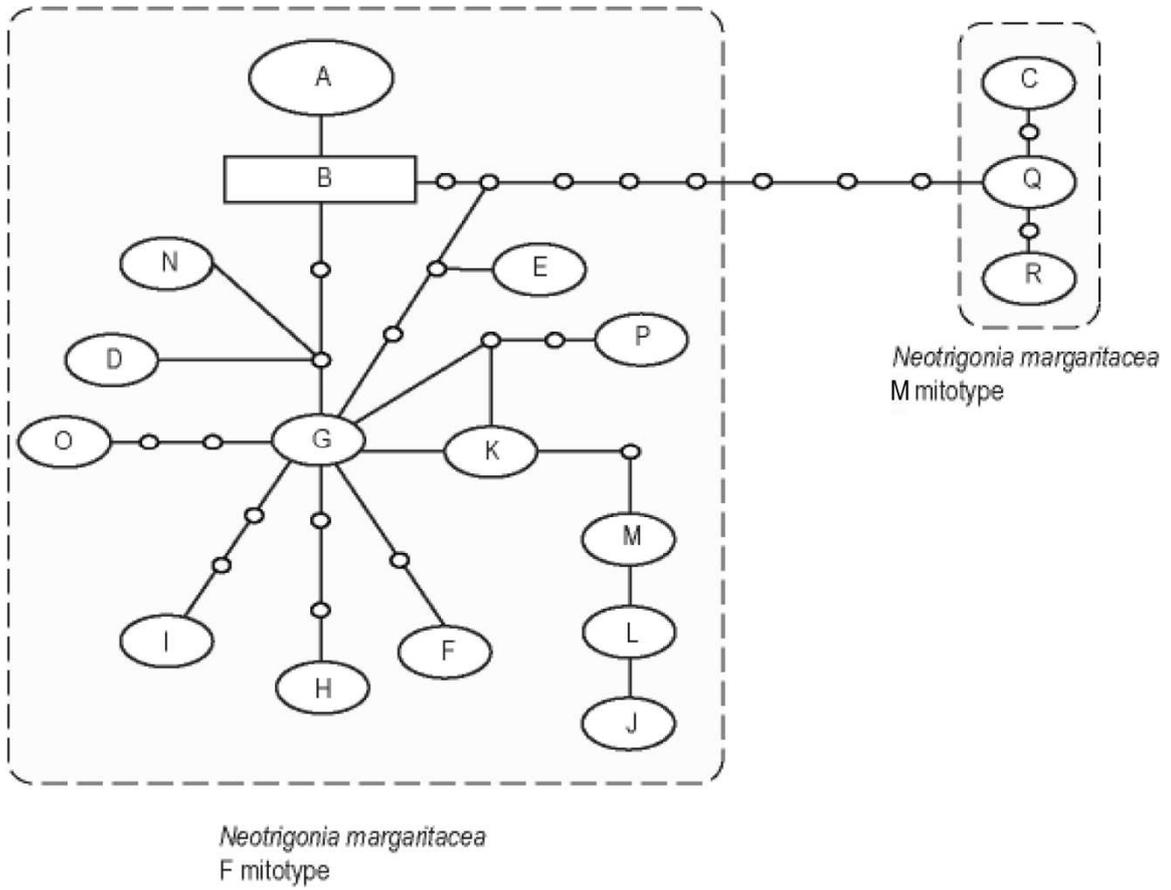
Bayesian analysis tree based on Cytochrome C oxidase subunit I for the Unionoidea and Trigonioidea taxa. A subset of less distant outgroups were used during this analysis. Only MP jackknife >70 values are shown above the line and posterior probabilities (pp) > 0.95 values are shown below the line. Due to highly divergent *Musculista senhousia* sequences, long branches were detected for these terminals (Appendix III). Terminal names correspond to NCBI accession numbers listed in Table 4.1 and Table 4.2. Male, M, and female, F, types are marked according to available information. *Neotrigonia margaritacea* GenBank sequences are annotated by an asterisk*.

Figure 4.4

A maximum parsimony consensus tree based on 16S rDNA gene for Palaeoheterodonta bivalve taxa. Only MP jackknife > 70 and posterior probabilities (pp) > 0.95 values are shown. Terminal names correspond to NCBI accession numbers listed in Table 4.1 and Table 4.2. Male, M, and female, F, types are marked according to available information. *Neotrigonia margaritacea* GenBank sequences are annotated by an asterisk*.

Figure 4.5

The most parsimonious transformation shows that DUI is the ancestral state of all Bivalves. The DUI character was mapped onto a bivalve phylogeny by Giribet and Wheeler (2002), where black branches indicate DUI presence and light grey DUI absence. The presence of DUI was recorded for Mytiloidea, Unionoidea, Tellinoidea, Solenoidea, Veneroidea and now Trigonioidea. Absence was recorded for *Arca noae*, *Venus verrucosa*, *Callista chione*, *Crassostrea virginica*, *C. gigas* and Etheriidae (see text for references). All other bivalves were scored as unknown and are indicated by a question mark by on the tips of the tree (?).



Figure

4.1

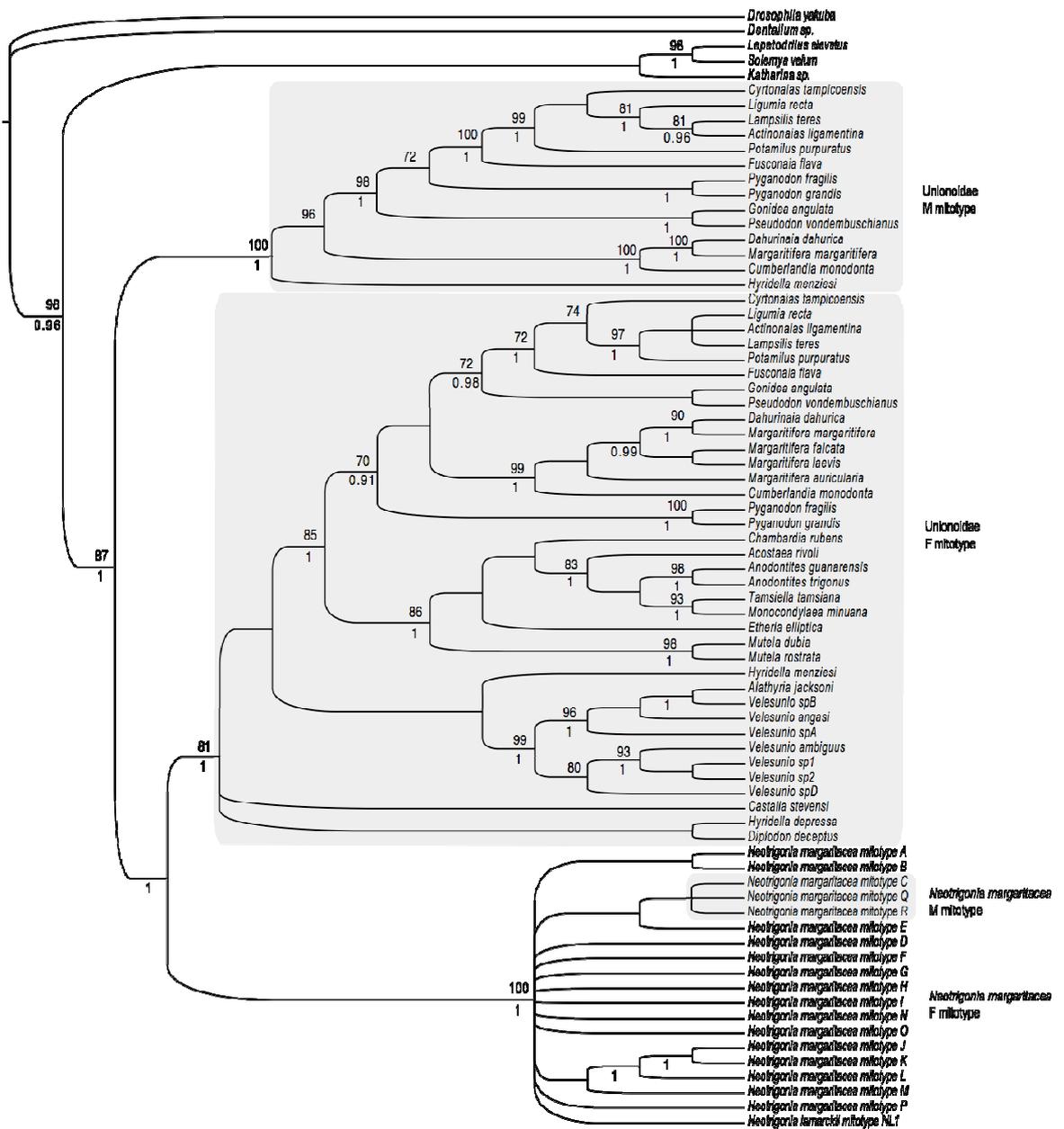


Figure 4.2

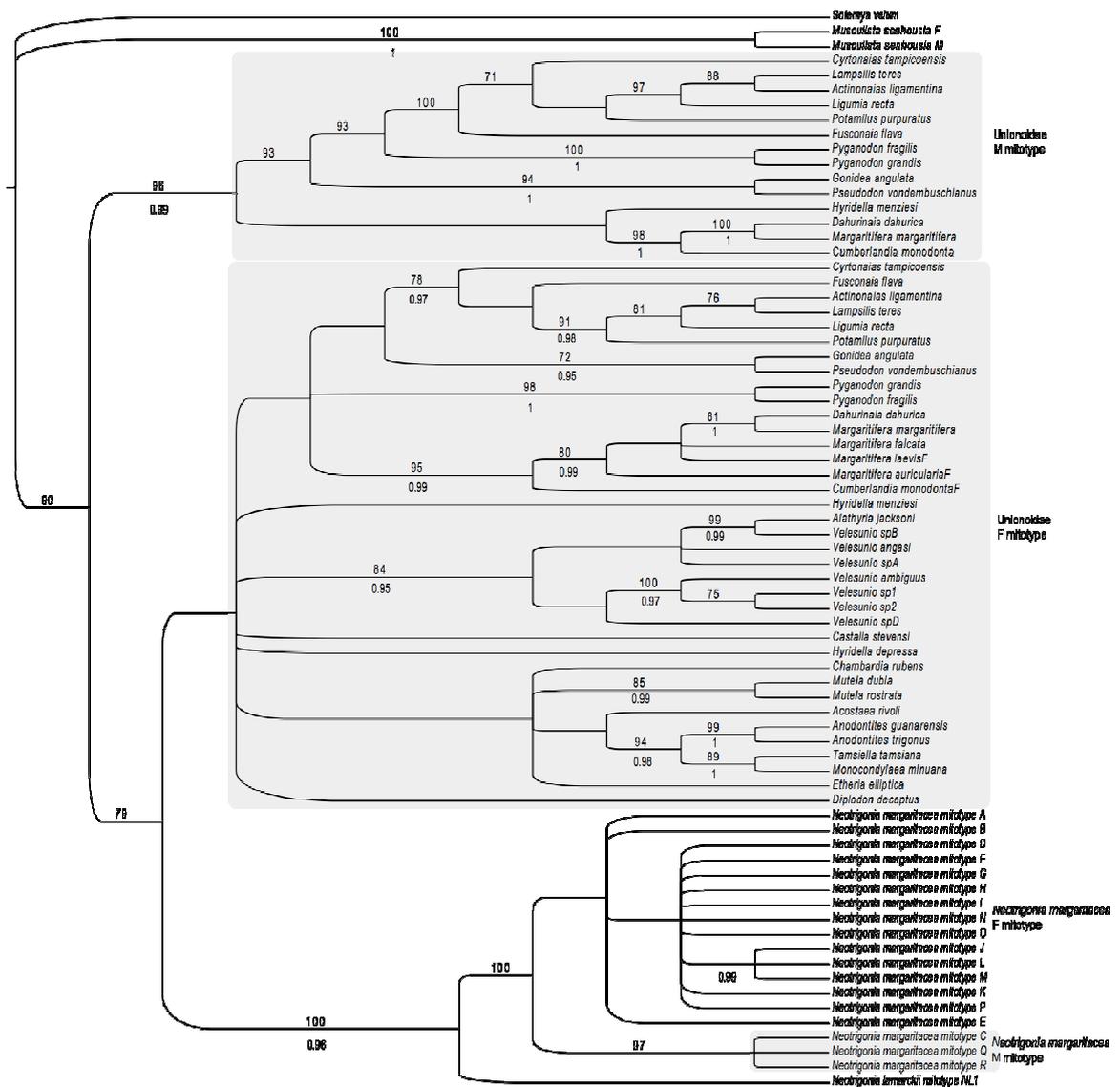


Figure 4.3

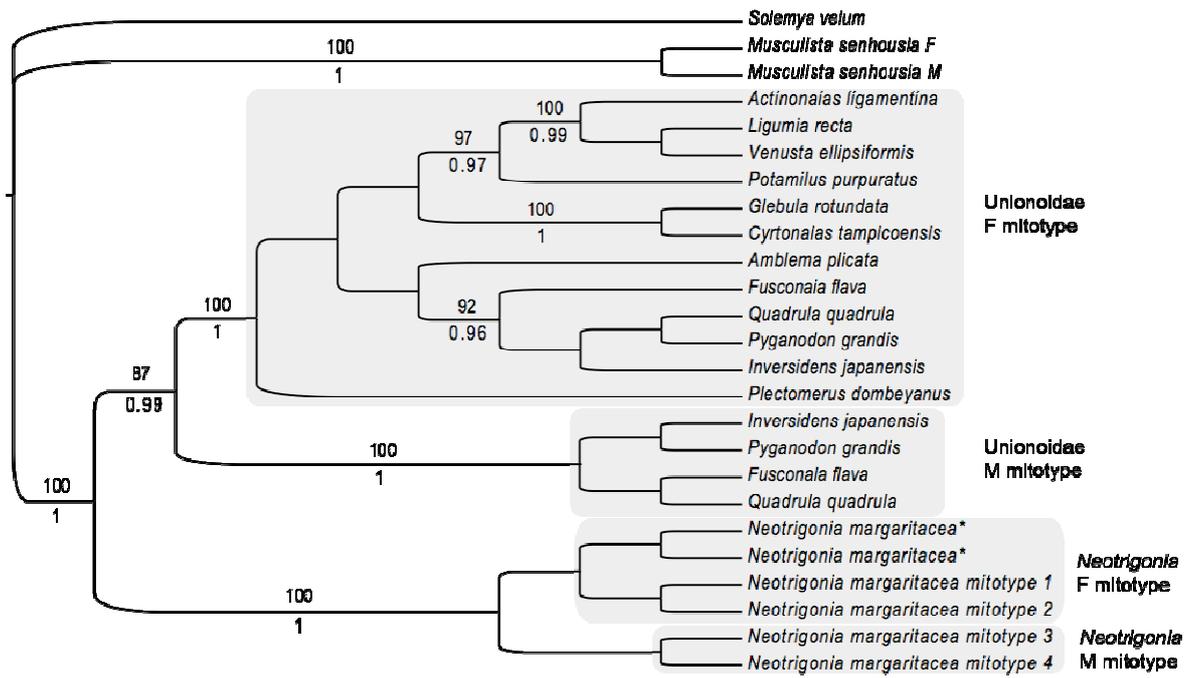


Figure 4.4

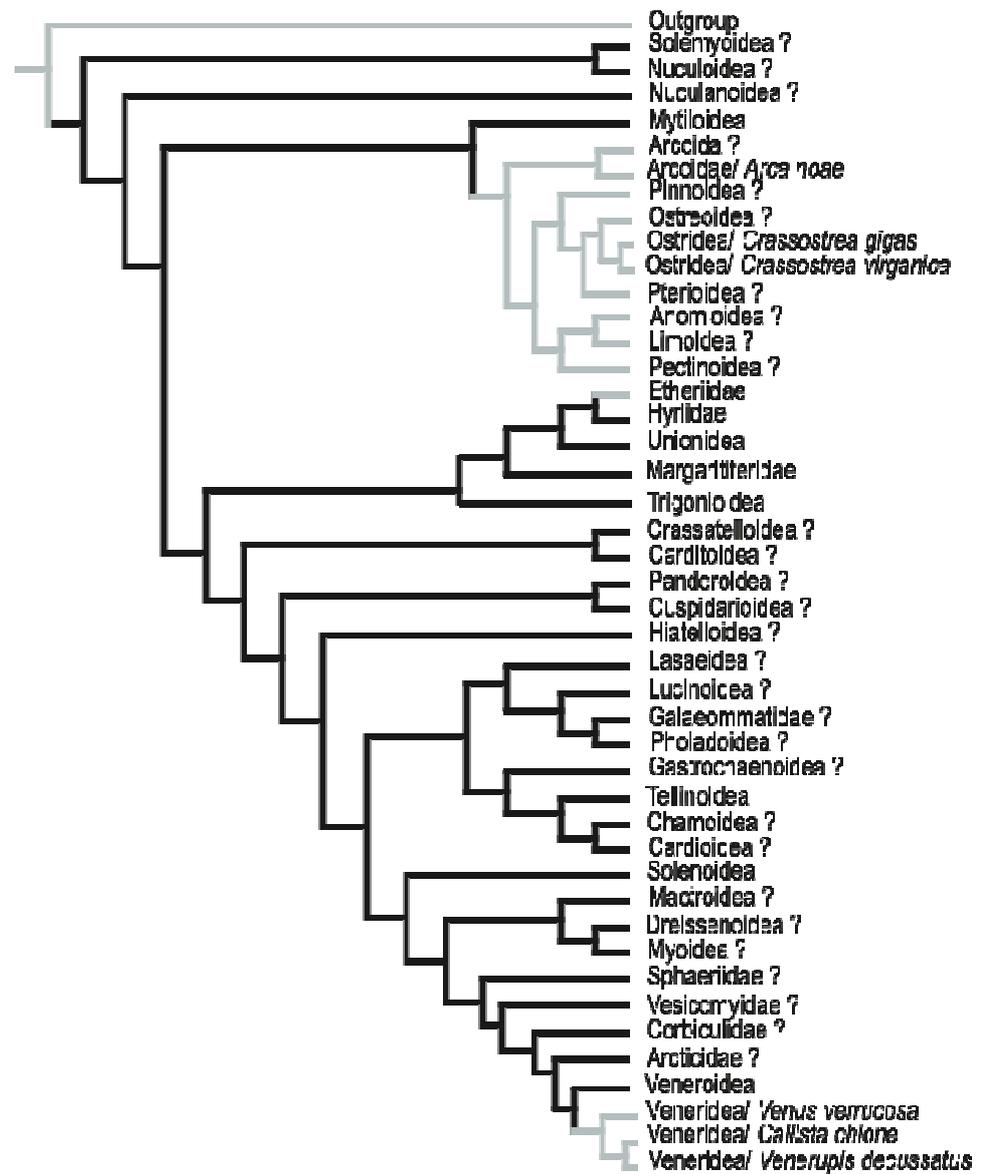


Figure 4.5

Ultrastructure of oogenesis and the ovary of *Neotrigonia margaritacea* (Lamarck, 1804) (Bivalvia, Mollusca)

Ana Glavinic

Flinders University, Adelaide, PO Box 2100, Adelaide, SA 5001, Australia.

Author for correspondence: ana.glavinic@flinders.edu.au, telephone +61 8 82015747

Keywords: oocytes, previtellogenesis, vitellogenesis, postvitellogenesis, trickle spawning, tachitictic pattern, bivalve reproduction.

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Abstract

Oogenesis is described in the Australian marine bivalve, *Neotrigonia margaritacea*, from specimens sampled over a five month period. The structure of oocytes and gonad tissue are described for the first time in this genus, using electron microscopy and histology. The ovary was found to contain oocytes in various developmental stages throughout the study period. Oocytes develop from oogonia derived from protogonia and then undergo three distinct stages of oogenesis; previtellogenesis, vitellogenesis and postvitellogenesis with mature oocytes. Mature oocytes are large in size and have a thick vitellogenic layer. The vitellogenic layer has a single passage, a micropyle, which could provide a barrier to polyspermy. The spawning mode is inferred based on gonad tissue and oocyte structure. We hypothesise that *Neotrigonia margaritacea* can be considered as sequentially tachitictic, extended reproductive activity, using trickle (continuous) spawning over an extended summer season.

Introduction

Broadcast spawning is a common reproductive strategy among marine invertebrates, in which both males and females release their gametes into the water column. Fertilization takes place externally in the water column, to increase the chance of gamete interaction. Synchronous spawning commonly occurs in anthozoans (Harrison *et al.*, 1984; Babcock *et al.*, 1986, 1992; Brazeau and Lasker, 1989) and various echinoderms (Pearse *et al.*, 1988; Babcock *et al.*, 1992; Lamare and Stewart, 1998), but broadcasting also occurs in other marine invertebrates such as polychaetes (Caspers, 1984; Hardege, 1999; Watson *et al.*, 2000), gastropods (Counihan *et al.*, 2001) and marine bivalves (Babcock *et al.*, 1992; Minchin, 1992; Grant and Creese, 1995). While some freshwater bivalves such as the zebra mussel, *Dreissena polymorpha* (Veneroidea), are also broadcast spawners with external fertilization (Burky, 1983; Misamore *et al.*, 1994), the majority of bivalves belonging to Unionoidea fertilize internally by inhaling freely-spawned sperm and retaining their larvae. Unionoidea is the sister group to the once diverse marine lineage Trigonoidea, the only extant forms of which belong to *Neotrigonia* (Tevesz, 1975; Stanley, 1984; Morton, 1987; Giribet and Wheeler, 2002). Unionoidea and Trigonoidea are currently grouped as Palaeoheterodonta, and knowledge of the reproductive strategy of *Neotrigonia* is of considerable relevance to inferring plesiomorphic traits for Paleoheterodonta as a whole. Presently there is nothing known about the spawning method of *Neotrigonia*, the sole remaining genus of Trigonoidea.

Bivalves have been classified according to two reproductive patterns; tachitictic, with short and limited reproductive periods, and braditictic, with extended period of reproductive activity (Fretter, 1984). In externally-fertilising bivalves, such as *Anadara trapezia* (Arcoidae) (Hadfield and Anderson, 1988) and the mytilid *Mytilus edulis* (Humphreys, 1962; Newell *et al.*, 1982, Pipe, 1987) a single spawning event may occur during the summer when water temperature and food supply are high. However *Anomia discripta* (Anomiidae) has two spawning peaks, a minor in summer and a major in autumn, while *Venerupis crenata* (Veneridae) has a prolonged trickle spawning season (Hadfield and Anderson, 1988). Although reproductive activity of *Venerupis crenata* decreases during cooler winter months, this species is still considered braditictic as regeneration and maturation of gonads occurs continuously throughout the year (Hadfield and Anderson, 1988). Both patterns have been recognized in brooding unionoids, with bradytictia seen as a derived condition,

having evolved twice independently in Anodontini and in Lampsilini from the tachytictia condition (Heard, 1998). Within Unionoidea the family Margaritiferidae are considered relatively basal; however, they can either spawn in a single event and release larvae in summer (Bauer, 1987) or may have multiple reproductive events each year and therefore are considered as sequentially tachitictic (Smith, 2001). The distinction between tachitictic and bradytictic Unionoidea bivalves is that bradytictic mussels continue to brood their larvae long after metamorphose (Heard, 1998). Measures used to infer the reproductive pattern of bivalves include gonad volume, oogenesis duration and spawning modes are frequently used (Sastry, 1979; Smith, 2001).

Oogenesis in bivalves is classified as follicular (Pipe, 1987) because the bivalve oocytes develop within acini that make up the female gonad. Each acinus is surrounded by connective tissue with haemocoelic sinus and intermittent myoepithelial cells (Eckelbarger and Davis, 1996; Pipe, 1987; Dorange and Le Pennec, 1989; Al-Mohanna *et al.*, 2003). While it is a continuous process, three phases of oogenesis are generally distinguished; previtellogenesis, vitellogenesis and postvitellogenesis (or mature) oocytes. In general the three phases can be classified by the level of uptake of vitellogenin or yolk protein via microvilli (De Gaulejac *et al.*, 1995). It has been reported that the remaining un-spawned oocytes in many molluscs undergo degeneration, involving the breakdown of the plasma membrane and reabsorption of the components in the gonads, resulting in decreased gonad volume (Eckelbarger and Davis, 1996; Pipe, 1987; Dorange and Le Pennec, 1989; Al-Mohanna *et al.*, 2003). The timing and the mechanism of this process is not entirely understood, but it is suspected that it coincides with the end of breeding season, which is typically controlled by changes in environmental temperature (Sastry, 1979; Behzadi *et al.*, 1997).

This study concentrates on *Neotrigonia margaritacea* (Lamarck, 1804), which is an endemic southern Australian bivalve. This species occurs in subtidal depths, in sandy substrate, often in areas with strong water currents (Tevesz, 1975; Stanley, 1984). Morton (1987) argued that *Neotrigonia* species are gonochoric, although the female reproductive cycle of the species has never been studied. Previous studies have described early prodissoconch morphology to infer non-planktotrophic larval development (Ó Foighil and Graf, 2000) and spermatogenesis has been described (Healy, 1989). According to Healy (1989), *Neotrigonia* sperm ultrastructure is

similar to that of internally fertilizing and brooding unionoidean bivalves. Oogenesis in *Neotrigonia* has yet to be investigated. Therefore, a population of *Neotrigonia margaritacea* was sampled over a 5 month period to document oogenesis using light microscopy, scanning and transmission electron microscopy.

Methods

Samples of *N. margaritacea* were collected from the Adelaide region in the Gulf St Vincent, South Australia, 5 km seaward of Port Stanvac (S35.00250, E138.31487) at 18 m depth. The site was exposed to continuous currents and is composed of well-sorted coarse sand and shell grit. Adult samples were collected by SCUBA diving monthly, during the Summer period from the beginning of November 2005 until March 2006. Further sampling efforts were made during Autumn and Winter months but insufficient numbers were found to justify collection. Water temperature varied from 18°C to 22°C during the sampling period. Forty specimens in total were analysed, thirteen of which were male and twenty-seven were female. A Chi-square statistic was calculated to assess if observed sex ratio deviated significantly from expected 1:1 ratio for all samples used. To ensure maturity, only specimens larger than 2 cm width and 2.5 cm height were collected. Gonad tissue was examined under a dissecting microscope and spawning status was estimated according to the fullness of the gonad. Gonad smears were viewed under a light microscope to record presence of oocytes or sperm. For each monthly sample, the gonad tissue of five female specimens was separated from the somatic tissue via dissection under a dissecting microscope and then weighed (Appendix IV). The tissue samples were prepared for transmission electron microscopy (TEM, Philips CM200), scanning electron microscopy (SEM, Philips XL30) and light microscopy (LM, Olympus BH-2). Samples were all fixed for 24 hours using 3% glutaraldehyde in 0.2M phosphate buffer (filtered) pH 7.4, with 0.3M sucrose. For SEM, the gonad tissue was cut into 2 mm pieces, rinsed in buffer, dehydrated in an ethanol series and critical point dried using a Balzer CPD 030 critical point drier. Specimens were then mounted on stubs and sputter-coated with 6 nm of gold. For TEM, the gonad tissue was cut into 2 mm-thick pieces, and fixed for 2 h at 4°C then rinsed for 15 min in three changes of buffer and post fixed in 1% osmium for 80 min at 4°C. Specimens were rinsed in buffer and dehydrated through an ethanol series, and embedded in low-viscosity Spurr's resin. For LM, tissue was embedded in paraffin wax following

dehydration and transfer to xylene. Sections 5-7 μm thick were cut through the ovary, stained with haematoxylin and eosin (Thompson, 1966), mounted in DPX on microscope slides, and examined using an Olympus BH-2 microscope.

Light microscopy and TEM data were used for describing oogenesis stages and only LM slides were used to assess oocyte size frequency distribution. Oocyte size was determined by measuring the diameter of protogonia, oogonia, previtellogenic, vitellogenic and postvitellogenic oocytes. Twenty eggs were measured for each stage per individual specimen (Appendix IV). Only oocytes sectioned through the nucleus were measured. Statistical analysis was undertaken on SPSS version 15. A one- way ANCOVA was used to test monthly variations in oocyte size and gonad mass. To standardize gonad mass to shell length, shell length data was used as the covariate in the ANCOVA. One- way ANOVA was used to compare mean oocyte size across all stages. Tukey's post hoc tests were performed to establish significant variation in gonad mass and oocyte size, between months. The total number of oocytes (all stages included) was also counted from five acini per specimen and mean percent of oocytes in each stage was calculated.

Results

General morphology of gonad tissue and oocytes

Neotrigonia margaritacea is a gonochoric species though based on sample size of 40 specimens, the sex ratio was significantly different from a 1:1 ratio (Chi square $\chi^2= 4.9$, $P= 0.038$). The gonad was found in the viscera above the foot and ventral to the stomach. The ovary consists of a series of highly branched and globulated clusters of acini, surrounded by a thin acinal wall, and contains developing oocytes during oogenesis (Fig. 5.1). All mature oocytes were found in the acinal lumen and developing oocytes or previtellogenic and postvitellogenic cells were in close contact with the acinal wall (Fig. 5.1, Fig. 5.2d and e, Fig. 5.3). TEM and LM enabled categorization of five developmental stages of oocytes (Figs. 5.4, 5.5, 5.6, 5.7). The gonad tissue, examined across all months in summer, contained all developmental stages of oocytes including protogonia, oogonia, previtellogenic, vitellogenic and post vitellogenic stages (Fig. 5.4). Mature oocytes were characterized by a dramatically larger diameter and spherical shape (Fig. 5.2f, 5.3e and f). As oocytes increased in diameter, they aggregated in the centre of the lumen (Fig. 5.4c, d). In mature ovaries, the lumen was filled with large oocytes (Fig. 5.4c).

Protogonia

N. margaritacea protogonia (germ cells), were located along the internal walls of the acini (Fig. 5.3a, b and Fig. 5.5a, b). They were irregular in shape and measured between 3-6 μm (mean \pm SD = $3.91 \pm 0.90 \mu\text{m}$, $n=20$). Protogonia, visualized using LM (Fig. 5.3a, b), had a large irregularly-shaped nucleus in comparison to the total cell size. Some mitochondria and golgi apparatus were visible using TEM (Fig. 5.5b).

Oogonia

Oogonia were present in the wall of acini, one or two cell layers into the lumen (Fig. 5.3a, b). Oogonia measured from 6-10 μm in size (mean \pm SD = $7.03 \pm 1.18 \mu\text{m}$, $n=20$) (Fig. 5.3b) and had a basophilic nucleus and lighter basophilic cytoplasm (Fig. 5.3b). With TEM they often had a large nucleus and the same cytoplasmic organelles as protogonia (Fig. 5.5a, b). Some cortical granules, membrane-bound granules, were visible within plasma membranes (Fig. 5.5c).

Previtellogenic oocytes

Previtellogenic oocytes were irregular in shape. The size of the oocyte reached 50 μm (mean \pm SD = $31.54 \pm 11.35 \mu\text{m}$, $n=20$) and the nucleolus within is 5 μm in diameter (Fig. 5.3c and Fig. 5.6a). Light microscopy showed previtellogenic oocytes had a basophilic nucleus and lightly eosinophilic cytoplasm (Fig. 5.3c). The nuclear membrane was well-defined and numerous mitochondria and rough endoplasmic reticulum were visible under TEM (Fig. 5.6a). Previtellogenic oocytes were still in close proximity to one another but there were no visible desmosomes. Vast numbers of cortical granules were now aggregated in cortical cytoplasm (Fig. 5.6a). Microvilli and the vitelline coat were not present at this stage.

Vitellogenic oocyte

These oocytes were larger, $\sim 100 \mu\text{m}$ in diameter (mean \pm SD = $92.12 \pm 3.72 \mu\text{m}$, $n=20$) and continued to grow by accumulating yolk granules (Fig. 5.3d and Fig. 5.6b). The vitellogenic oocytes were intensely eosinophilic with HandE staining (Fig. 3d,e). Vitellogenic oocytes were found in the lumen of acinus. The point of attachment to the acinal wall was visible and is referred to here as a stalk (Fig. 5.4c). Yolk granules were 1-5 μm in diameter, light grey and of the same density as the

vitelline layer, while lipid granules were smaller, about 1 μm in diameter, and dense in consistency so they appeared black in the TEM images (Fig. 5.6b and c). The nucleus was round and 50 μm in diameter with a nucleolus of 10 μm diameter (Fig. 5.6b). Microvilli appeared for the first time at this stage (Fig. 5.7a). The vitelline layer was 3-4 μm thick and the perivitelline space was narrow 1-2 μm . Numerous mitochondria, Golgi apparatus and endoplasmic reticulum were visible and more abundant than in earlier stages (Fig. 5.6b).

Postvitellogenic oocytes

Postvitellogenic oocytes range in size from 200-284 μm (mean \pm SD = 200.33 \pm 47.8 μm , $n=20$) (Fig. 5.3f and Fig. 5.6c). The oocytes at this stage were located in the centre of the acinus lumen (Fig. 5.4c,d), but were still connected to the wall of acini by a stalk (Fig. 5.4f). The cytoplasm was extremely eosinophilic (Fig. 5.3f) and TEM (Fig. 5.6c) showed that there were numerous yolk and lipid granules. The nucleus was homogeneous and the nucleolus appeared dense. Mitochondria and rough endoplasmic reticulum appeared through the ooplasm. The perivitelline space between microvilli and the vitelline layer was large, reaching 15 μm (Fig. 5.7b). The thickness of the vitelline layer was up to 20 μm and the layer is furrowed (Fig. 5.2e and Fig. 5.7c). The vitellogenic layer was not continuous around the oocyte, a break occurred at the apical region forming a gap, or micropyle, of around 10 μm width (Fig. 5.2f, Fig. 5.4f and Fig. 5.7c, d).

Oocyte size frequency distribution and gonad tissue mass

The maximum gonad mass occurred in November followed by December (Fig. 5.8a). These levels were followed by a trend of decreasing gonad tissue mass in January, February and March. The one-way ANCOVA for gonad mass was significant ($F=4.67$, $P=0.017$, $r^2=0.024$), indicating that gonad mass differs between months over the summer period and was significantly correlated to shell length. The maximum gonad weight was 0.099 g and maximum post-vitellogenic oocyte diameter recorded was 284 μm , both from females sampled in November.

One-way ANOVA showed that monthly variations in mean oocyte size were not significant across all stages of development: protogonia ($F=0.38$, $P=0.99$), oogonia ($F=0.38$, $P=0.77$), previtellogenic ($F=0.75$, $P=0.77$), vitellogenic ($F=0.99$, $P=0.58$), and postvitellogenic ($F=0.57$, $P=0.96$) oocytes, indicating that females

carried all stages of oocytes during the sampling period. However, the mean percent of different stages of oocytes did vary among months (Fig. 5.8b). The highest mean percent of postvitellogenic oocytes was recorded in November and December, when the lowest mean percent of protogonia was found (Fig. 5.8b). The histogram shows that mean percent of oogonia and previtellogenic oocytes did not differ between months (Fig. 5.8b).

Discussion

A slightly female-biased sex ratio in *Neotrigonia margaritacea* is recorded. This is not unusual for bivalves and it is similar to a number of freshwater species (Heard, 1975; Bauer, 1987; Byrne, 1998; Garner *et al.*, 1999; McIvor and Aldridge, 2007). The general features of the *N. margaritacea* gonad and oogenesis processes are similar to the general pattern found among other bivalves, including Pteriomorphia: e.g., *Mytilus edulis* (Humphreys, 1962; Pipe, 1987) *Pecten maximus* (Dorange and Le Pennec, 1989), *Pinna nobilis* (De Gaulejac *et al.*, 1995), *Atrina pectinata* (Fang and Qi, 1988), *Brachidontes virgiliae* (Bernard *et al.*, 1988), Veneroidea: *Amiantis umbonella* (Al-Mohanna *et al.*, 2003); and the closest relatives to *Neotrigonia*, Unionoidea: e.g., *Elliptio complanata* (Won *et al.*, 2005). Oogenesis occurs in an acinus, starting with protogonia developing into oogonia, previtellogenic, vitellogenic and then postvitellogenic oocytes. Mature oocytes are large and surrounded by a thick jelly coat. These mature postvitellogenic oocytes were found throughout the sampling period, suggesting trickle spawning occurs in *N. margaritacea*, so the species can be considered to be sequentially tachitictic, following the definition of Fretter (1984).

The previtellogenic stage is characterized by the presence of a defined nucleus, cortical granules and a large number of mitochondria, numerous ribosomes and rough endoplasmic reticulum. This has been previously interpreted as initialization of major synthetic activity, such as oogenesis, in other bivalves (De Gaulejac *et al.*, 1995). The nuclear envelope is well formed and possesses numerous pores allowing transfer of nutrients for the growing oocyte. During the vitellogenic stage, *N. margaritacea* oocytes grow and accumulate organelles and their products. However, the mechanisms supplying the material needed for the growing oocyte in bivalves remains largely unknown (De Gaulejac *et al.*, 1995) and was not elucidated here. In the bivalves *Mytilus edulis* (Pipe, 1987) and *Crassostrea gigas* (Suzuki *et al.*, 1992), auto-synthetic formation of yolk protein was suggested because of the

presence of large and numerous rough endoplasmic reticulum and Golgi bodies in the cytoplasm. Similar observations were made for *N. margaritacea*, suggesting autotrophic vitellogenesis may occur in this species. The actual formation of yolk granules has been relatively well studied in bivalves and a number of different production sites have been reported. In the gastropod *Aplysia depilans* (Bolognari and Licata, 1976), as well as the bivalves *Mytilus edulis* (Humpreys, 1962), and *Pinna nobilis* (De Gaulejac *et al.*, 1995), formation of yolk granules occurs by merging of ribonucleoprotetic granules, glucidic elements and lipid globules, within the Golgi apparatus. In *Neotrigonia margaritacea*, we observed numerous whorls of rough endoplasmic reticulum and Golgi body in the cytoplasm of the vitellogenic oocytes, surrounding small yolk granules and mitochondria. Golgi apparatus has been proposed as a suitable site for coalescence due to compartmental organization of Golgi complex (Rothman, 1985). In other studies on molluscs, formation of granules has been associated with both Golgi apparatus and rough endoplasmic reticulum (Anderson, 1969; Taylor and Anderson, 1969; de Jong-Brink *et al.*, 1976; Humpreys, 1967; Pipe, 1987).

In *N. margaritacea*, mitochondria are first produced and observed in protozoa, and multiply during the previtellogenic stage. At the later stages of oogenesis in *N. margaritacea* mitochondria are still present in the cytoplasm, but not as numerous as in earlier stages, suggesting that mitochondrial activity is reduced. There is also evidence for transformation of mitochondria into yolk granules for a number of bivalve species (Humphrey, 1962, 1967; Favard and Carass, 1958; Fang and Qi, 1988), although neither the transformation of mitochondria into yolk granules or an intermediate stage was observed for *N. margaritacea*. Large lipid droplets were not observed before the vitellogenic stage in *N. margaritacea*, and mitochondria and lipid droplets were still present in the postvitellogenic stage.

The mature oocytes of *N. margaritacea* are almost 300 μm in size, with 50% of this diameter attributed to the vitelline layer. The vitelline layer is an accessory structure composed of glycoprotein jelly, consistent with other marine invertebrates (Thomas *et al.*, 1999). Progressive thickening of the vitelline layer is apparent in vitellogenic and postvitellogenic developmental stages of the *N. margaritacea* oocyte. Diverse roles for the vitelline layer in marine invertebrate eggs have been reported by Lillie (1914), Bohus Jansen (1953), Hagstrom (1956), Ohtake (1976), Foltz (1995), Thomas and Bolton (1999), Thomas *et al.*, (1999) and Bolton *et al.*, (2000), such as

gamete protection, increasing egg target size and reducing polyspermy. We propose that the primary role of the vitelline layer of *N. margaritacea* is to provide a barrier to polyspermy (Hagstrom, 1956).

Large spawned oocytes are a potential target for many sperm, thus increasing fertilisation problems related to polyspermy. Polyspermy often occurs in free spawning marine invertebrates, where penetration of more than one sperm leads to abnormal cleavage and death, as described for *Crasostrea gigas* (Gould and Stephano, 2003). One of the principal mechanisms that may be employed to prevent polyspermy is modification of the oocyte extracellular layer to prevent sperm binding and/or penetration (Gould and Stephano, 2003). This complex mechanism is a combination of chemical and physical processes acting coherently at the time of vitelline coat formation and oocyte spawning. The increased thickness of the vitelline coat acts as a physical barrier, and in some bivalve species it is enhanced by the release of jelly from cortical granules (Gould and Stephano, 2003). This causes the elevation and hardening of the coat forming a sperm block, as has been observed in many marine invertebrates after the single sperm has fertilized the egg (Gould and Stephano, 2003). At fertilization, no significant exocytosis of cortical granules was reported for the bivalves *Crassostrea* (Alliegro and Wright, 1983) and *Mytilus* (Humphreys, 1967). However, there are reports for some species of bivalves where numerous cortical granules are present in the oocytes and exocytosis occurs before fertilization when the egg is shed into sea water (Pasteels, 1965). Based on this evidence, it is speculated that cortical granules in *N. margaritacea* oocytes contribute to the formation of the vitelline layer enhancing its strength and size prior to fertilization.

In *N. margaritacea*, the vitelline coat is furrowed in appearance and it is not continuous. A micropyle present in the *N. margaritacea* vitelline coat is the remnant of a stalk, or a site of detachment from the acinal wall. Similar micropyles have been found in the Unionoidean sister group to *Neotrigonia*, such as *Margaritifera margaritifera*, *Unio terminalis*, *Unio elongatus* and *Anodonta anatina* (Beams and Sekhon, 1966; Focarelli *et al.*, 1988; Hansten *et al.*, 1997; Cek and Sereflisan, 2006). The micropyle supports an apparent sperm passageway, which together with the shear thickness of the vitelline layer is most likely to reduce the chances of polyspermy. This was also shown to occur in *Unio elongatus* (Focarelli *et al.*, 1988). The opening through the vitelline layer, is a suitable size to act as a sperm passageway in *N.*

margaritacea because it is up to 10µm wide and is therefore larger than a single sperm, which in diameter is at most 2.5 µm across (Healy, 1989). This micropyle nevertheless limits the number of sperm able to fertilize the egg. The furrowing of the vitelline layer could act to guide the sperm towards the micropyle.

Similarly to the closely-related freshwater unionid group Margaritiferidae (Grande *et al.*, 2001), which have been identified as sequentially tachitictic (Smith, 2001), *Neotrigonia margaritacea* appears to use trickle spawning over the summer period studied here. While spawning was not directly observed, this reproductive strategy can be inferred as the gonad mass decreased gradually and significantly over the sampling period, whilst the adult shell size sampled and average mature oocyte size did not significantly differ. This indicates the presence of oocytes ready to spawn throughout the summer, suggesting sequentially tachitictic reproductive pattern. This inference was supported by light microscopy and transmission electron microscopy, where gonad ultrastructure revealed some oocytes at each developmental stage within the ovary in each month sampled. An asynchrony in development was also detected in gametogenic cycle of the trickle spawning *Venus verrucosa* (Veneridae), where histological studies have revealed the presence of at least two development stages within the same gonad (Tirado *et al.*, 2003). However a single species can change its reproductive strategy from one year to another, as in the case of heterodont bivalve *Spisula solidissima* (Family Mactridae), from a short synchronous spawn to a long, partially asynchronous or several partial spawns (Bricelj and Malouf, 1980; Kanti *et al.*, 1993). In comparison to the oocytes of the unionids *Margaritifera margaritifera* and *Unio elongatulus*, which at most reach 83 and 150 µm in diameter respectively (Focarelli, 1988; Grande *et al.*, 2001), *N. margaritacea* produces larger oocytes, supporting the conclusion that *Neotrigonia* has lecithotrophic larval development (Ó Foighil and Graf 2000), while Unionoidea have glochidia parasitic larvae (Watters and O'Dee, 1999).

In conclusion this study has reported in detail the different stages of oogenesis and provided the information on the spawning mode for *N. margaritacea*. The ultrastructure of oocytes and the process of oogenesis in *N. margaritacea* is similar to other bivalves. However, the mode of spawning is different for the two groups comprising the Palaeoheterodonta, where freshwater Unionoidea are brooding animals with internal fertilization and can be both braditictic or tachitictic, while the marine

Neotrigonia appear to fertilize externally and is sequentially tachitictic using extended trickle spawning.

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Figure 5.1

Diagrammatic representation of oogenesis in *Neotrigonia margaritacea* based on light and electron microscopy observations. The various stages of ovogenesis are protogonia, oogonia, followed by previtellogenic, vitellogenic and postvitellogenic oocytes.

Figure 5.2

Scanning electron microscopy of gonad tissue acinus and postvitellogenic oocyte of *Neotrigonia margaritacea* A through to E, gonad wall (Gw), connective tissue (Ct), acinus (Ac), post vitellogenic oocytes (PV) and vitelline layer (VI). (F) Light microscopy image of postvitellogenic freely spawned oocyte showing micropyle (Mp) and vitelline layer (VI). Scale bar on A to D is 200 μm and E and F is 50 μm .

Figure 5.3

Light microscopy images showing different ovogenesis stages of *Neotrigonia margaritacea*; A) protogonia (Pr) closely located to the acinal wall; B) oogonia (Oo) showing a distinct nucleus; C) previtellogenic oocytes (PVo) nucleus (Nu) and distinct nucleolus (n); D) vitellogenic oocytes (Vo); E) late vitellogenic oocytes with eosinophilic cytoplasm; and F) post vitellogenic oocytes (PV) with visible vitelline layer (VI). Scale bar for A, B and C is 10 μm , for D and E is 50 μm and F is 100 μm .

Figure 5.4

A portion of the *Neotrigonia margaritacea* ovary, showing individual acini at various stages of development; A) early stages of ovogenesis with proportionally more protogonia (Pr) and oogonia (Oo) visible near the acinal wall (Aw); B) all stages of ovogenesis present in one acini, postvitellogenic (PV) and vitellogenic (Vo); C) vitellogenic and post vitellogenic oocytes are numerous and aggregate in the lumen (Lu) of the acinus, nucleus visible (n); D) pre spawned acinus full of postvitellogenic oocytes and notably less protogonia and oogonia; E) post spawning acinus is half empty, note degeneration of oocytes and increased number of protogonia, oogonia and previtellogenic oocytes. F) post vitellogenic oocytes still connected to the wall of the acini by a stalk (S). Scale bars A through to E are 200 μm and F scale bar is 100 μm .

Figure 5.5

Transmission electron microscopy images of protogonia and oogonia of *Neotrigonia margaritacea*; A) Oogonia (Oo) with protogonia (Pr) and previtellogenic oocyte (PvO); B) close up of protogonia showing nucleolus (Nu), mitochondria (Mc), Golgi body (Gb), plasma membrane (Pm) and energy stores (Es); C) close up of oogonia showing plasma membrane (Pm), cortical granules (Cg), nucleus membrane (Mb), mitochondria (Mc), endoplasmic reticulum (ER), Golgi body (Gb), nucleus (Nu), and nucleolus (n). Scale bar for A is 10 μm and for B and C it is 5 μm .

Figure 5.6

Transmission electron microscopy images of previtellogenic, vitellogenic and postvitellogenic oocytes of *Neotrigonia margaritacea*; A) previtellogenic oocytes (PvO) with large nucleus (Nu) mitochondria and a waste number of cortical granules (Cg) in cortical cytoplasm (Cc); B) close up of vitellogenic oocytes (Vo) showing mitochondria (Mc), endoplasmic reticulum (ER), microvilli (Mv), lipids (L), yolk granules (Y); C) post vitellogenic oocytes (PV) with large nucleus (Nu), microvilli (Mv) and extensive vitelline layer (VI). Scale bar for A and B is 5 μm and for C it is 10 μm .

Figure 5.7

Transmission electron microscopy images of *Neotrigonia margaritacea*; A) close up of previtellogenic oocytes (PVo) showing mitochondria (Mc), and microvilli (Mv) which are also depicted in B) and involved in lipid (L) transfer from cytoplasm to vitelline layer (VI). Note large vitelline space (vs) between postvitellogenic cytoplasm and vitelline layer; C) depicts post vitellogenic oocytes (Pv) with a furrowed vitelline layer (VI) above the opening, depicted in D) opening is a micropyle (Mp). Scale bar in A is 1 μm , in B 2 μm and in C and D it is 10 μm .

Figure 5.8

Temporal variation in *N. margaritacea* reproductive status over the summer months for A) gonad weight and B) mean proportion of oocytes in different stages of oogenesis.

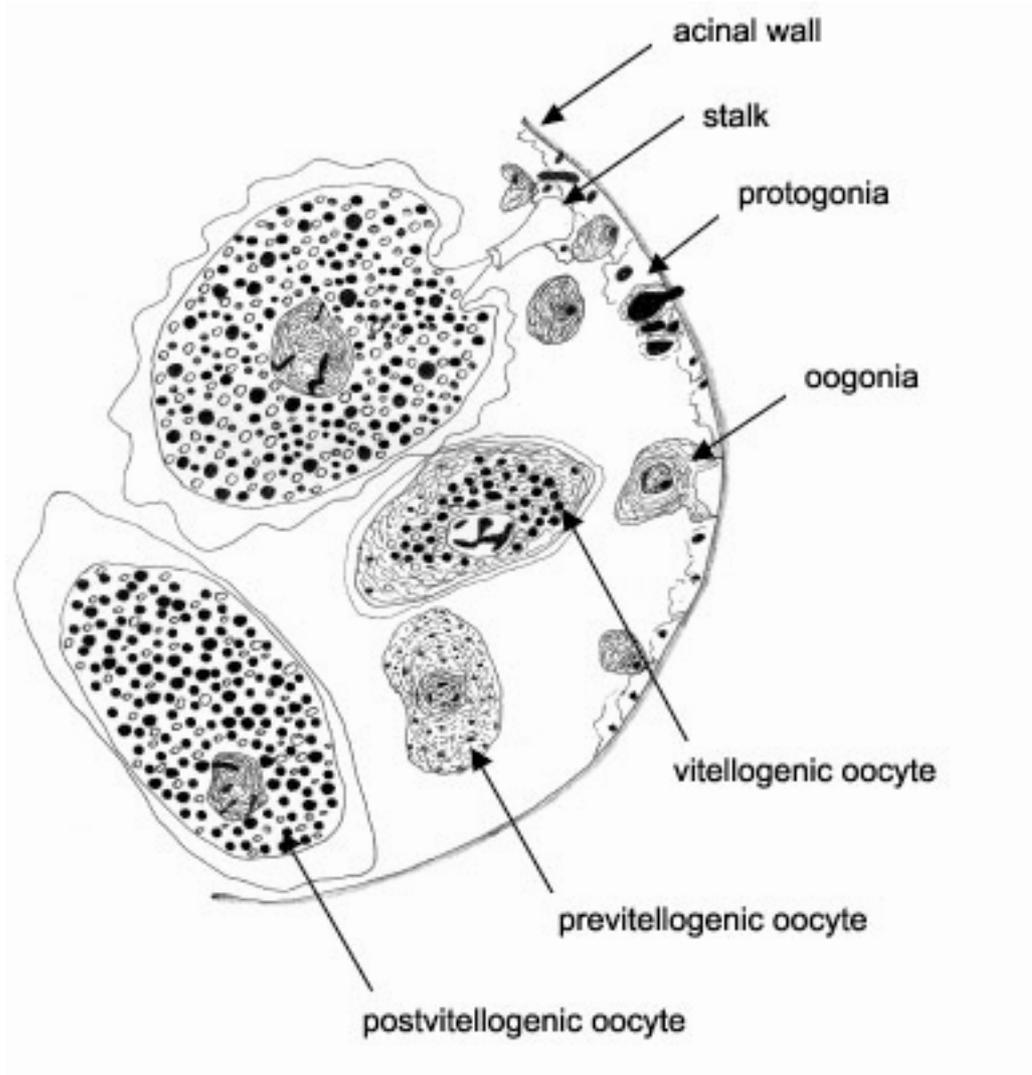


Figure 5.1

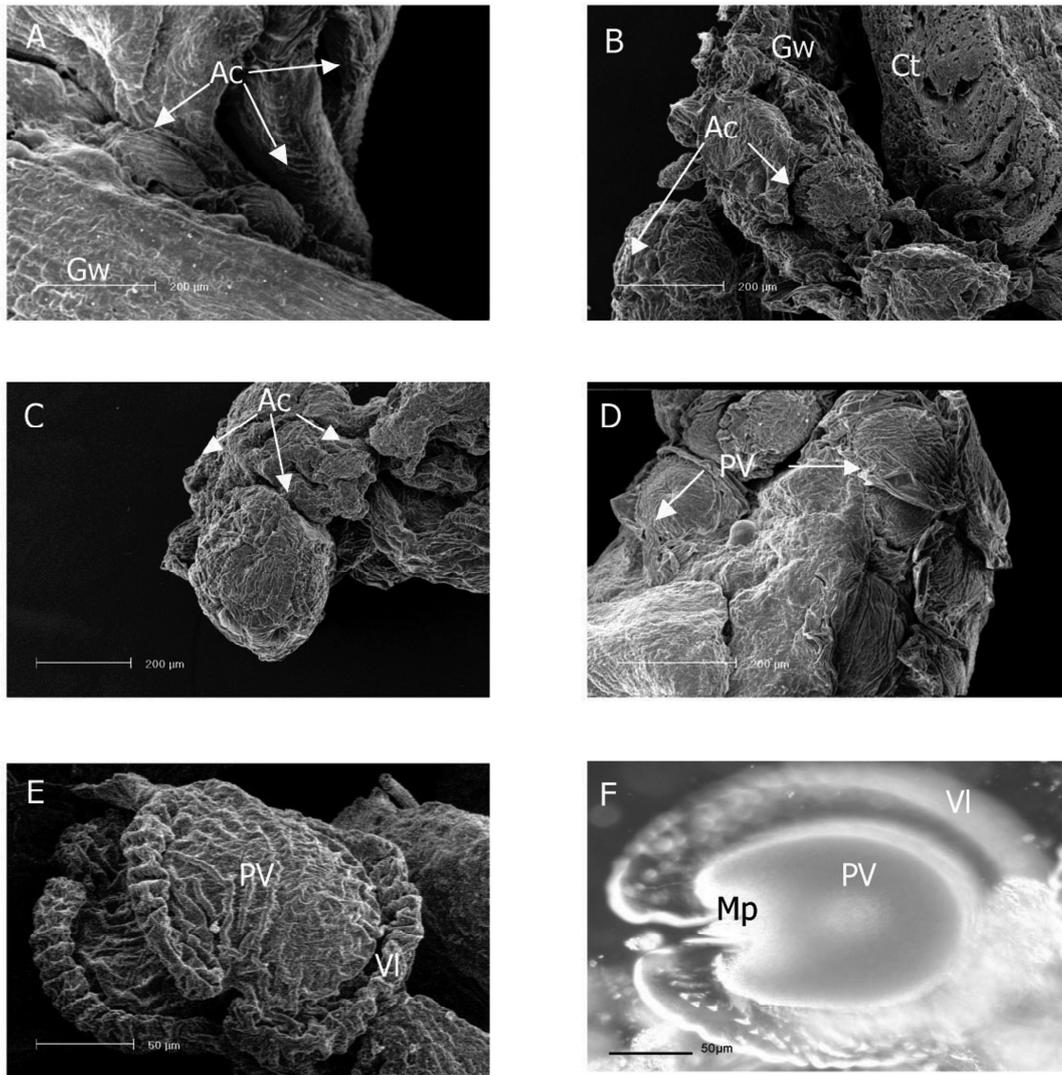


Figure 5.2

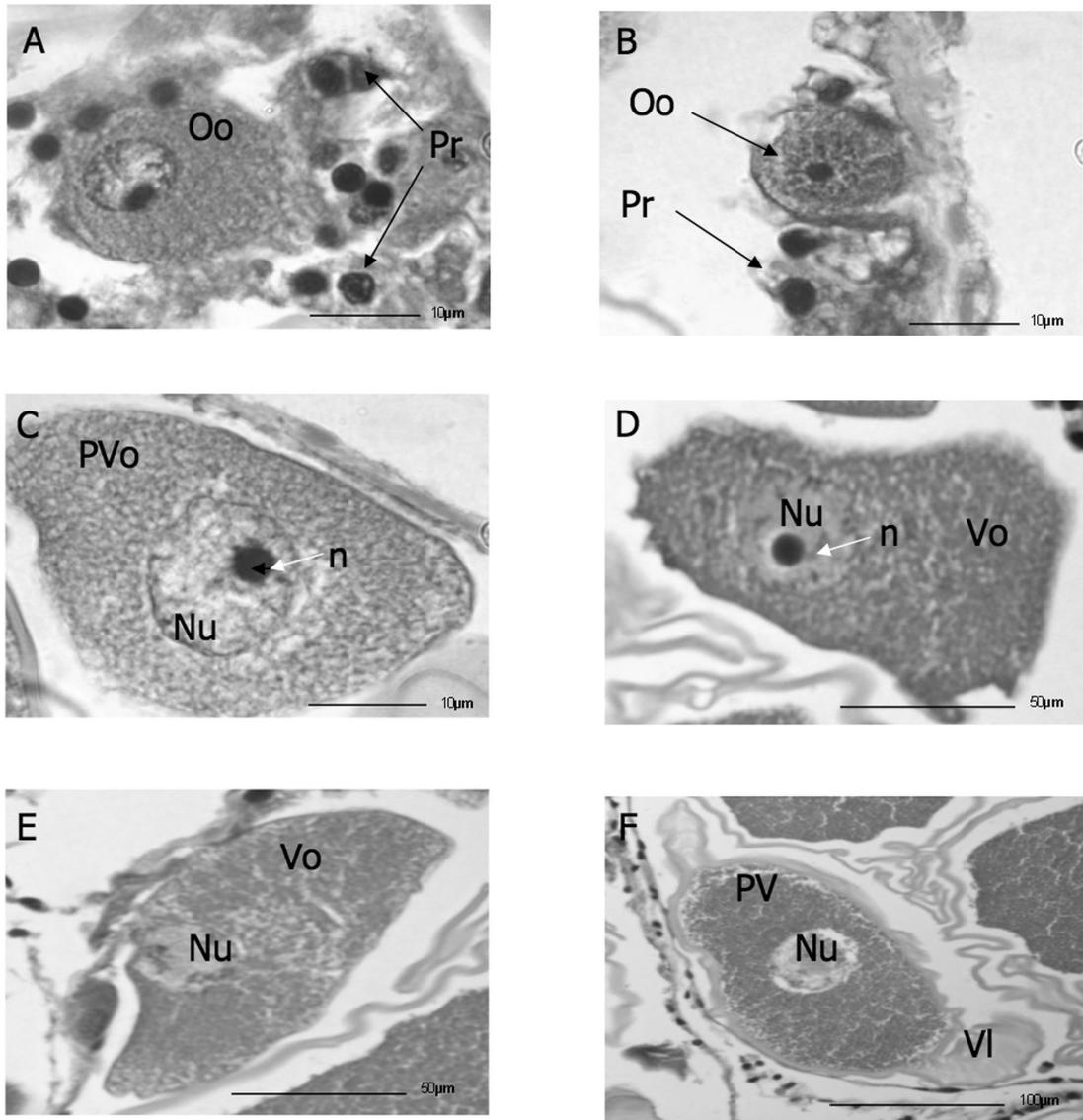


Figure 5.3

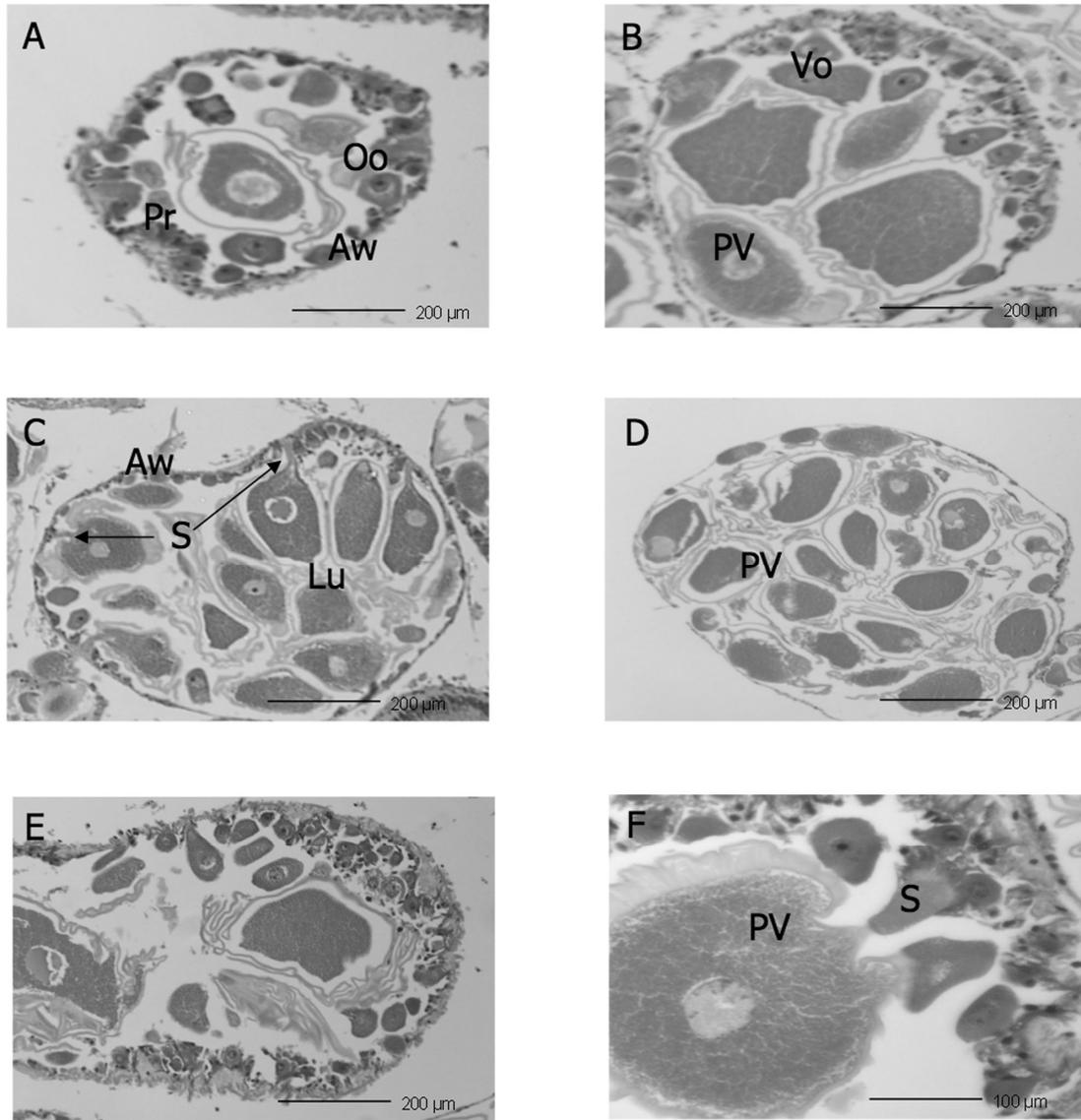


Figure 5.4

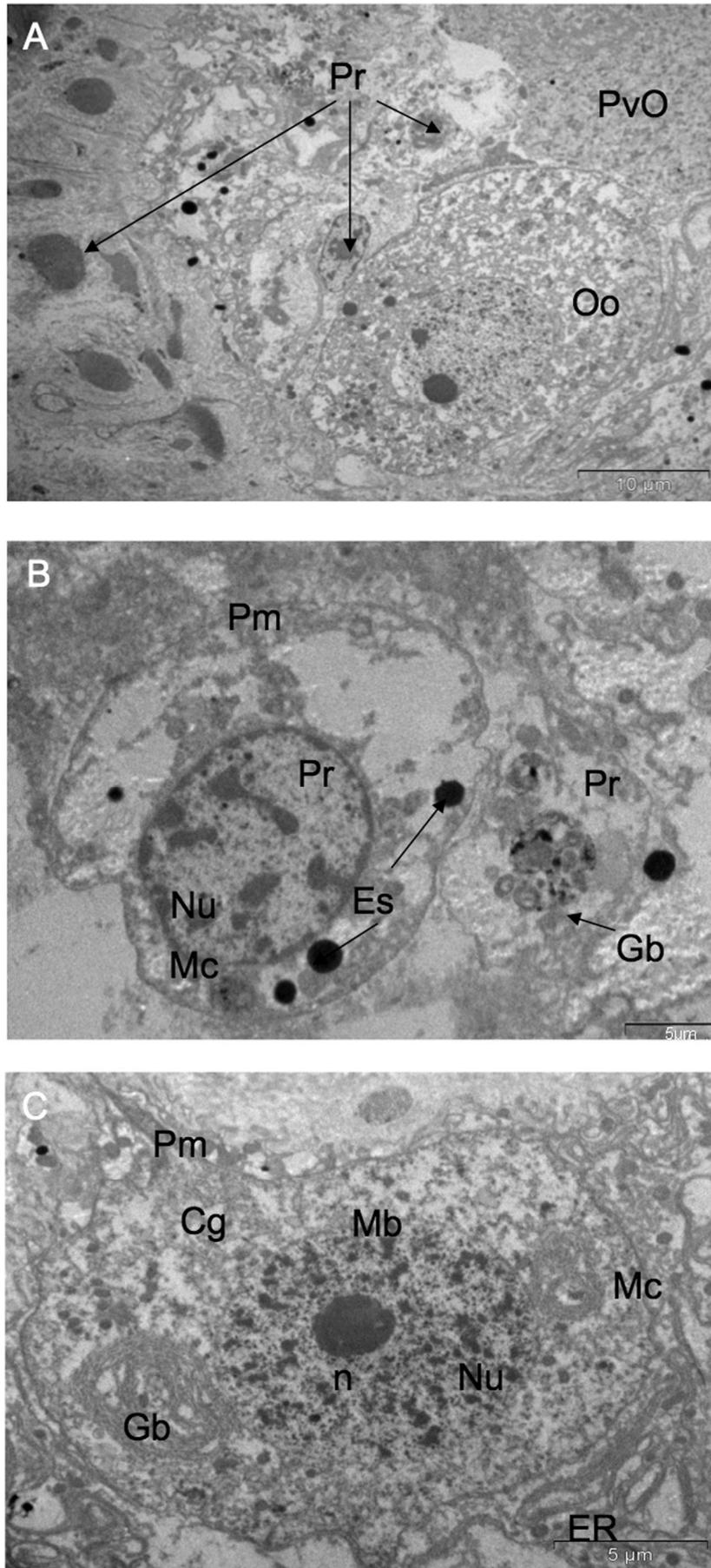


Figure 5.5

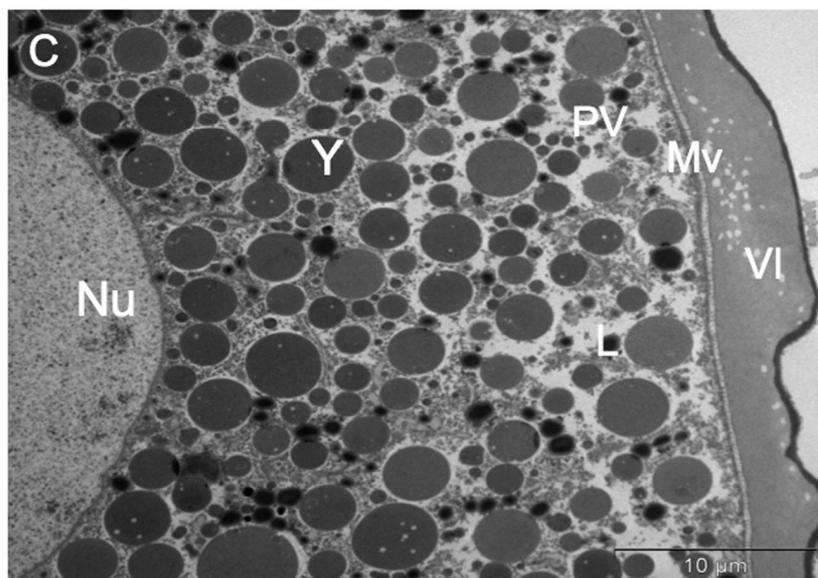
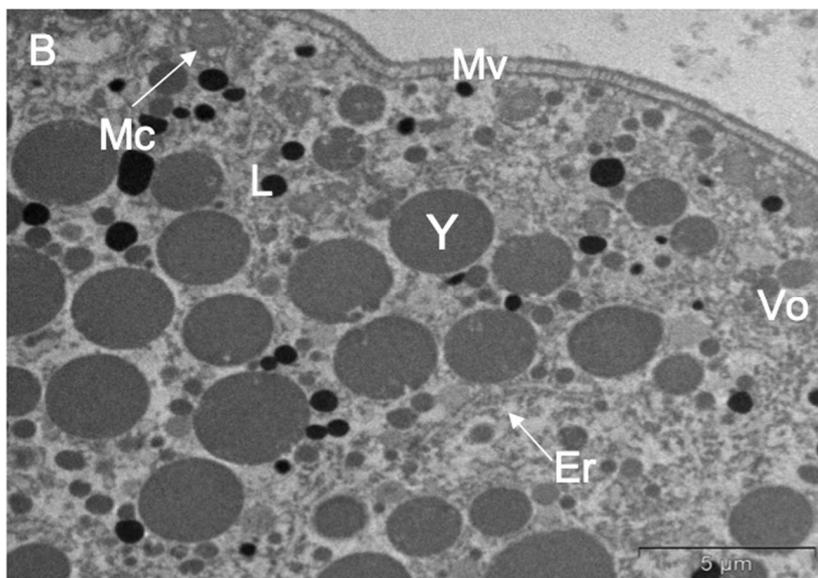
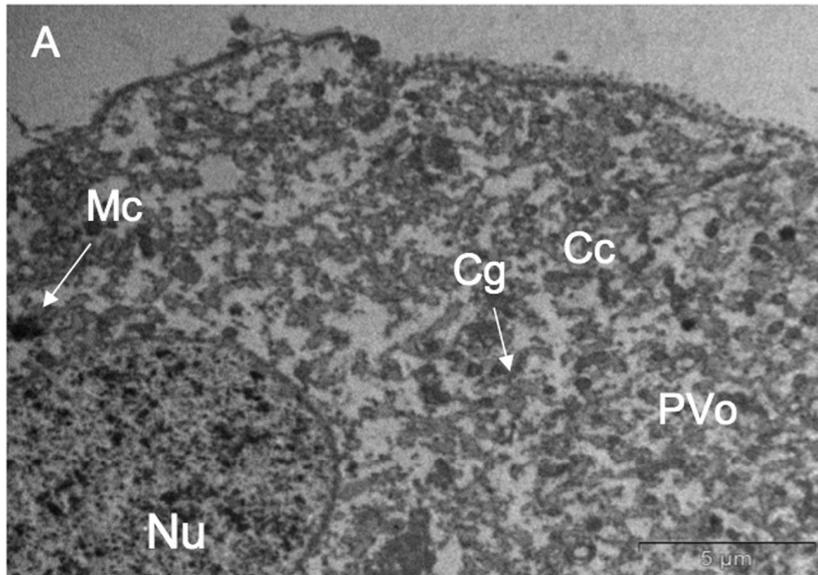


Figure 5.6

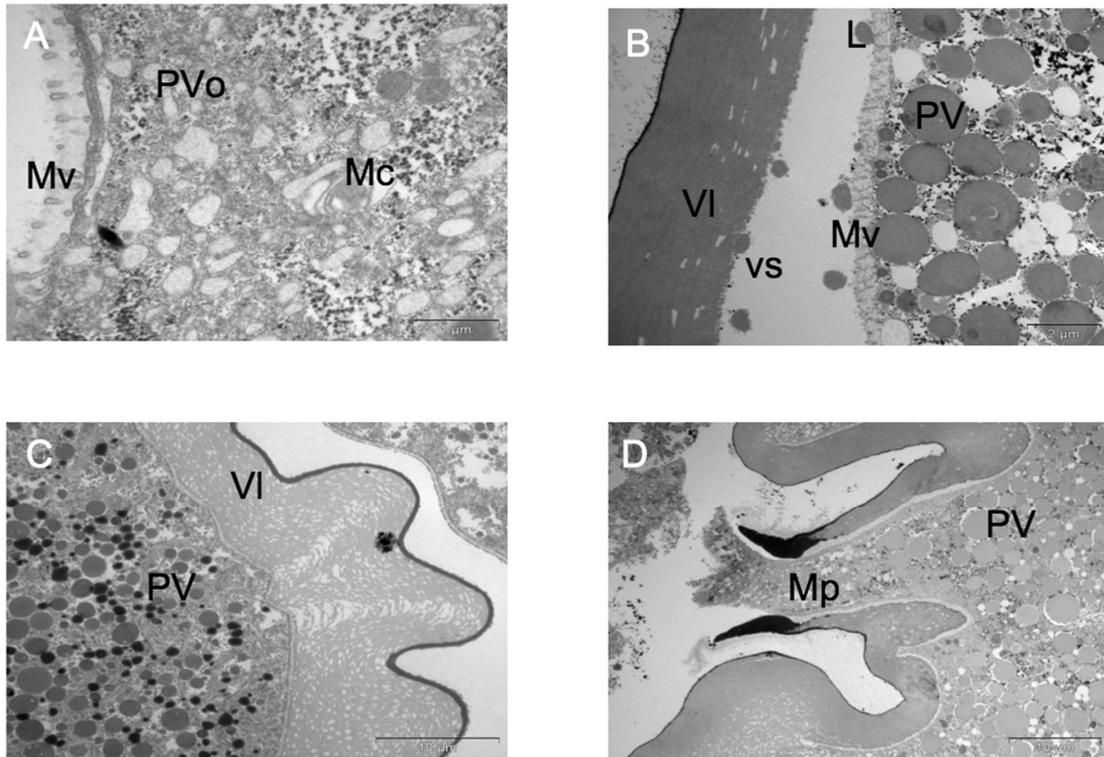


Figure 5.7

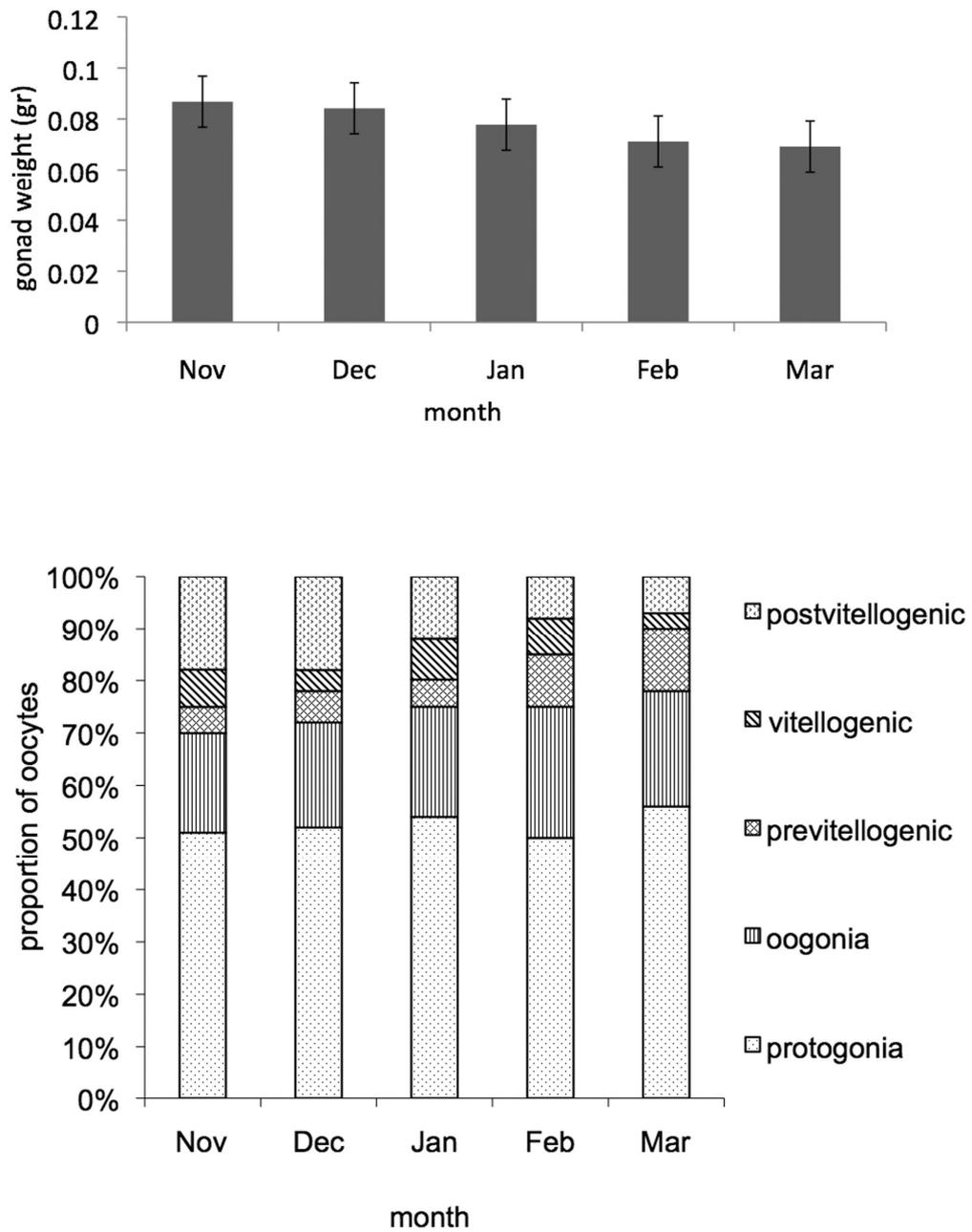


Figure 5.8

General Discussion

The research presented in this thesis was stimulated by a current gap in our knowledge in regards to the systematics and phylogeny of *Neotrigonia*. While museum collections, previous publications and current systematics have provided valuable information about some aspects of the evolutionary affinities of *Neotrigonia*, molecular and morphological analysis of freshly collected specimens has enabled me to examine phylogenetic relationships within the genus. From the results present in this thesis four important conclusions can now be drawn:

1. Molecular and morphological data supported the synonymization of *N. bednalli* with *N. margaritacea* (Chapters 2 and 3);
2. The Southern Australian *Neotrigonia margaritacea* has a strong genetic structure and high haplotypic diversity based on COI and ITS1 data (Chapter 3);
3. Doubly Uniparental Inheritance is present in *Neotrigonia margaritacea* and it has undergone a masculinization event (Chapter 4); and
4. The process of oogenesis is established for *Neotrigonia margaritacea* (Chapter 5) and sequentially tachitictic spawning is inferred as a reproductive mechanism.

Extant Species of *Neotrigonia*

Evidence from the morphological and molecular systematics approach of this research, along with newly-acquired knowledge of mitochondrial inheritance and life history, present a renewed perspective of evolution of the *Neotrigonia*. Specimens of *Neotrigonia* from museum collections world wide, newly-collected specimens and all available types were utilized to revise species status and distribution. This was a challenging and laborious task as many of the museum collections have not been curated since late 1960s. However, the Australian Museum *Neotrigonia* collection was last worked on in 1990's by Lamprell and Whitehead (1992), and was the most important collection in this study. The Australian Museum collection holds the largest number of specimens ranging across the seven extant nominal species. The results based on morphological characters do not reject species status for *N. gemma*, *N. lamarckii*, *N. uniophora*, *N. strangei* and *N. kaiyomaruae* but suggest that *N. bednalli* is a junior synonym of *N. margaritacea*.

Further research on molecular and morphological characters needs to be conducted for all extant species of *Neotrigonia* to confirm their species status. The species rank is used as a representation of an irreducible phylogenetic entity, thus representing a unique role in interpreting evolutionary pathways. This view dates back to Darwin (1859), who regarded evolution to equate to “the origin of the species”, and is supported by many other works that emphasized the evolutionary importance of the species rank (Dobzhansky, 1937; Cain, 1954; Mayr, 1963; Ereshefsky, 1992). In the past decade, much philosophical debate was concentrated around the species rank and what it means for interpreting evolutionary pathways for a particular group of organisms. This promoted an alternative view, where the species rank does not represent a valid taxonomic category, because the phenetic, cohesion and monophyletic species concepts do not delimit species-level taxa that are distinct from lower or higher taxa (Pleijel, 1999; Ereshefsky, 2000; Pleijel and Rouse, 1999, 2000; Lee, 2003). In contrast, interbreeding concepts delimit species-level taxa characterized by gene flow not found in higher taxa, and thus justify the continued use of the species category (Lee, 2003). Given the extensive use of the term ‘species’ across much scientific literature, both in systematic and ecological use, it would be an abrupt move to discontinue the species rank. Therefore Pleijel and Rouse (1999, 2000) and Lee (2003) suggested the use of combined morphological and molecular data in defining a species rank or that the species rank is replaced by a category of Least Inclusive Taxonomic Unit (LITU) based on all available information. Ideally this approach should be conducted on all remaining *Neotrigonia* species if we are able to collect fresh specimens from all type localities. This approach is adopted by the Assembling Tree of Life project (AToL) (AToL, 2010). The AToL project is designed to provide for more comprehensive scientific communication and information sharing about the evolution of life forms. At the moment the BivAToL, a large project investigating evolutionary relationships within the bivalves is underway (BivAToL, 2010). Therefore results from this thesis and all future taxonomic research on *Neotrigonia* should be shared to meet the goals of BivAToL and ultimately AToL.

Phylogeography

According to a very comprehensive review of the state of southern hemisphere phylogeography by Beheregaray (2008), the Australian scientific community is in the top-five most-productive countries in this discipline. However, when research is

classified based on the continental origin of the organisms, Australia comes up as the last continent (Beheregaray, 2008). The intention of current research on phylogeography of the *Neotrigonia* was not only to contribute to this scientific knowledge but also to spark an interest for future research in this part of the world. As a research subject the *Neotrigonia* is an ideal candidate, having rich fossil record and wide geographic distribution around the Australian continent (Chapter 2). It provides for an integrative field of research, which encompasses many areas of biology as well as the historical and geographical disciplines of earth science.

Species of *Neotrigonia* occur along the coast of Australian continent, with prevailing species diversity on the east coast (Chapter 2). Except for *N. kayiomaruae*, which occurs on the west coast, and *N. margaritacea*, which has a widespread southern distribution, all other *Neotrigonia* species occur on the east coast (Chapter 2). The biogeographical disjunction detected between western and eastern Australian species can be correlated with a palaeogeographical barrier in the Bass Strait region. This isthmus was exposed on numerous occasions for extensive periods over the last 3 my (Dartnall, 1974; Davies, 1974). Therefore this genus range appears to have a biogeographic structure similar to that found in several other phylogenetic studies of southern Australian marine invertebrates (e.g. O'Loughlin *et al.*, 2003; O'Hara and Poore, 2000; Kassahn *et al.*, 2003; Waters and Roy, 2003a,b; Waters *et al.*, 2004, 2005). This southern temperate disjunction can be compared to the Atlantic vs Gulf coasts of Florida (Avice, 2000), the Indian vs Pacific Ocean coasts of Northern Australia (Benzie, 1999a, b), or the Indian vs Atlantic Ocean coasts of South Africa (Lessios *et al.*, 2003).

In contrast, the distribution of *Neotrigonia margaritacea* stretches across the three main temperate provinces (*sensu* Bennett and Pope, 1953): Peronian (south-east Australia), a Flindersian (south-west) and a Maugean (Tasmania and southern Victoria) (Chapter 3). The present distribution of *Neotrigonia margaritacea* can be attributed to contemporary ocean currents and the importance of geological, climatic and hydrographic history. Only low levels of divergence exist between the South Australian, Victorian and Tasmanian populations of *N. margaritacea* (Chapter 3), with no data to support a vicariant event across Bass Strait. However, significant genetic structure exists between populations of *N. margaritacea* (Chapter 3). The level of divergence shown between the western (Port Lincoln and Gulf St. Vincent) and eastern populations (Bruny Island and Western Port) is no greater than any other,

indicating that recolonization may have originated from a westerly source population. The occasional reversal of the Leeuwin Current (Baines *et al.*, 1983; Kaempf *et al.*, 2009) reduces the potential for dispersal from west to east, creating a source and sink dynamic within western populations. This also seems likely given that the Bass Strait land bridge was last flooded from a westerly direction after the last glacial maximum (Lambeck and Chappell, 2001). Similar phylogeographic results have been reported by Helgen and Rouse (2006) for South Australian crinoid species.

This idea would have to be tested with further sampling of intermediate populations on either side of the Bass Strait land bridge. Acquiring live specimens was attempted, and at times was successful (Appendix I), but *Neotrignia* species occur in patchy subtidal distributions and are very difficult to find. Further phylogeographic research on *Neotrignia* would benefit from collection of all extant species in large-enough sample numbers from available populations and ideally from a larger number of populations over a smaller geographical scale. An approach like this would enable detection of shared and currently missing haplotypes, and would also provide a better indication of gene flow. This would enable a better understanding of the history of these populations.

Doubly Uniparental Inheritance

The presence of Doubly Uniparental Inheritance (DUI) in *Neotrignia* (Chapter 4) can potentially complicate haplotype studies, because divergence levels between male and female mitotypes from the same population due to DUI is greater than sequence divergence between females from different populations. This phenomenon of DUI is now known to occur in six bivalve taxa: Mytiloidea, Unionoidea, Tellinoidea, Solenoidea, Veneroidea (Skibinski *et al.*, 1994a,b; Zouros *et al.*, 1994a,b; Liu *et al.*, 1996; Hoeh *et al.*, 1996, 2002; Passamonti and Scali, 2001; Curole and Kocher, 2002; Serb and Lydeard, 2003; Passamonti, 2007; Theologidis *et al.*, 2008) and Trigonioidea (Chapter 4). Doubly uniparental inheritance in the *Neotrignia* is most likely a result of a masculinization event. This result is most similar to the process of DUI previously described for the genus *Mytilus* (Hoeh *et al.*, 1997) but the sequence of events that led to masculinization remain unknown. One possible mechanism is supported by a study on mtDNA transmission in *Mytilus* (Skibinski *et al.*, 1994b), where some males failed to pass either F or M mitotype to their sons. Thus there is direct evidence that in the absence of an M mitotype in a

male, the F mitotype will assume the role of the M mitotype in the sperm (Skibinski *et al.*, 1994b). This explains a small level of divergence between gonad and somatic F mitotype and gonad-derived recently masculinized M mitotype. Although the information existing so far on this process is limited to a few cases, our understanding could be further improved by taking larger sample numbers and monitoring consecutive generations from a designated population.

According to the phylogenetic results in Chapter 4, *Neotrigonia margaritacea* and *N. lamarckii* do not affiliate according to gender. This result could be misinterpreted as an independent origin of DUI in the genus *Neotrigonia*, where in fact the reversals in the route of mitotype transmission have resulted in new divergence of F and M mitotypes. This supports the likelihood of a masculinization event taking place in *Neotrigonia margaritacea*. The derived position of M mitotypes relative to F mitotypes (gonad and somatic tissue DNA) shown by phylogenetic results (Chapter 4, Fig. 4.2 and 4.3) and their close genetic proximity depicted in a parsimony network (Chapter 4, Fig. 4.1) suggest that the paternally-inherited M mitotypes were recently spawned from the female mitotype.

Confirmation of DUI presence in *Neotrigonia margaritacea* enables us to assume that DUI has been present within the Bivalvia for more than 250 million years. DUI is a unique plesiomorphic trait, and the phylogenetic results from this research are consistent with the hypothesis of Hoeh *et al.* (1998, 2002) and Walker *et al.* (2006), which is that DUI has been operating in the Palaeoheterodonta prior to the trigonioid-unionoid divergence. This further supports the hypothesis of single origin of DUI within the Bivalvia. It is accepted that a complex character, such as DUI, is more easily and frequently lost than gained (Budd and Jensen, 2000).

These results can only be confirmed by examination of, in first instance, gonad- and somatic-derived DNA for both sexes of *N. lamarckii*, and then, if possible, the same analysis should be conducted for all *Neotrigonia* species. Also additional genes, such as the cytochrome *c* oxidase subunit II gene (MCox2e) or the tRNA histidine (TrnH), which have been informative for other taxa (Theologidis *et al.*, 2008; Doucet-Beaupre *et al.*, 2010), should be analysed for *Neotrigonia*. It has also been shown that total genome analysis would be beneficial because DUI can be defined by a large amount of nucleotide substitution that falls within the coding region, in addition to divergence levels between male and female mitotype sequences (Brenton *et al.*, 2006).

Oogenesis

Routinely the reproductive anatomy and larval characteristics of living specimens are used to broaden our understanding of bivalve evolution, in particular, of the Paleoheterodonta group, which contains both brooders and spawners (Graf and Cummings, 2006). Chapter 5 describes the oogenesis of *Neotrigonia* for the first time, using histologic and microscopic techniques to clearly outline characteristics of the protogonia, oogonia, previtellogenic, vitellogenic and postvitellogenic oocytes. The general features of the *N. margaritacea* gonad and oogenesis processes are similar to most bivalves from the subclasses Pteriomorpha (Albertini, 1985; Pipe, 1987; Fang and Qi, 1988; Bernard *et al.*, 1988; Dorange and Le Pennec, 1989; De Gaulejac *et al.*, 1995), Veneroidea (Al-Mohanna *et al.*, 2003), and Unionoidea (Won *et al.*, 2005). In comparison to the oocytes of *Margaritifera margaritifera* and *Unio elongatulus*, which at most reach 83 and 150 μm in diameter respectively (Focarelli *et al.*, 1988; Grande *et al.*, 2001), *N. margaritacea* produces larger oocytes of 200 μm in diameter. *Neotrigonia margaritacea* oocytes are thus on the whole larger in size than Unionoidea oocytes, with larger nucleus and cytoplasm holding yolk and lipid granules. This would provide nutrients for prolonged oocyte survival during trickle spawning, and lecithotrophic larval development (Ó Foighil and Graf, 2000). I attempted larval development experiments following Sastry (1979) but with very little success, mainly due to small numbers of mature individuals and a lack of synchrony in male and female spawning in the laboratory. Future research should address larval development and therefore successfully identify all stages of reproductive development in *Neotrigonia margaritacea*. Furthermore information about larval morphology and traits could also be developed into phylogenetically-informative characters. This would further explore the portion of variable larval traits that are homoplastic (i.e. they require convergences, parallelisms, or character reversals) with respect to specific molecular-based genealogical hypotheses (Strathmann and Eersisse, 1994).

Conservation implications of these findings

Species names are also important tools in conservation. As pointed out by Dall (1997), species selected for conservation actions are almost always those that can be recognized and have definite names. Likewise groups selected for monitoring of distribution and diversity tend to be those with robust taxonomy (New, 1999).

Haywood (1994) pointed out that there are political advantages of splitting rather than lumping species together, so as to create longer, more impressive species lists. However, excessive division or splitting, introduces a level of ignorance to the conservation approach, as many named species tend to be recognized as equivalent simply by designation, rather than by any degree of ecological or evolutionary differentiation (Haywood, 1994). Amalgamation of different forms, or lumping, in the conservation approach, often supported by legislation, represents a species as though they are invariant entities without recognising varying levels of genetic variability or adaptive capability across their geographical range (New, 1999). This research indicates that *Neotrigonia margaritacea* has a broad distribution throughout southern Australian seas and, although the *N. bednalli-margaritacea* complex has been reduced to one named species, it exhibits a clear genetic structure between populations indicative of limited gene flow (Chapter 3).

Phylogeographic analyses have also played an important role in defining evolutionary significant units (ESU), a unit of conservation below the species level (i.e. genetic biodiversity) that is often defined on the grounds of unique geographic distribution and mitochondrial genetic patterns (Moritz, 1994). The Australian legislation addresses conservation of genetically-distinct populations through the federal *Environmental Protection and Biodiversity Conservation Act 1995* (EPBCA) (Dall 1997), which focuses upon matters of so-called National Environmental Significance. The *Neotrigonia margaritacea* population in Port Stanvac is reproductively isolated from the nearest other populations found in Port Lincoln and Western Port (Chapter 3) and therefore this should be considered as an important site for the preservation of marine biodiversity. However, the EPBCA is firstly limited by only applying to Commonwealth-owned lands and therefore excludes all coastal waters within 3 nautical miles of the coast – including the SA gulfs. Secondly, the legislation only allows for listing of rare, endangered and threatened species, populations and ecological communities, via a complicated process of nomination and investigation by the national Threatened Species Committee (that can take years).

An important part of the assessment process for threatened species listing is identifying key threatening processes (KTP) (Fig. 6.1), which may cause native species or population to become eligible for listing. The Port Stanvac population of *Neotrigonia margartiacea* has been exposed to loss of habitat through beach-sand replenishing by one of the local councils and was temporarily listed as being “sub

fossil” (Shepherd and Thomas 1982). This population may be exposed to future threats caused by a desalination plant being built at Port Stanvac or ongoing wastewater disposal at Christies Beach. Currently, the Commonwealth government is assessing the giant cuttlefish (*Sepia apama*) population at Port Lowly (Spencer Gulf, SA) for listed status due to potential threats caused by a proposed desalination plant and over-fishing. Although this species is broadly distributed around the Australian coast and generally not considered endangered at that species level, it has a unique genetic structure (Kassahn *et al.*, 2003). Therefore my information about the clear genetic structure of *Neotrigonia margaritacea* populations (Chapter 3), its small or sparse population size, and how it has changed in status over time may enable a trigger for proper assessment of this species, resulting in appropriate conservation action (Fig. 6.2).

A large portion of the known *Neotrigonia margaritacea* range is in South Australian State waters. Therefore the conservation guidelines set by the State government Department for Environment and Heritage within its ‘No Species Loss’ policy, could protect this biodiversity of South Australian waters where the federal EPBCA may be ineffective. Legislation addresses a conservation approach based on genetic structure of both populations and species. In theory, the results of this thesis could be used to set priorities for conservation based on genetic data rather than artificial species limits, which may be genetically meaningless. To effectively assess a species or a population before it can be included on threatened species list, all relevant Commonwealth and State legislation needs to be addressed as illustrated in Fig 6.1. As an example Fig. 6.1 could be applied to the conservation assessment of *Neotrigonia margaritacea* population from Port Stanvac (Fig. 6.2). Initially, this population was considered as a population of ‘conservation concern’ before any other legislative processes were implemented. Following this assessment process a number of ongoing KTPs have been identified, which could potentially lead to species richness decline (see Fig. 6.2). On this basis, I suggest that the *Neotrigonia margaritacea*, population from Port Stanvac (see Fig. 6.2) should be given further conservation attention in South Australia.

Conclusions

Returning to my thesis aims, this research has enhanced the general knowledge regarding the evolutionary affinities of *Neotrigonia* species. The number

of extant species belonging to the genus is established. The species delimitation and phylogeography are established within a stable phylogenetic reconstruction. Doubly Uniparental Inheritance is detected within *Neotrigonia*. Finally, the process of oogenesis is determined for *Neotrigonia margaritacea*.

In the broad aspect of bivalve systematics, this research supports previous findings by Giribet and Wheeler (2002) in that the monophyly of the Palaeoheterodonta, comprising the Unionoidea and Trigonioidea, is confirmed. The most significant synapomorphies identified to further corroborate the monophyly are: similarities in morphology of the oocytes and reproductive tissue; as well as the oogenesis process (Chapter 5); and, in molecular terms, the presence of DUI (Chapter 4). In conclusion, this thesis advances scientific understanding about *Neotrigonia* in regards to current systematics, phylogeny, phylogeography, mitochondrial inheritance pattern and aspects of reproduction, especially oogenesis.

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Figure 6.1

Flow diagram showing a series of decisions and actions involved in the conservation assessment process. Species or populations of conservation ‘concern’ could be selected based on either State or Commonwealth legislation. Flow diagram connects activities and decision making processes that are conducted by the government bodies or brought forward by scientific research.

Figure 6.2

Flow diagram showing how Fig. 6.1 could be applied to the conservation assessment process for *Neotrigonia margaritacea* in Port Stanvac. The flow diagram connects activities and decision making processes that are currently in place indicated by a solid line, while dashed connecting lines are dependent on future research outcomes.

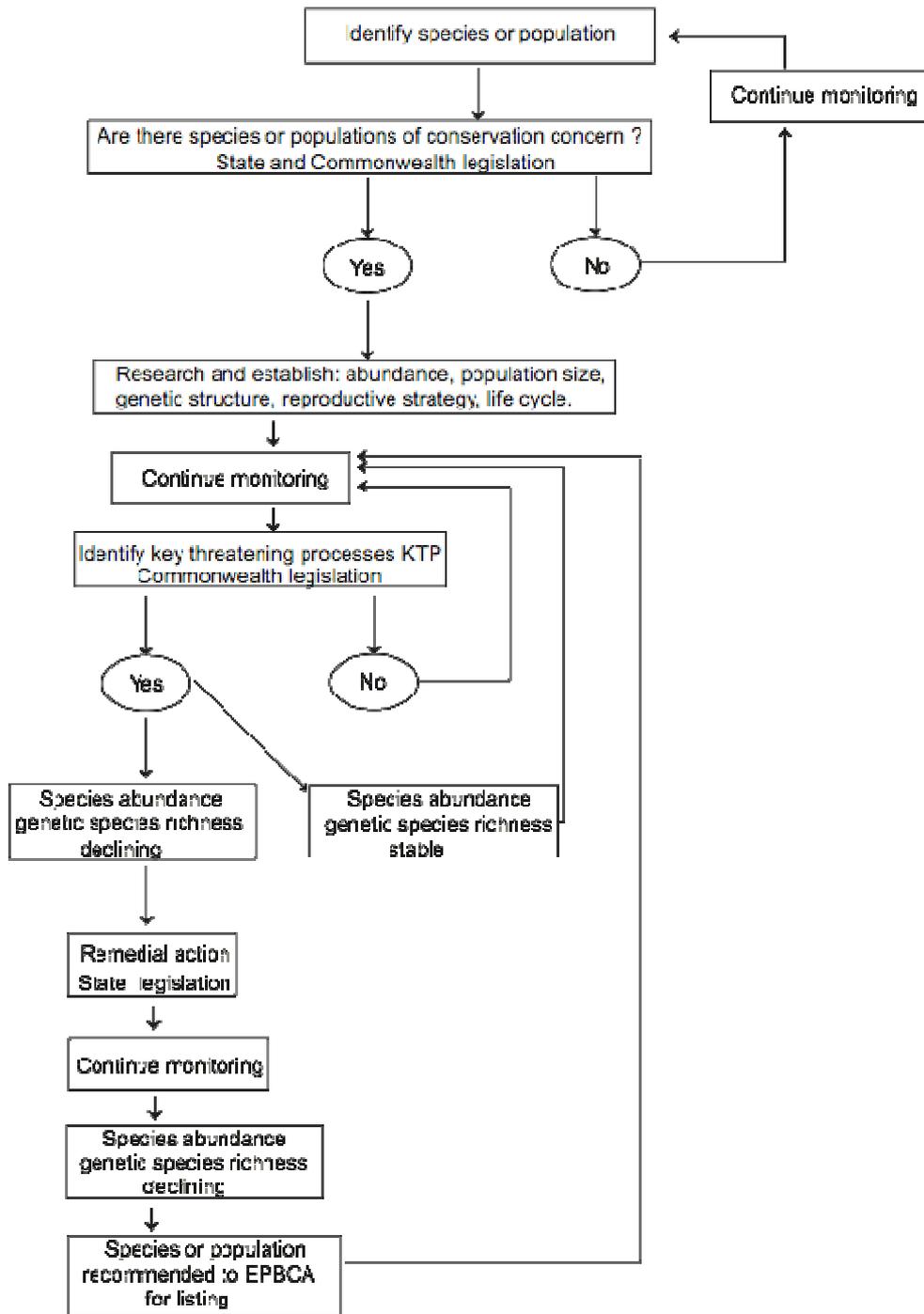


Figure 6.1

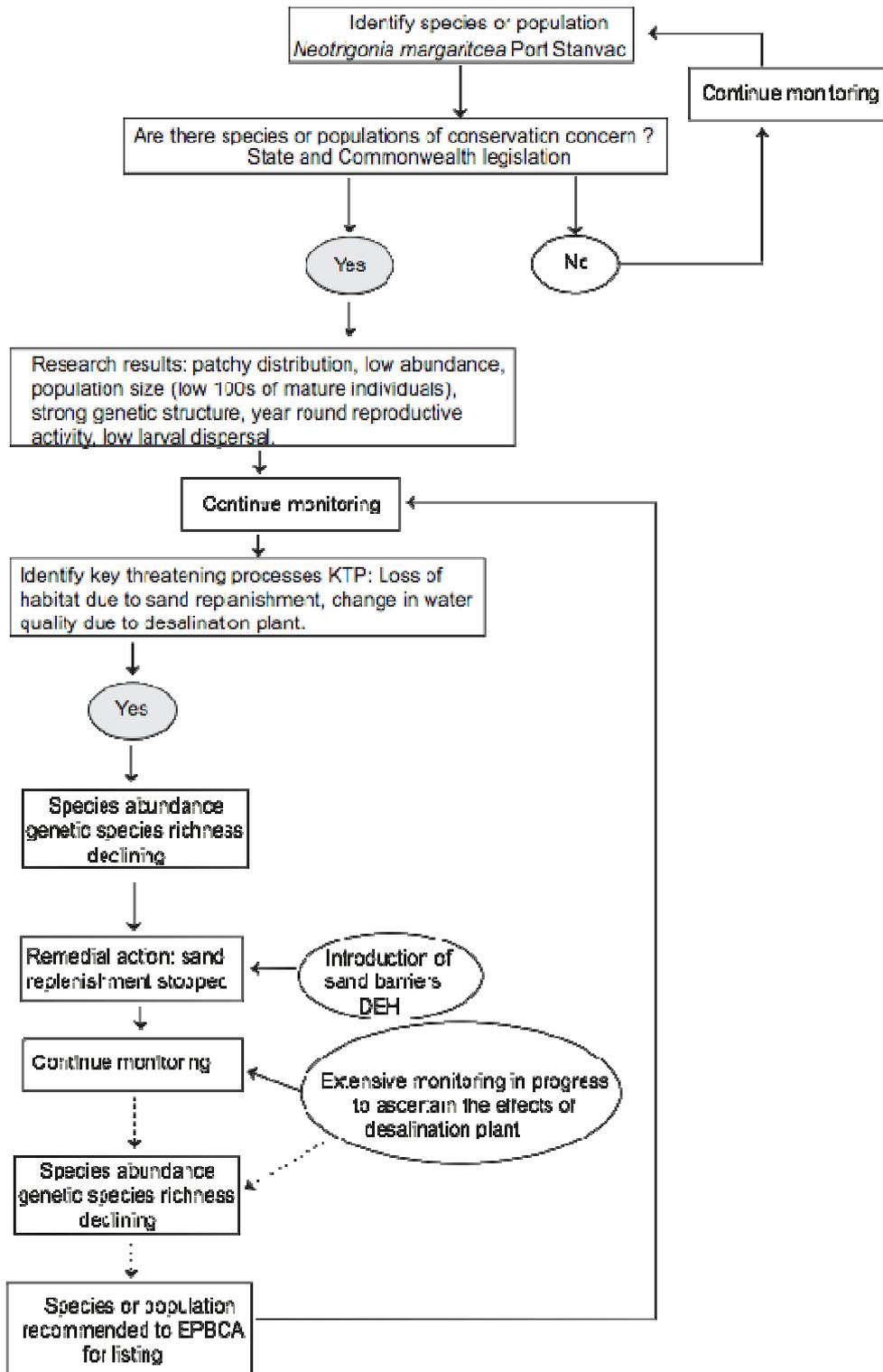


Figure 6.2

Appendix I

Appendix 1.1

Table 1.1. Listing of all collected *Neotrigonia* specimens and all locations where collection was attempted either by SCUBA diving, dredging and beach combing. Multiple SCUBA diving attempts were conducted at all locations. Some locations were explored extensively such as Brighton, Port Stanvac and Port Noarlunga SA, where diving was conducted in grid pattern over 6 months. L = Live; D = Dead shells.

State	Location	Latitude	Longitude	Collection method	Specimens obtained	Targeted specimen
Queensland	North Stradbroke Island	27°25'17.26"S	153°32'21.45"E	SCUBA diving	none	<i>N. lamarckii</i>
		27°25'19.10"S	153°32'57.09"E	SCUBA diving	none	<i>N. lamarckii</i>
		27°25'34.52"S	153°32'55.98"E	SCUBA diving	none	<i>N. lamarckii</i>
		27°23'25.12"S	153°38'4.11"E	dredging	3 L	<i>N. lamarckii</i>
	Heron Island	23°25'59.25"S	151°54'15.42"E	SCUBA diving	none	<i>N. uniophora</i>
NSW	Port Jackson	33°51'12.07"S	151°15'21.12"E	SCUBA diving	4D	<i>N. margaritacea</i>
		33°50'56.34"S	151°15'32.80"E	SCUBA diving	5D	<i>N. margaritacea</i>
		33°50'50.97"S	151°15'56.30"E	SCUBA diving	2D	<i>N. margaritacea</i>
		Eden	37° 4'54.15"S	149°56'55.60"E	SCUBA diving	none
Victoria	Western Port	38°21'10.58"S	145°14'26.81"E	SCUBA diving	20L	<i>N. margaritacea</i>
	Port Fairy	38°24'21.92"S	142°14'25.98"E	SCUBA diving	none	<i>N. margaritacea</i>
South Australia	Robe	37°10'27.41"S	139°42'45.38"E	SCUBA diving	none	<i>N. margaritacea</i>
	Kingscote	35°37'2.15"S	137°41'37.42"E	SCUBA diving	none	<i>N. margaritacea</i>
	American River	35°41'55.99"S	137°42'35.87"E	dredging	none	<i>N. margaritacea</i>
	Kings Beach	35°40'4.75"S	138°18'52.32"E	SCUBA diving	none	<i>N. margaritacea</i>

Appendix

	Victor Harbour, The Bluff	35°36'21.06"S	138°36'34.88"E	SCUBA diving	none		<i>N. margaritacea</i>
	Port Noarlunga	35° 8'59.80"S	138°27'43.46"E	SCUBA diving	3L	<i>N. margaritacea</i>	<i>N. margaritacea</i>
	Port Stanvac	38°21'10.58"S	145°14'26.81"E	SCUBA diving	15L	<i>N. margaritacea</i>	<i>N. margaritacea</i>
				dredging	40L	<i>N. margaritacea</i>	<i>N. margaritacea</i>
	Brighton	35° 1'4.51"S	138°30'38.98"E	SCUBA diving	>100D	<i>N. margaritacea</i>	<i>N. margaritacea</i>
	Wool Bay	34°59'41.31"S	137°45'37.94"E	SCUBA diving	none		<i>N. margaritacea</i>
	Edithburgh	35° 5'7.38"S	137°45'3.72"E	SCUBA diving	none		<i>N. margaritacea</i>
	Marion Bay	35°14'17.29"S	136°58'58.54"E	SCUBA diving			<i>N. margaritacea</i>
	Wallaroo	33°53'24.74"S	137°30'30.41"E	dredging	3D	<i>N. margaritacea</i>	<i>N. margaritacea</i>
	Port Lincoln	35° 9'42.79"S	135°50'41.58"E	dredging	48L	<i>N. margaritacea</i>	<i>N. margaritacea</i>
Western Australia	Albany	35° 7'37.01"S	118° 3'53.38"E	dredging	1D	<i>N. margaritacea</i>	<i>N. kaiyomarue</i>
	Margaret River	33°57'7.42"S	114°57'3.17"E	SCUBA diving	none		<i>N. margaritacea</i>
	Rottneest Island	32° 1'11.44"S	115°32'23.37"E	SCUBA diving	none		<i>N. margaritacea</i>
		31°59'11.56"S	115°32'7.23"E	SCUBA diving	none		<i>N. margaritacea</i>
	Geralton	28°48'3.86"S	114°35'18.20"E	SCUBA diving	none		<i>N. margaritacea</i>
	Monkey Mia	25°47'30.64"S	113°43'19.01"E	Beach combing	none		<i>N. uniophora</i>
	Denham	25°55'44.40"S	113°32'4.80"E	Beach combing	none		<i>N. uniophora</i>

Appendix 1.2

Standard Operating Procedure for Hand held dredge/Dredging

Purpose:

Describes the procedure used to catch and transport invertebrates from a dredge site to FUSA with as little mortality as possible.

Policy:

This method is recommended for the collection and transport of benthic invertebrates from a dredge site to laboratories in the School of Biological Sciences at Flinders University, SA.

Responsibility:

Research scientist and assistants, animal care specialist, researchers, university programs students, university programs coordinator, boat skipper.

Equipment description

Standard dredging methods that use a large dredge (2 m² opening exceeding 500kg in weight) have been shown to represent a significant pulse disturbance to the substrate and infauna (Cheshire and Miller 1996). Therefore I have designed a dredge cage manufactured by the School of Biology workshop, to be a hand-held, light weight and small in frame. In total, rope and weights included, it is 5 kg in weight. The frame is made of steel, 50 cm wide, 30 cm deep and 80cm high and is covered with galvanised mesh size of 1cm² (Fig.1.1). This dredge, of such small proportions, creates a minimum impact on substrate, does not have the ability to dig in and rip up the seagrass, but is effective in picking up benthic fauna.

Materials:

Dredge cage

Buckets

Flowing sea water

Procedures:

Note: Dredges generally capture invertebrates, however, some small species of bottom fish are also caught. The dredge is hand held, and generally small piece of sampling equipment so that it causes minimal disruption to the bottom sediments as well as injury to animals living on the bottom. The following method is used to minimize the impact on the animals.

1. A small dredge size is used to minimize bottom destruction. The length of time dredging should not exceed 5 minutes from the time the dredge is on the bottom to the time that the net is to be pulled up. Boat speed is maintained at 2 knots during dredging time. Shorter dredge time prevents animals being crushed or suffocating in dredge.
2. Once the dredge is brought on board, the contents are transferred into a large sea tray with flowing seawater. The animals should be spread out and sorted through immediately in order to prevent continued crushing and suffocation. It is useful to have a bucket of clean cold water in the sorting table in which to place fragile organisms.
3. Animals that are not targeted, such as fish, should be immediately returned to the water at the same site they were collected. The skipper of the boat will manoeuvre the boat to the dredge site if the boat has drifted.

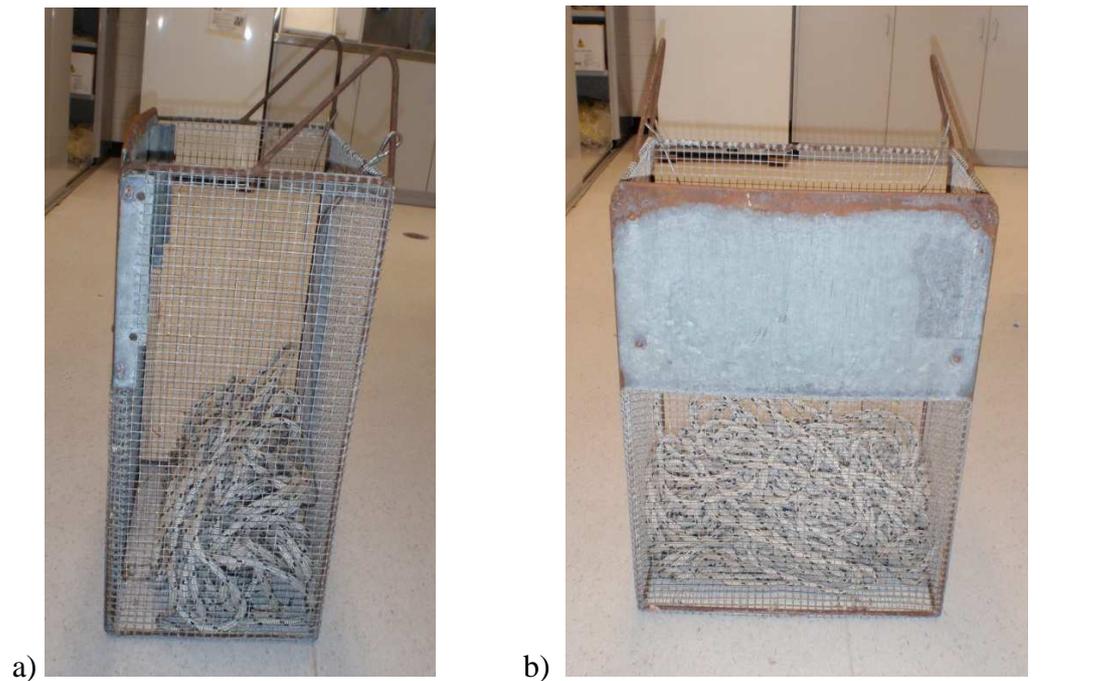


Figure 1.1.Dredge used for benthic sampling. a) side view and b) bottom view of a dredge.

References

Cheshire A.C. and Miller D.J. 1996. The potential impact of beach sand replanishment dredging on the Adelaide northern metropolitan beaches. A report to the Coast Protection Branch, South Australian Department of Environment and Natural Resources.

Appendix II

Australian connectivity interface CSIRO

Access: <http://www.csiro.com/connie2/>

Introduction

The Australian Connectivity Interface or *Aus-Connie* has been developed as a tool for environmental scientists to investigate the large-scale patterns of spatial connectivity around Australia. Specifically, it provides the user with an estimate of the probability that any two regions are connected by modelled ocean circulation over a specified dispersion period.

I attempted using this interface to predict larval dispersal for *Neotrigonia margaritacea* and by doing so investigate possible connectivity between populations tested in Chapter 3.

Method

Connectivity between all four populations (Chapter 3), was tested over five months in which *Neotrigonia margaritacea* are suspected to be reproductively active (Chapter 5). Therefore interface was run five times for each population, where populations were marked as source. The dispersal time was hypothetically chosen as 10 days which is also the minimum parameter for this analysis. Vertical level corresponds to depth at which organisms occur and on average this was 15m. The connectivity statistics were based on the year 2006, also the year sample collection was performed. This data set is the representative of an average current activity, wave action and potential seasonal influences, such as storms and winds.

Results

The Australian Connectivity Interface generates results in the form of digital maps. Dispersal is estimated and expressed in percentages. Dispersal area is highlighted on the map.

Results show lack of connectivity between any of the populations tested, over all five months (Fig. 2.1). Port Lincoln population could be exposed to effects of ocean

current the most, in comparison to other populations. Predicted larval dispersal area for the Port Lincoln is the greatest in the January. Similar level of larval dispersal is predicted for Gulf St. Vincent and Brunny Island populations while predictions for larval dispersal from Western Port population are minimal and do not change over five months. These results are expected as the location of Western Port population is in sheltered gulf system.

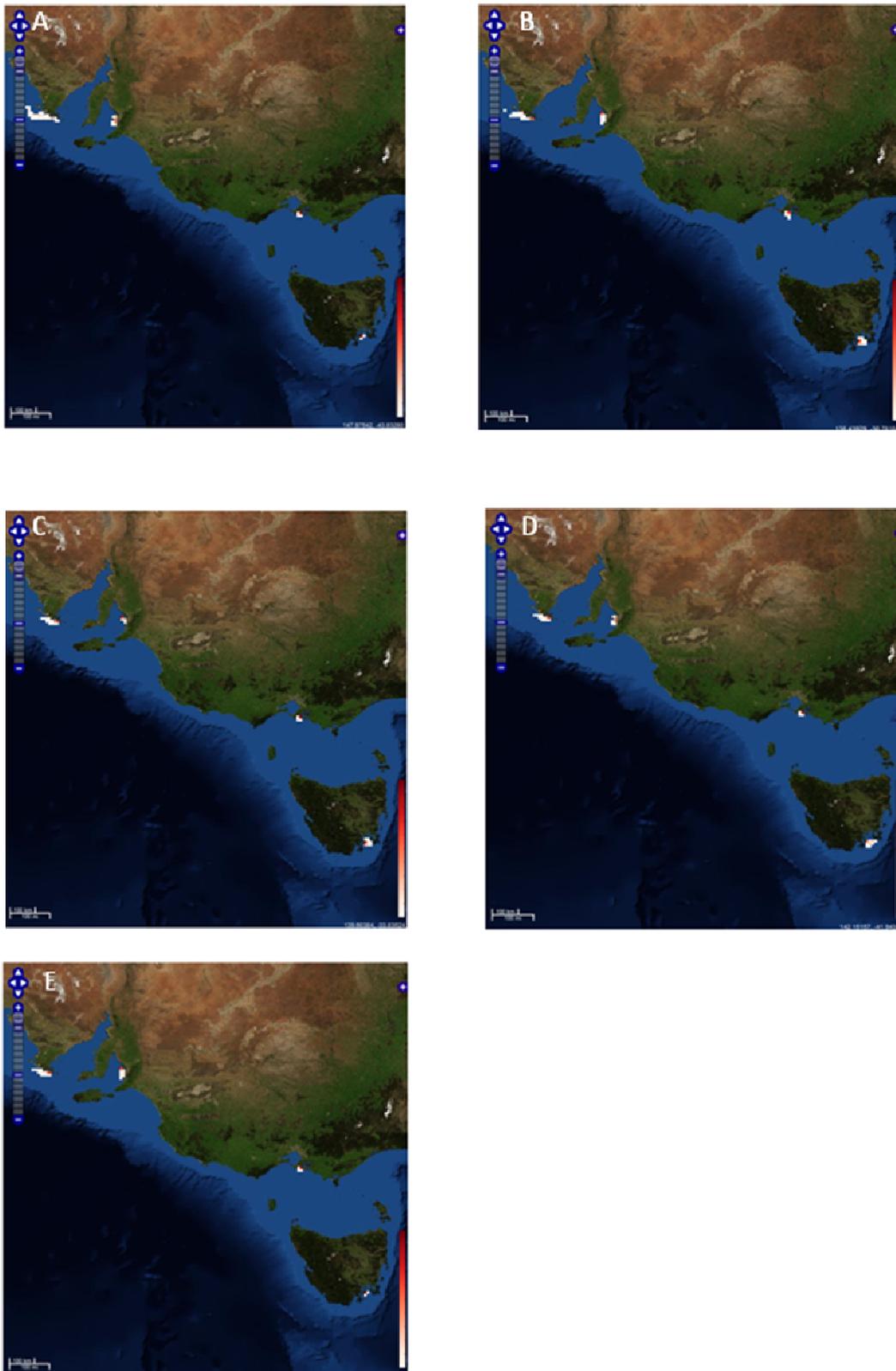


Figure 2.1. Compiled results from the Australian connectivity interface: A) January, B) February, C) March, D) November and E) December. There is no connectivity between populations, and in general very little dispersal due to ocean currents within all populations.

Appendix III

Appendix III is a collection of all trees generated as a result of data exploration and multiple analysis conducted on COI and 16s rDNA data sets (Chapter 4). Bayesian analysis and maximum parsimony analyses were conducted to explore origin of DUI within *Neotrigonia* and its evolution within Palaeoheteronta.

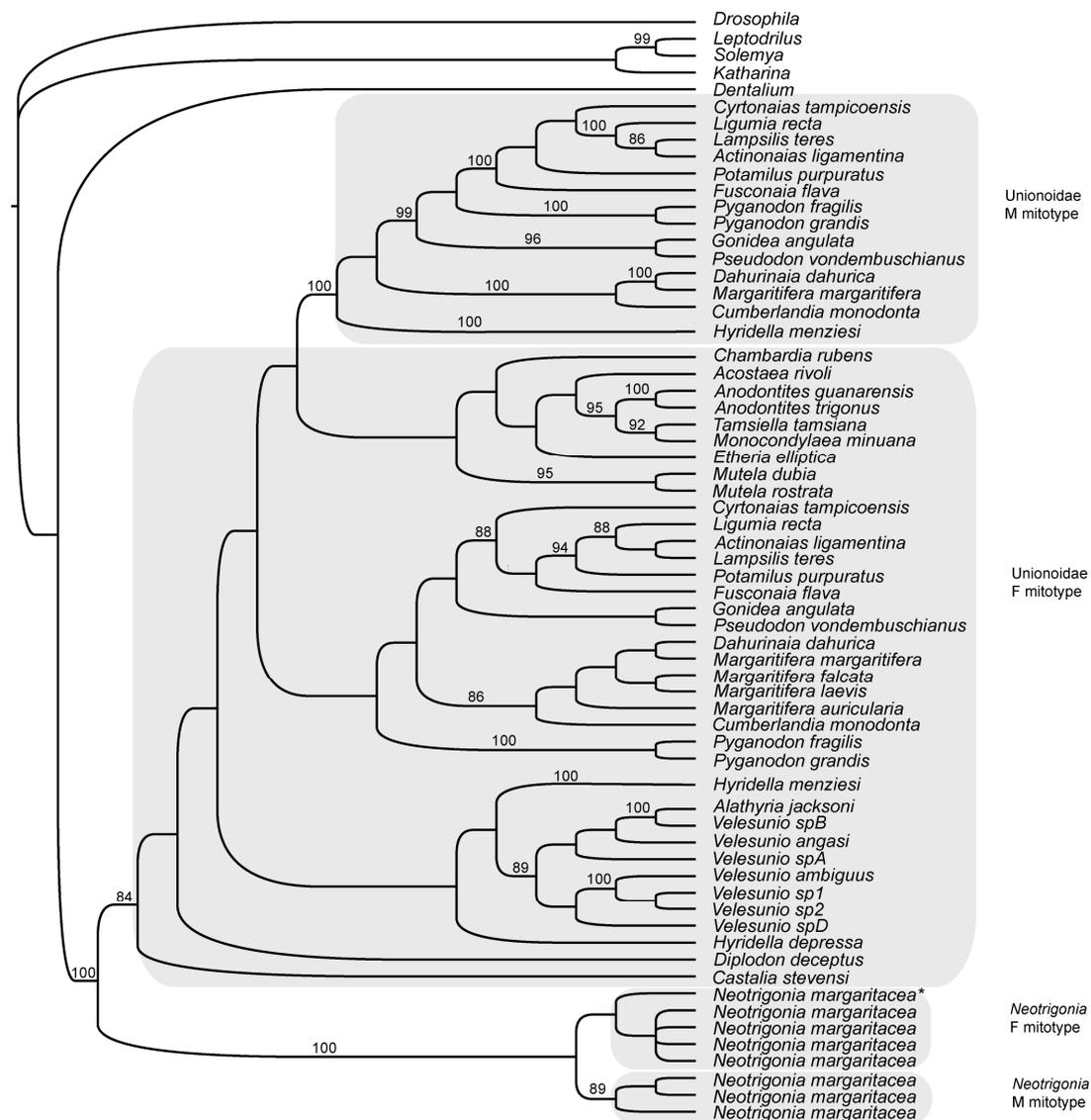


Figure 3.1. A maximum parsimony consensus tree based on Cytochrome C Oxidase subunit I using outgroup taxa as in Hoeh *et al.* (2002). MP jackknife > 85 values are shown. Terminal names correspond to NCBI accession numbers listed in Table 4.1 and Table 4.2. Male, M, and female, F, types are marked according to available information, *Neotrigonia margaritacea* GenBank sequences are annotated by an asterisk*.

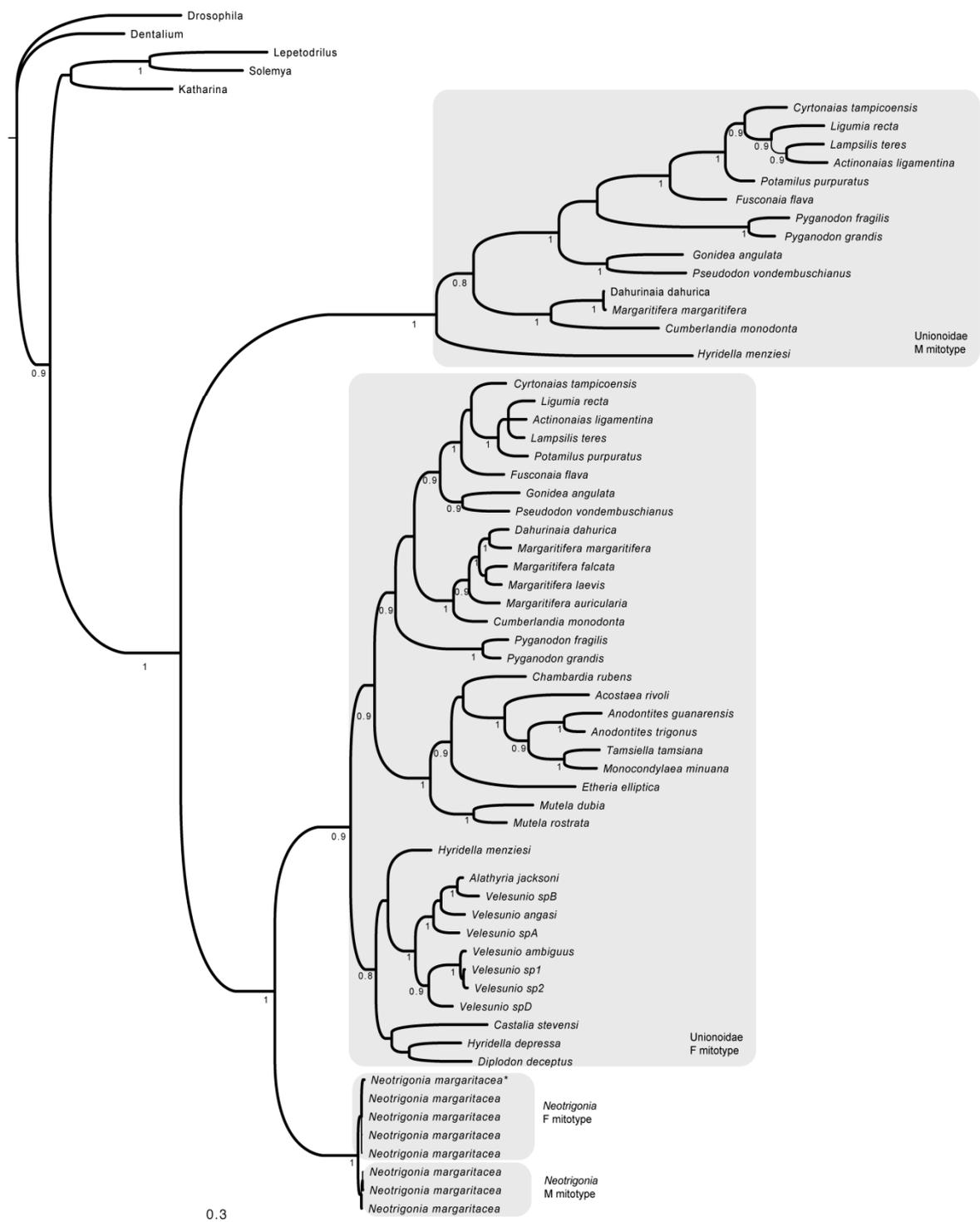


Figure 3.2. Bayesian analysis tree based on Cytochrome C Oxidase subunit I for the Unionoidae and Trigonioidae taxa with outgroup taxa as in Hoeh *et al.* (2002). Posterior probabilities (pp) > 0.95 values are shown. Terminal names correspond to NCBI accession numbers listed in Table 4.1 and Table 4.2. Male, M, and female, F, types are marked according to available information. *Neotrigonia margaritacea* GenBank sequences are annotated by an asterisk*.

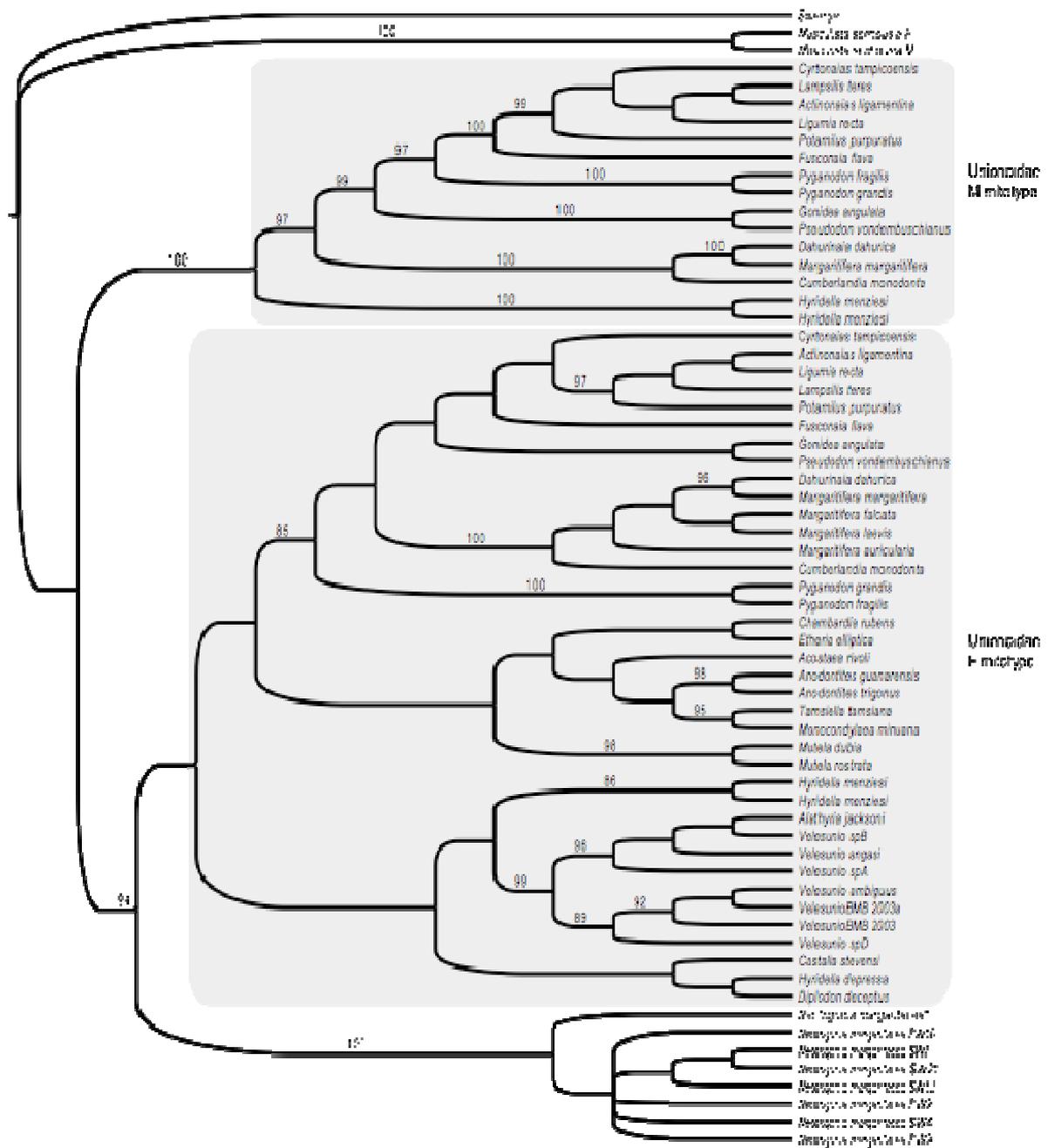


Figure 3.3. A maximum parsimony consensus tree based on Cytochrome C oxidase subunit I for the Unionoidae and Trigonioidea taxa, RY coded and less distant outgroups were used during analysis. Only MP jackknife > 85 values are shown. Terminal names correspond to NCBI accession numbers listed in Table 4.1 and Table 4.2. Male, M, and female, F, types are marked according to available information. *Neotrigonia margaritacea* GenBank sequences are annotated by an asterisk*.

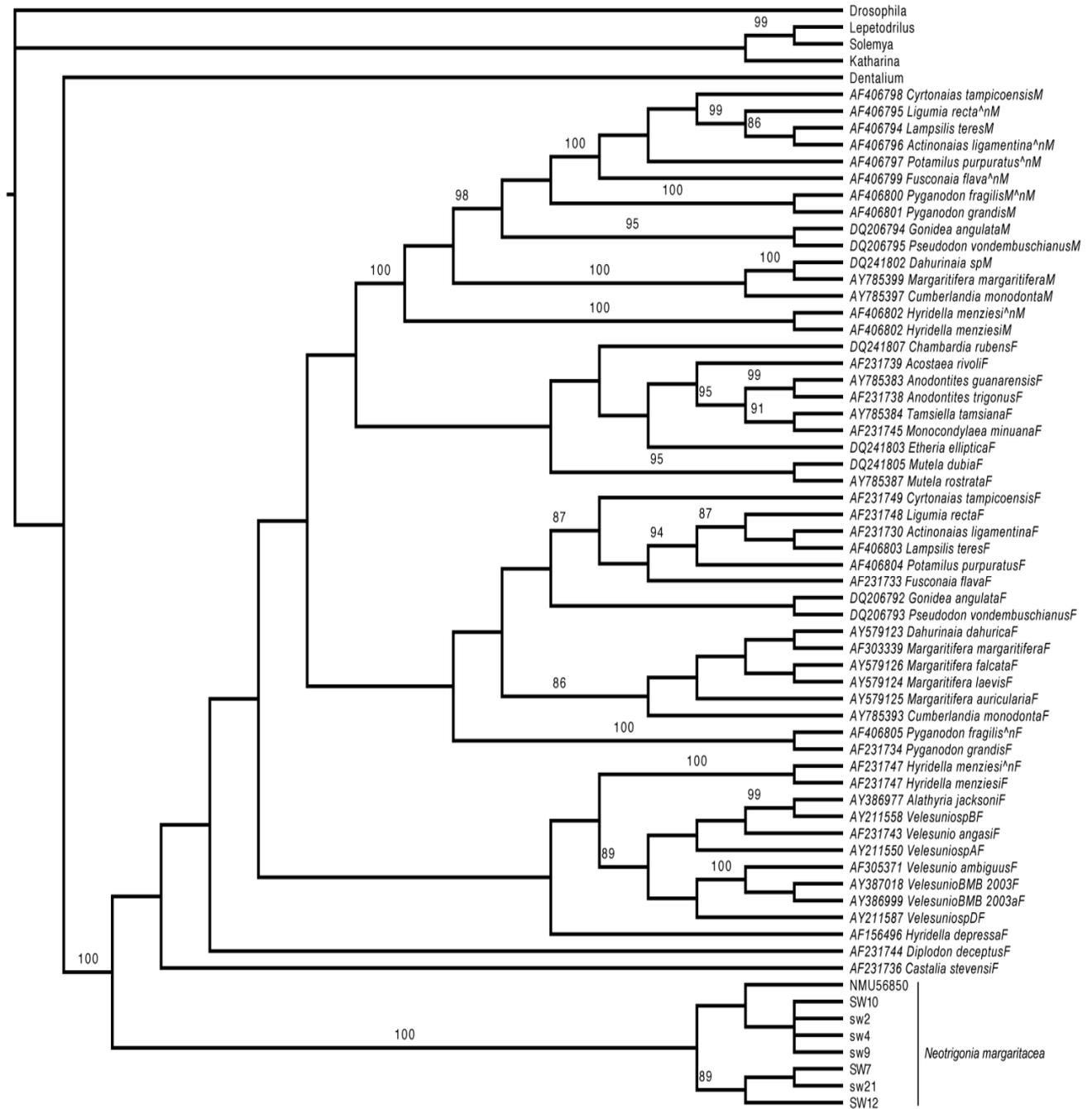


Figure 3.4. A maximum parsimony consensus tree based on Cytochrome C Oxidase subunit I using outgroup taxa as in Hoeh *et al.* (2002). MP jackknife > 85 values are shown. Terminal names correspond to NCBI accession numbers listed in Table 4.1 and Table 4.2. Male, M, and female, F, types are marked according to available information.

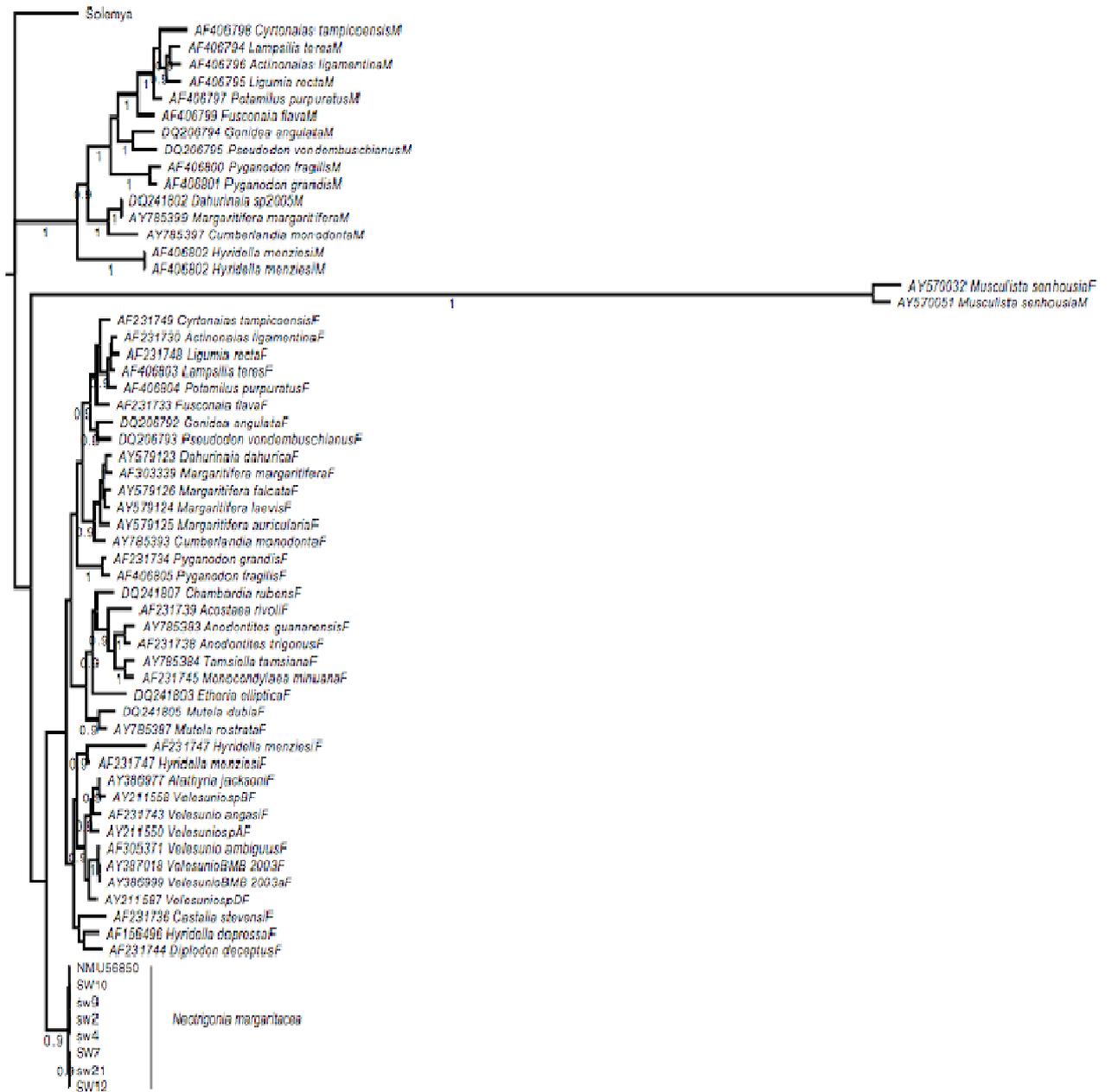


Figure 3.6. Bayesian analysis tree based on Cytochrome C Oxidase subunit I for the Unionoidae and Trigonioidae using less distant outgroup taxa. Posterior probabilities (pp) > 0.95 values are shown.

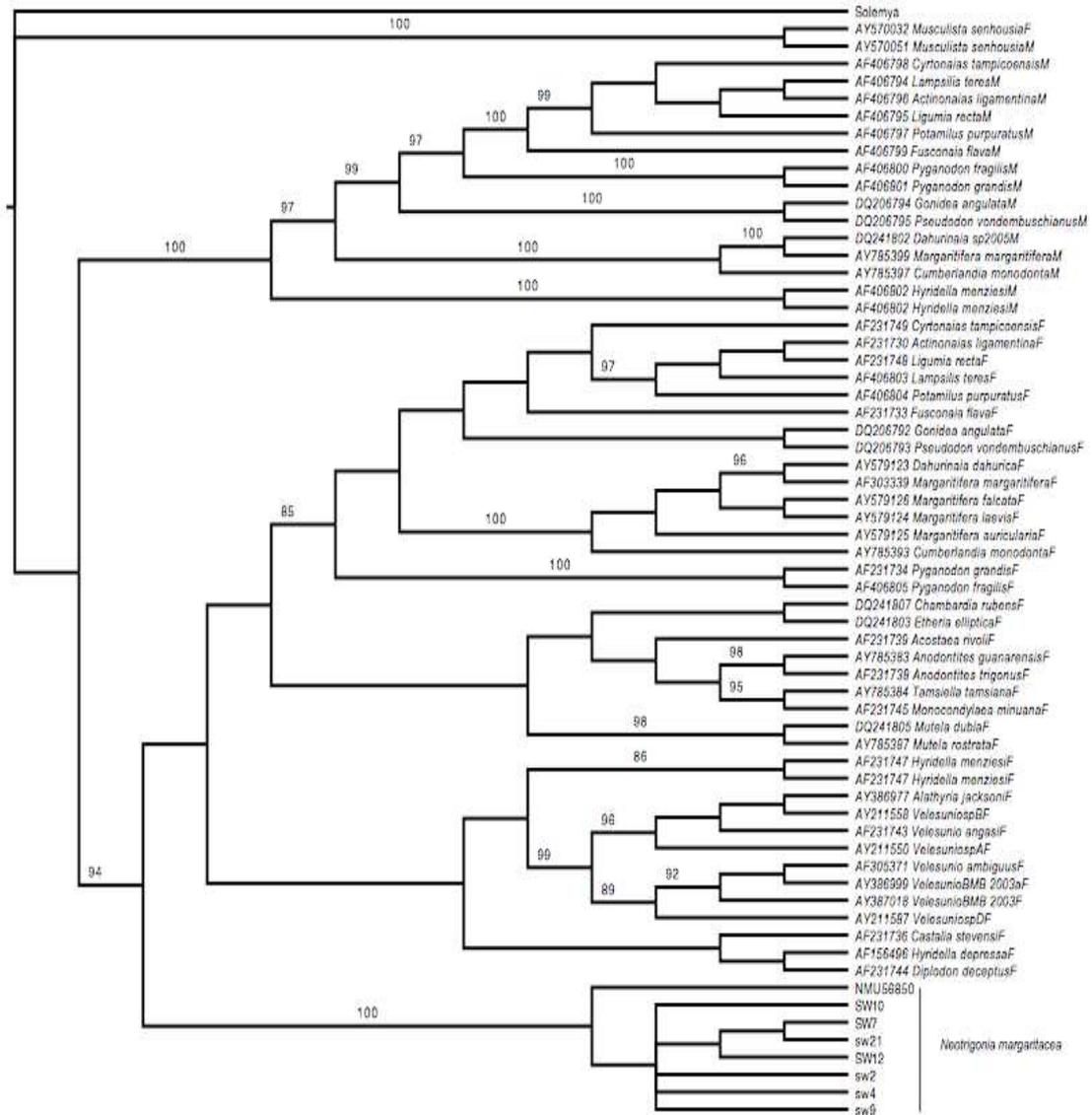


Figure 3.8. A maximum parsimony consensus tree based on Cytochrome C Oxidase subunit with less distant outgroups and RY coded. MP jackknife > 85 values are shown. Terminal names correspond to NCBI accession numbers listed in Table 4.1 and Table 4.2. Male, M, and female, F, types are marked according to available information.

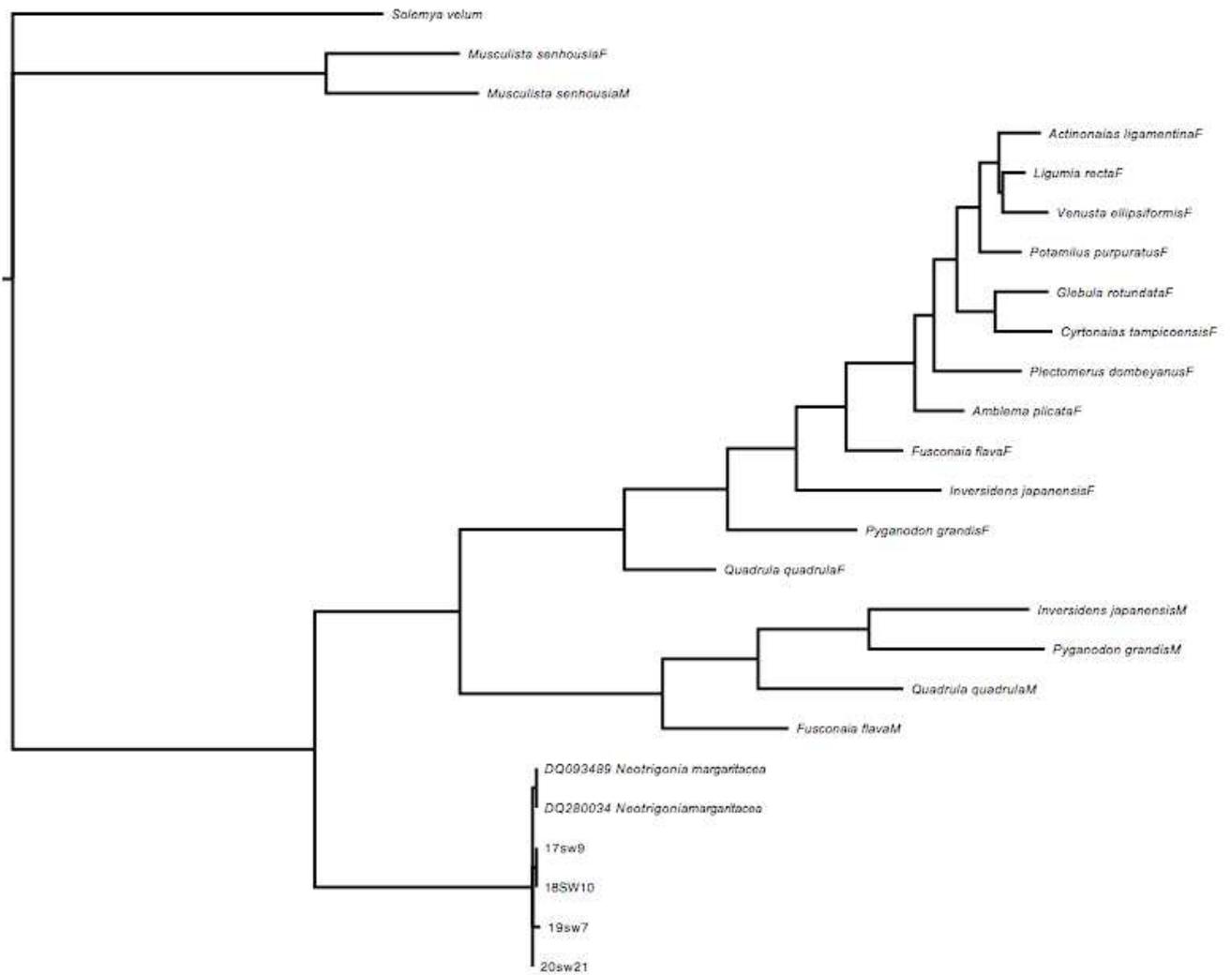


Figure 3.10. A maximum parsimony consensus tree based on 16S RNA for the Unionoidae and Trigonioidea.

Appendix IV

Table 4.1. Raw data of diameter measurements for protogonia in each sampling month.

Protogonia	November	December	January	February	March
	3.4	3.2	3.4	3	4.1
	3.6	3.5	3.6	5	3.5
	5.6	3.4	3.2	1	4.3
	6.1	4.1	3.5	3.4	4.6
	3.8	3.5	3.4	3.8	5.5
	4.3	4.3	4.1	4.1	3.5
	5.8	4.6	3.5	4.1	3.2
	2.7	5.5	4.3	3.5	4.3
	3.1	3.5	4.6	4.3	5.8
	3.2	3.2	5.5	4.6	2.7
	3.5	3.1	3.5	5.5	3.1
	3.4	3.3	3.2	3.5	3.2
	4.1	6.1	3.1	3.2	3.5
	3.5	5.2	3.5	4.8	3.4
	4.3	4.1	4.3	3.3	4.1
	4.6	3.5	4.6	6.1	3.5
	5.5	3.1	5.5	3.4	3.6
	3.5	3.3	3.5	3.6	5.1
	3.2	3.2	3.2	3.2	3.4
	3.1	4.2	3.1	3.5	5.2
total	80.3	77.9	76.6	76.9	79.6
mean	4.015	3.895	3.83	3.845	3.98
stdev	1.006962603	0.866617745	0.743462738	1.069419419	0.869119336

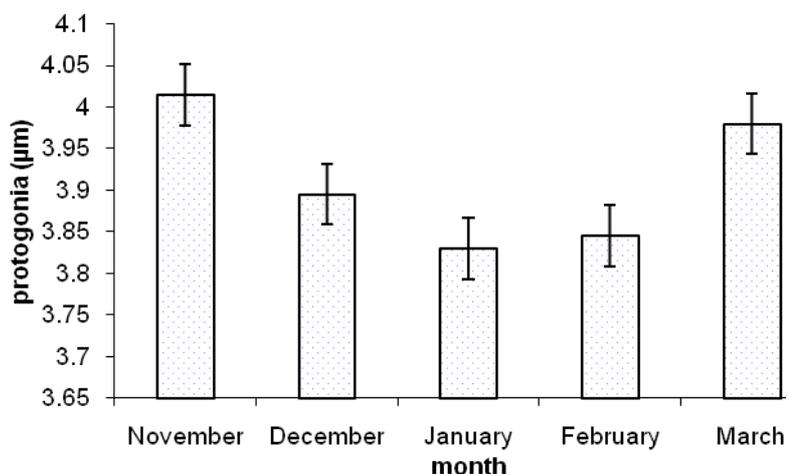
**Figure 4.1.** Comparison of raw data of diameter measurements for protogonia in each sampling month.

Table 4.2. Raw data of diameter measurements for oogonia in each sampling month.

Oogonia	Novembar	Decembar	January	February	March
	6	10	7.6	10	9.6
	6.1	9	8.2	8.9	7.6
	8.6	9.4	9	9.2	8.2
	7.2	6.4	9.3	9.4	7.8
	7	8.5	9.7	9.6	9.8
	7.3	7.6	8.6	7.6	6.4
	6.2	8.2	7.2	8.6	8.5
	6	9	9.4	6.9	7.6
	8	9.3	6	6	8.2
	8	9.7	6.1	6.1	9
	9	8.6	8.6	8.6	9.3
	10	7.2	7.2	7.2	9.7
	10	9.4	7	7	8.6
	8.9	6.8	7.3	7.3	7.2
	9.2	8.9	10	10	10
	9.4	10	9.6	9.6	8.9
	9.6	10	8.5	7.6	9.2
	7.6	7.9	8.5	8.2	9.4
	8.6	8.3	7.9	7.8	9.6
	6.9	6.9	7.3	9.8	7.6
total	159.6	171.1	163	165.4	172.2
mean	1.596	8.555	8.15	8.27	8.61
stdev	1.353980487	1.121781947	1.168669959	1.28107685	1.003100457

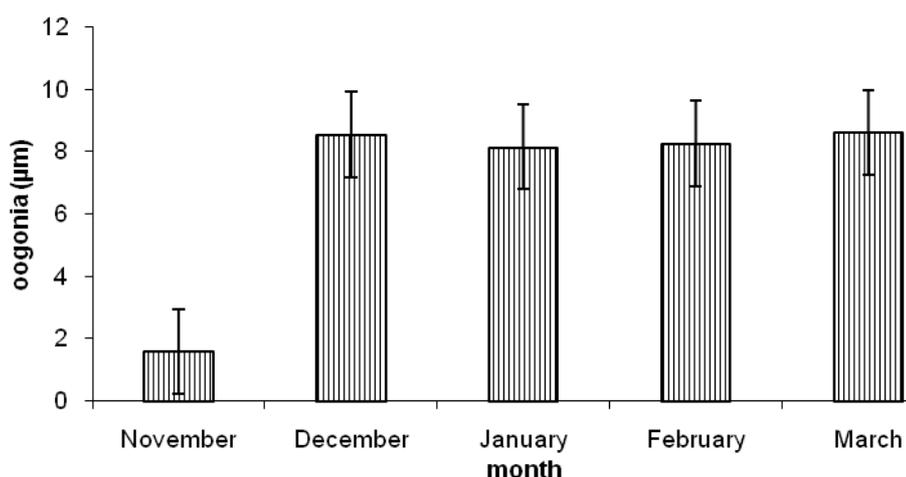
**Figure 4.2.** Comparison of raw data of diameter measurements for oogonia in each sampling month.

Table 4.3. Raw data of diameter measurements for previtellogenic in each sampling month.

Prevetellogenic	November	December	January	February	March
	20.6	43	28	36.8	50
	22.8	48	39	50.2	50
	36.8	4.2	25	55	49
	50.2	28	40	49.3	25
	55	33	49	15	40
	49.3	29	43	16	49
	15	45	38	18.9	43
	16	46	37	22.3	38
	18.9	28	22	25	37
	22.3	31	28	51	24
	25	39	51	44	36
	40	40	44	48	52
	49	22	48	37	43
	43	28	37	36	23
	38	51	36	41	27
	37	44	41	39	29
	24	48	39	43	31
	36	37	28	48	32
	52	36	37	4.2	23
	43	41	49	28	34
total	693.9	721.2	759	707.7	735
mean	11.565	36.06	37.95	35.385	36.75
stdev	13.07389826	11.09805104	8.331771784	14.34709855	9.925379484

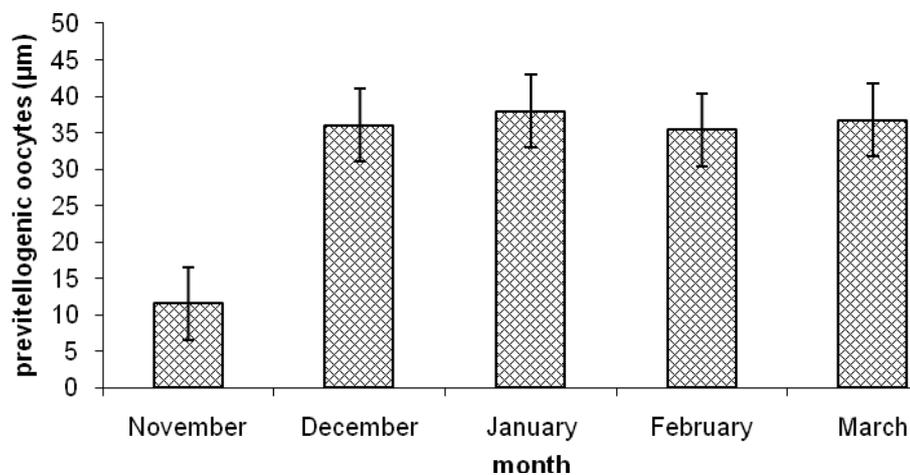


Figure 4.3. Comparison of raw data of diameter measurements for previtellogenic oocytes in each sampling month.

Table 4.4. Raw data of diameter measurements for vitellogenic oocytes in each sampling month.

Vitellogenic	November	December	January	February	March
	89	97	83	83	102
	73	110	110	92	98
	100	112	94	77	73
	102	94	76	89	96
	98	86	89	73	82
	132	76	83	100	106
	86	95	92	102	94
	78	98	77	98	73
	63	94	91	132	89
	115	96	73	86	83
	79	89	95	89	92
	86	73	96	73	77
	94	100	113	100	91
	108	102	86	102	73
	98	98	78	98	95
	76	132	63	132	73
	75	86	115	86	100
	83	94	79	95	102
	94	76	86	69	98
	109	89	94	99	132
total	1838	1897	1773	1875	1829
mean	91.9	94.85	88.65	93.75	91.45
stdev	16.53035485	13.42140866	13.36639541	16.61285172	14.53661439

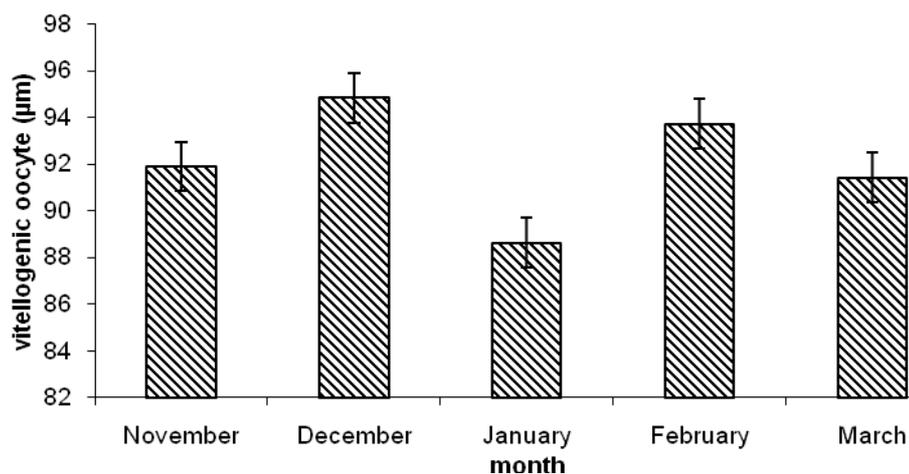


Figure 4.4. Comparison of raw data of diameter measurements for vitellogenic oocytes in each sampling month.

Table 4.5. Raw data of diameter measurements for postvitellogenic oocytes in each sampling month.

Postvitellogenic	November	December	January	February	March
	172	208	320	172	126
	192	172	172	208	203
	208	160	136	152	168
	280	252	252	232	198
	284	196	200	196	206
	264	312	224	228	213
	272	240	216	160	166
	240	200	108	132	184
	112	164	120	148	165
	192	252	152	224	207
	172	284	126	200	228
	208	264	203	164	160
	152	272	168	252	132
	232	240	198	284	148
	196	112	206	264	224
	228	192	213	272	200
	160	172	166	240	164
	132	208	184	112	252
	148	168	165	192	284
	224	208	207	229	264
total	4068	4276	3736	4061	3892
mean	203.4	213.8	186.8	203.05	194.6
stdev	49.897157	49.832984	49.152394	48.6009259	42.4641024

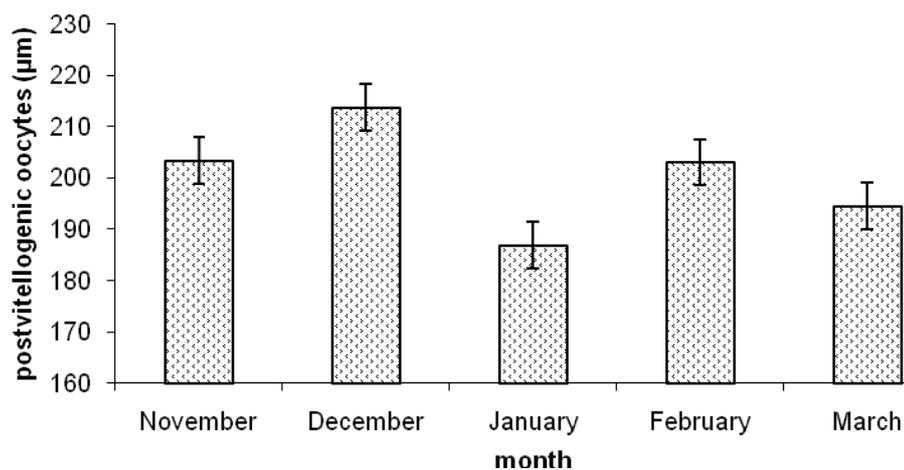


Figure 4.5. Comparison of raw data of diameter measurements for postvitellogenic oocytes in each sampling month.