CHAPTER 3

Phylogeny of the genus *Naso* inferred from molecular data (Section A)

3.1 Introduction

To understand patterns and processes that may have been responsible for the evolution of a reef fish group, relationships and ancestry among species of a group need to be established. To resolve such issues a phylogenetic approach is required. By inferring the inter-specific phylogeny of a specific group, it is possible to explore the mechanisms that underlie the evolutionary patterns of species diversification and cladogenesis. Therefore, this chapter examines interspecific relationships within the selected genus *Naso* (unicornfish) and includes a broader genus- level phylogeny of the Acanthuridae (surgeonfishes), so that the monophyly and position of *Naso* relative to other acanthurid genera can be established.

Species of the genus *Naso* exhibit considerable morphological and ecological diversification (Winterbottom and McLennan 1993; Borden 1998). Previous studies (Winterbottom 1993; Winterbottom and McLennan 1993; Borden 1998) identified distinctive morphologies associated with benthic foraging within reef habitats, and streamlined scombriform morphologies associated with pelagic foraging in open water. A major focus of these earlier studies, recently summarized by Brooks & McLennan (2002), has been an estimation of the phylogenetic relationships of species with these different foraging modes. The literature (Winterbottom and McLennan 1993; Borden 1998; Randall

2001; Brooks and McLennan 2002) contains a number of specific hypotheses that explore the relationships amongst these foraging modes, their ecological significance and the temporal patterns of diversification, which were based largely on morphological characters. This section of the study is designed to re-evaluate these hypotheses using a more comprehensive phylogeny based on molecular markers. A total of 3 markers was used to generate a comprehensive species-level phylogeny of all *Naso* species. To determine the placement and infer the root of the genus *Naso* within the family Acanthuridae, an additional 18 acanthuroid species (from 5 genera) were sequenced using a nuclear marker. Producing a complete phylogeny of the genus *Naso* allowed for the examination of the following four hypotheses, that can be developed from the work of previous authors.

1. The morphological tree of Borden (1998) is congruent with the molecular tree generated in this study.
2. Benthic foraging on macroscopic algae is plesiomorphic in the genus *Naso* (pelagic foraging is derived) (Winterbottom 1993; Winterbottom and McLennan 1993; Borden 1998; Brooks and McLennan 2002).
3. The presence of frontal horns and other cephalic structures does not reflect evolutionary relationships within the genus *Naso* (Borden 1998).
4. All members of the subgenus *Axinurus* (*N. minor*, *N. thynnoides* and *N. caeruleacauda*) constitute a single morphologically defined group within the genus *Naso* (Smith 1966; Randall 1994; Randall 2001).

With the development of molecular techniques, the scope to infer evolutionary relationships within and between taxa has become more accessible (Hillis et al. 1996). A number of tropical reef fish phylogenies have been inferred from molecular data (e.g.

Bernardi and Crane 1999; Tang et al. 1999; Bernardi et al. 2000; Craig et al. 2001; Tang 2001; Wang et al. 2001; Lo Galbo et al. 2002; Streelman et al. 2002; Clements et al. 2003; Ruber et al. 2003; Thacker 2003). Most of these examined high-level relationships using mtDNA (sequencing 1 - 4 gene regions) rather than species-level relationships. Some of the studies (e.g. Bernardi et al. 2000; Streelman et al. 2002; Ruber et al. 2003) examined specific features, such as foraging modes, in relation to the phylogeny obtained, an issue also examined and addressed in this study. Two genus-level molecular phylogenies for the family Acanthuridae (surgeonfishes) have been published (Tang et al. 1999; Clements et al. 2003). However, these studies used only a few species of *Naso* (unicornfish), the genus that is the focus of this study.

3.1.1 General information on Naso

The suborder Acanthuroidei consists of six families of tropical fishes displaying a high level of structural and morphological diversity (Tyler et al. 1989; Winterbottom and McLennan 1993; Randall 2002). Most species are deep-bodied laterally compressed fishes with a benthic foraging mode, feeding on a variety of sessile and motile invertebrates and marine plants and are strongly associated with reef environments. However, a significant minority of acanthuroid fishes display a contrasting life-style, foraging in open water for planktonic and small nektonic prey. The most distinctive pelagic foraging taxon is represented by the monotypic genus *Luvarus* (Family Luvaridae) confined to open waters (Tyler et al. 1989). Pelagic foraging has also been recorded from the family Acanthuridae, especially in the genera *Naso* and *Acanthurus* (Jones 1968; Randall 2002). The family Acanthuridae comprises 6 extant genera containing 80 species, *Naso* (19 recognised

species), *Prionurus* (7), *Paracanthurus* (1), *Zebrasoma* (7), *Acanthurus* (37) and *Ctenochaetus* (9) (Randall 2002). Relationships amongst the six genera are well documented from morphological (Winterbottom and McLennan 1993) and molecular (Tang et al. 1999; Clements et al. 2003) studies. Moreover, the morphological reconstruction of generic relationships within the suborder (Winterbottom and McLennan 1993) is congruent with the reconstruction based on mitochondrial sequences (Clements et al. 2003). Two primary issues remain. Firstly, there is a need to clarify the patterns of evolutionary diversification within genera. This requires species-level phylogenies, especially in the genera *Naso* and *Acanthurus* where the component species exhibit considerable ecological diversification (Winterbottom and McLennan 1993; Borden 1998). Secondly, the temporal pattern of diversification, especially in these genera, needs further clarification (Clements et al. 2003) (see chapter 4).

The 19 described species in the genus *Naso* (the only extant member of the subfamily Nasinae) exhibit a variety of morphologies, including striking extensions of the frontal region of the head either as horns or bulbous protuberances (Smith 1966; Randall 2002). In addition, body proportions vary amongst the different species, ranging from relatively deep-bodied forms with an extended snout to relatively slender streamlined forms with body proportions similar to those of scombrid fishes (here termed scombriform morphology). Smith (1966) in a taxonomic survey of the subfamily Nasinae divided the genus *Naso* into three genera, *Naso, Axinurus* and *Callicanthus*, based on a number of morphological features: two peduncular plates and frontal horn/protuberance in *Naso*; a single peduncular plate in *Axinurus*; dentition and the absence of a frontal horn in *Callicanthus*. In subsequent studies of the Nasinae, Randall (1994; 2001) did not retain

these genera but noted that species placed by Smith (1966) in *Axinurus* were morphologically distinct from other members of the genus *Naso*.

A recent allozyme study by Dayton (2001) using a limited number of *Naso* species (*N. caesius*, *N. hexacanthus*, *N. lituratus*, *N. unicornis* and *N. thynnoides*), indicated a strong sister-relationship between *N. caesius* and *N. hexacanthus* with *N. thynnoides* as the basal species, and the remaining two species unresolved. Dayton (2001) suggested that the subgenus *Axinurus* (represented by *N. thynnoides*) may form a monophyletic group within the genus *Naso*, and tentatively indicated that this group may be basal to the remaining species of *Naso*.

3.1.2 Foraging modes of Naso

Winterbottom & McLennan (1993) argued that benthic foraging targeting macroscopic algae is plesiomorphic in the Acanthuridae (Brooks and McLennan 2002) and pelagic foraging (plankton feeding) derived. They considered that planktivorous foraging evolved independently from herbivorous ancestors in both *Naso* and *Acanthurus* but that in the absence of species-level phylogenetic hypotheses it could not be determined how many times planktivory originated within *Naso.* Borden (1998) developed a species level phylogeny of *Naso* based on 15 species and 13 character states (3 osteological, 10 based on soft tissue). He also concluded that i) herbivory (benthic foraging) was ancestral in *Naso*, a view reflected in the basal position of a suite of deep-bodied reef-associated species in his phylogeny; ii) pelagic foraging (zooplanktivory) was a derived condition appearing once in his phylogeny; iii) horn development was “haphazard” occurring at a number of points within the phylogeny. However, Borden (1998) also noted that the phylogeny was only

partially resolved and that further studies and increased taxon sampling were required to resolve existing polytomies and species-level relationships associated with pelagic foraging. The internal structures associated with pelagic foraging in Nasinae have been described by Tyler (1970) and Tyler et al. (1989). These are primarily associated with the caudal propulsive unit. Key features are: a narrow caudal peduncle; caudal fin with a high aspect-ratio; fusion of the hypural bones into a single plate with bases of the caudal fin rays overlapping the hypural bone (hypurostegy) resulting in a consolidated caudal skeleton similar to that of scombrids and istiophorids (Tyler et al. 1989). The pattern of occurrence suggests that these features have evolved repeatedly in other open-water pelagic fishes, indicative of convergence to common requirements of open-water foraging.

3.2 Materials and Methods

3.2.1 Sampling

Specimens for this study were either collected by spearing from a wide geographic range (see acknowledgments) or acquired from museum collections (Table 3.1).

At least 2 individuals per species with the exception of *Naso reticulatus* and *Luvarus imperialis* (Table 3.1) were analysed. In addition to samples from the 19 recognised *Naso* species, I had samples from one undetermined, possibly new (but undescribed) species of *Naso*. Tissue samples (pectoral fin) of freshly speared fish were immediately placed into 80% ethanol or salt-saturated 20% DMSO (dimethyl sulfoxide). Muscle tissue of museum specimens had been preserved either in 95-100% ethanol, or had been fixed in 10%

formalin prior to preservation in 55% isopropanol. DNA extractions from fresh specimens and formalin fixed museum species PCR and direct sequencing are described in Chapter 2.

3.2.2 Phylogenetic Analysis

Two different categories of molecular markers were used to explore the acanthurid phylogeny: one nuclear marker (ETS2) and two mitochondrial (mt) markers (16S and cytochrome *b*). ETS2 is an intron from a nuclear oncogene. It was originally used as a conserved mammalian single-locus DNA marker (Lyons et al. 1997), but the primers, based on flanking exonic regions, also amplify fish DNA. This marker has been successfully used for serranids (van Herwerden et al. 2002). I used ETS2 by itself to confirm the relationship between all species of *Naso* and the remaining extant acanthurid genera. A nuclear marker has not featured previously in acanthurid studies, which used only mtDNA (Tang et al. 1999; Clements et al. 2003).

All three markers (ETS2, 16S & cyt *b*) combined were used to obtain a detailed resolution of species relationships within the genus *Naso*. All sequences (forward and reverse) were edited in GeneDoc (Nicholas and Nicholas 1997) prior to alignment using ClustalW in DAMBE (Xia 2000).

Gaps were treated as missing data.

Table 3.1: All 38 species sampled for this study, including museum specimens*. Location of capture or catalogue no's (museum species) are listed together with GenBank accession no's for each marker.

Species	Location	Accession ETS2	Accession 16SrRNA	Accession Cyt *b*
Acanthurus blochii	Lizard Isl., GBR	AY264685	AY264594	AY264632
Acanthurus dussumieri	Lizard Isl., GBR	AY264686	AY264595	AY264633
Acanthurus nigricans	Lizard Isl., GBR	AY264687	AY264596	AY264634
Acanthurus nigrofuscus	Lizard Isl., GBR	AY264688	AY264597	AY264635
Acanthurus nubilus	Kimbe Bay, PNG	AY264689	AY264598	AY264636
Acanthurus olivaceus	Lizard Isl., GBR	AY264690	AY264599	AY264637
Acanthurus pyroferus	Lizard Isl., GBR	AY264691	AY264600	AY264638
Acanthurus triostegus	Lizard Isl., GBR	AY264692	AY264601	AY264639
Acanthurus xanthopterus	Lizard Isl., GBR	AY264693	AY264602	AY264640
Ctenochaetus binotatus	Lizard Isl., GBR	AY264694	AY264603	AY264641
Ctenochaetus striatus	Lizard Isl., GBR	AY264695	AY264604	AY264642
*Luvarus imperialis**	AMS I38647002	AY264678	AY264587	AY264625
Naso annulatus	Lizard Isl., GBR	AY264696	AY264605	AY264643
Naso brachycentron	Amirante, Seychelles	AY264697	AY264606	AY264644
Naso brevirostris	Lizard Isl., GBR	AY264698	AY264607	AY264645
*Naso caeruleacauda**	ROM 67197	AY264699	AY264608	AY264646
Naso caesius	Lizard Isl., GBR	AY264700	AY264609	AY264647
Naso elegans	Amirante, Seychelles	AY264701	AY264610	AY264648
Naso fageni	Ningaloo Reef, WA	AY264702	AY264611	AY264649
Naso hexacanthus	Lizard Isl., GBR	AY264703	AY264612	AY264650
Naso lituratus	Lizard Isl., GBR	AY264704	AY264613	AY264651
Naso lopezi	SUAKREM	AY264705	AY264614	AY264652
Naso maculatus	Hawaii	AY264706	AY264615	AY264653
*Naso mcdadei**	LFNMMST-62	AY264707	AY264616	AY264654
Naso minor	SUAKREM	AY264708	AY264617	AY264655
*Naso reticulatus**	BPBM 23428	AY264709	AY264618	AY264656
Naso thynnoides	LaDigue, Seychelles	AY264710	AY264619	AY264657
Naso tonganus	Lizard Isl., GBR	AY264711	AY264620	AY264658
Naso tuberosus	Amirante, Seychelles	AY264712	AY264621	AY264659
Naso unicornis	Lizard Isl., GBR	AY264713	AY264622	AY264660
Naso vlamingii	Lizard Isl., GBR	AY264714	AY264623	AY264661
Naso undetermined	One Tree Isl., GBR	AY264715	AY264624	AY264662
Paracanthurus hepatus	Seychelles	AY264680	AY264589	AY264627
Prionurus microlepidotus	Broughton Isl., NSW	AY264681	AY264590	AY264628
Prionurus scalprum	Taiwan	AY264682	AY264591	AY264629
Zanclus cornutus	Lizard Isl., GBR	AY264679	AY264588	AY264626
Zebrasoma scopas	Lizard Isl., GBR	AY264683	AY264592	AY264630
Zebrasoma veliferum	Lizard Isl., GBR	AY264684	AY264593	AY264631

AMS: Australian Museum Sydney, Australia; **ROM**: Royal Ontario Museum, Canada; **BPBM**: Bernice P. Bishop Museum, Hawaii; **LFNMMST**: National Museum of Science and Technology, Taiwan, **SUAKREM**: Silliman University Angelo King Center for Research and Environmental Management, Philippines.

Saturation of nucleotide substitution was checked using a test by Xia et al. (2003), implemented in DAMBE version 4.2.8 (Xia 2000). This test calculates if the observed index of substitution saturation (Iss) of nucleotide sequences is significantly lower than the critical value (Iss.c) calculated for the same sequences where Iss.c = the value at which saturation occurs (Xia et al. 2003). The null hypothesis of this test is that there is saturation (Iss ≥ Iss.c). The overall genus-level (38 taxa) relationship was inferred from ETS2 sequences using three different methods: maximum likelihood (ML), parsimony (MP) implemented in PAUP* 4.0b10 (Swofford 1998), and a Bayesian inference (BI) approach implemented in MrBayes version 3.0B4 (Huelsenbeck and Ronquist 2001). The best substitution model for all ML and BI analyses were identified using a likelihood approach implemented in Modeltest 3.06 (Posada and Crandall 1998). Sequence data of all three markers were combined for members of the genus *Naso* and tested using the partition homogeneity test (see Results, also following Yoder et al. 2001) for congruence of tree lengths from each data partition, implemented in PAUP* 4.0b10. Trees of *Naso* were inferred using the same 3 methods. The subtree-pruning-regrafting (SPR) branch-swapping algorithm was used in the ML analyses (150 bootstrap replicates). Parsimony searches utilised a heuristic search (1000 bootstrap replicates, 50% majority rule consensus of all equally parsimonious, shortest trees) with the tree bisection reconnection (TBR) branch-swapping algorithm. The Bayesian analysis was performed using a Markov chain Monte Carlo (MCMC) search with 4 chains for 1 million generations. Trees were sampled every 100 generations, and the first 100,000 generations were discarded as burn-in. At the generic level (38 species) *Zanclus cornutus* and *Luvarus imperialis* and at the species-level (21 species) *Zebrasoma scopas* were specified for outgroup rooting of phylogenetic trees. All resulting topologies were tested for significant difference from the best tree selected by

PAUP* ($p \leq 0.05$ significance level, -lnL for ML, tree length for MP) using Shimodaira-Hasegawa (ML) or Kishino-Hasegawa (MP) tests implemented in PAUP* 4.0b10 (Swofford 1998).

3.2.3 Hypothesis testing

With the exception of Dayton et al. (1994), Dayton (2001) and Clements et al. (2003), all hypotheses of species relationships within the genus *Naso* are developed from studies based on morphological data (Winterbottom and McLennan 1993; Borden 1998). I examined these hypotheses by testing whether topologically constrained trees were consistent with the hypotheses below. This was done, by evaluating the constrained trees against the molecular sequences. If constrained trees were significantly worse than the best tree inferred from the molecular sequences, then the hypothesis was rejected.

Hypothesis 1: *The morphological tree of Borden (1998) is congruent with the molecular tree generated in this study*. In order to compare these trees, four species (*N. tonganus*, *Naso* undetermined, *N. elegans* and *N. reticulatus*) were excluded from the combined molecular data set so that it included the same taxa as the morphological tree. Borden's (1998, Fig. 7 p.109) tree topology was evaluated against the molecular data using MacClade 4.03 (Maddison and Maddison 2001). The resulting tree length was compared to the most parsimonious trees of the molecular analysis using the pairwise Kishino-Hasegawa test (KH-) and nonparametric tests (Wilcoxon signed-ranks and sign test) implemented in PAUP* 4.0b10 (Swofford 1998).

Hypothesis 2: Benthic foraging on macroscopic algae is plesiomorphic within the genus Naso (pelagic foraging is derived) (Winterbottom and McLennan 1993; Borden 1998).

Hypothesis 3: The presence of frontal horns and other cephalic structures does not reflect evolutionary relationships within the genus Naso (Borden 1998).

Hypothesis 4: All members of the subgenus Axinurus (N. minor, N. thynnoides and N. caeruleacauda) constitute a single morphologically defined group within the genus (Smith 1966; Randall 2001) *and are basal to other Naso species* (Dayton 2001). Hypotheses 2 – 4 were tested by entering trees with appropriate "backbone" constraints into PAUP* (see Appendix 1) and allowing the program to find the best trees given the constraint, using a heuristic search under parsimony.

Hypothesis 2 was tested by constraining all 8 benthic foraging species (Table 3.2) to form a monophyletic clade. Similarly, to test hypotheses 3 and 4, the four horned species and all members of the subgenus *Axinurus* (Table 3.2) respectively were constrained into monophyletic clades. Length of the constrained trees were compared against that of the most parsimonious unconstrained tree using the KH- and nonparametric tests implemented in PAUP* 4.0b10 (Swofford 1998).

Table 3.2: Adult foraging modes (dominant) for all *Naso* species with source references.

Species	Adult foraging mode	Source reference (Foraging mode)
Naso annulatus	Benthic & pelagic[2]	(Choat et al. 2002)
Naso brachycentron	Benthic[1,2]	(Choat and Clements 1998)
Naso brevirostris	Benthic & pelagic[2]	(Choat et al. 2002)
Naso caeruleacauda	Pelagic[3]	(Randall 2002)
Naso caesius	Pelagic	(Randall 2002)
Naso elegans	Benthic[1]	(Randall 2002)
Naso fageni	Benthic[1]	This study, macroalgae
Naso hexacanthus	Pelagic	(Choat et al. 2002)
Naso lituratus	Benthic[1]	(Clements and Choat 1995)
Naso lopezi	Pelagic	(Randall 2002)
Naso maculatus	Pelagic*	(Randall 2002)
Naso mcdadei	Benthic[1]	(Randall 2002)
Naso minor	Pelagic[3]	(Randall 2002)
Naso reticulatus	Pelagic*	
Naso thynnoides	Pelagic[3]	(Randall 2002)
Naso tonganus	Benthic[1]	(Randall 2002)
Naso tuberosus	Benthic	This study, macroalgae
Naso unicornis	Benthic[1,2]	(Choat et al. 2002)
Naso vlamingii	Benthic & pelagic	(Choat et al. 2002)
Naso undetermined	Pelagic*	

[1] Species constrained into a benthic clade (*Hypothesis 2*); [2] horned clade (*Hypothesis 3*) and [3] *Axinurus* clade (*Hypothesis 4*).
* probably feeding on pelagic matter.

3.3 Results

One sequence per species was deposited in GenBank, because both individuals analysed for a species had the same sequence. The alignment of the nuclear marker, ETS2 had 419 bp of which 27% were parsimony-informative. The first 81 sites of ETS2 were from the coding region (exon) and the remaining 338 bp (including alignment gaps) were from the intron. Gaps tended to be genus-specific. Only 2 gaps needed to be inserted to align sequences from *Naso* species, but as many as 30 gaps were required in the case of some *Acanthurus* species.

The two mtDNA alignments, 16S and cyt *b* were 574 bp (including gaps) and 339 bp (no gaps) respectively. ETS2 combined with both mtDNA markers produced 1332 bp of sequence with approx. 10% parsimony-informative sites.

3.3.1 Genus-level phylogeny

ETS2 sequences were not saturated with a site saturation (Iss) value of 0.214, which was significantly lower ($p<0.0001$) than the critical saturation value (Iss.c) of 0.791. The same topology was generated for the 38 taxa whether analysed by maximum parsimony (MP), maximum likelihood (ML) or Bayesian inference (BI) of phylogeny. For MP, 259 trees were equally parsimonious with a tree length of 320, high consistency (CI: 0.772) and retention (RI: 0.927) indices. For ML (HKY + Γ substitution model) analysis, 9823 trees were generated, the best tree (selected by PAUP and checked by SH-test) had a log likelihood score of lnL -2356.171. The genus *Naso* was monophyletic and formed a sister group to the remaining 5 acanthurid genera (*Paracanthurus*, *Zebrasoma*, *Prionurus*, *Acanthurus* and *Ctenochaetus*) (Figure 3.1). This pattern of generic relationships is similar to that described by Clements et al. (2003). Overall, bootstrap support for the main clades was high for all analyses (≥ 91) (Figure 3.1). The generic configuration was retrieved in association with the full phylogeny of *Naso* and provides a basis for determining the early history of the genus. While bootstrap support within the genus *Naso* was relatively weak for MP and ML analyses, Bayesian posterior probabilities generated stronger values for all major clades (Figure 3.1).

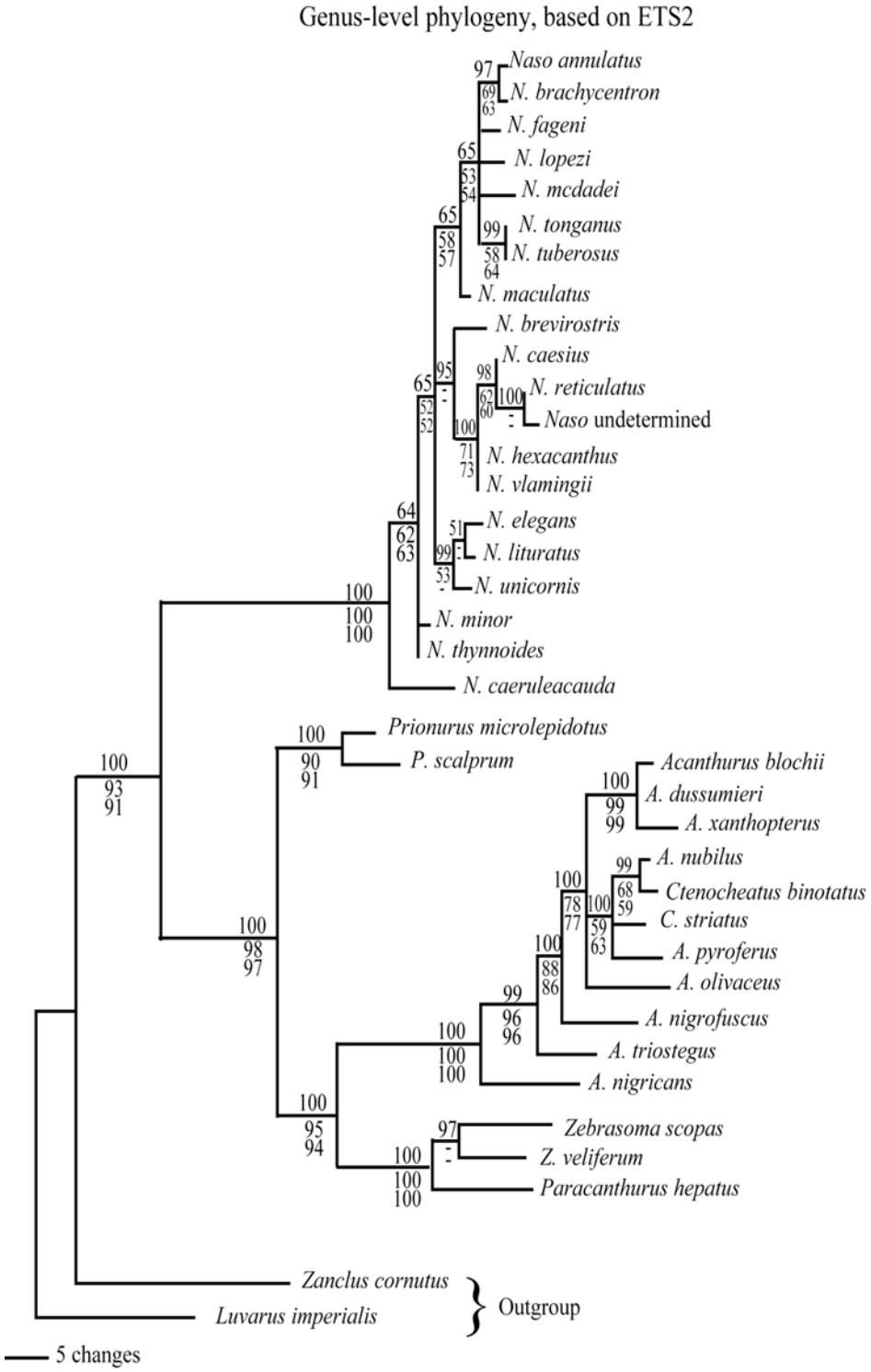

Figure 3.1: Phylogenetic tree of 38 acanthuroid species obtained by ML analysis of ETS2 sequences. Tree is outgroup-rooted with Livarus and Zanclus. Bayesian posterior probablities (in percent) are indicated above branches, bootstrap values (> 50) for MP and ML below branches (upper and lower values respectively).

Members of the subgenus *Axinurus* (*N. caeruleacauda*, *N. thynnoides* and *N. minor*) were basal to the remaining *Naso* species.

3.3.2 Naso *species-level phylogeny*

All three markers were combined for the species-level phylogeny. The partition homogeneity test score was low (P=0.02) for all 3 markers (21 species) suggesting that there are incongruent trees between partitions and that data partitions should not be combined. When only the 2 mtDNA markers were combined, the partition homogeneity test score P=0.3, indicated no incongruence between the 2 mtDNA markers. The only conflict between the nuclear (ETS2) and the 2 mtDNA markers was in the placement of *N. reticulatus* (compare Figure 3.1 to Figure 3.2). I therefore ran the combined data set again excluding *N. reticulatus* and obtained a partition homogeneity test score of P=0.41. A single base change (A to G) produced this difference in placement of *N. reticulatus* with ETS2. Therefore, all 3 markers were combined despite the low partition homogeneity score (see also Yoder et al. 2001).

No saturation was evident in the combined sequences, with Iss < Iss.c (0.1785 < 0.7779, $p<0.0001$).

The MP analysis produced 2 equally parsimonious trees of length 519, a CI: 0.617, RI: 0.640. The ML analysis (GTR + I + Γ substitution model) generated 185 trees of which the best had a log likelihood score lnL -4455.934. BI retrieved a similar topology (Figure 3.2) as MP and ML. The combined sequences resolved inter-specific relationships within

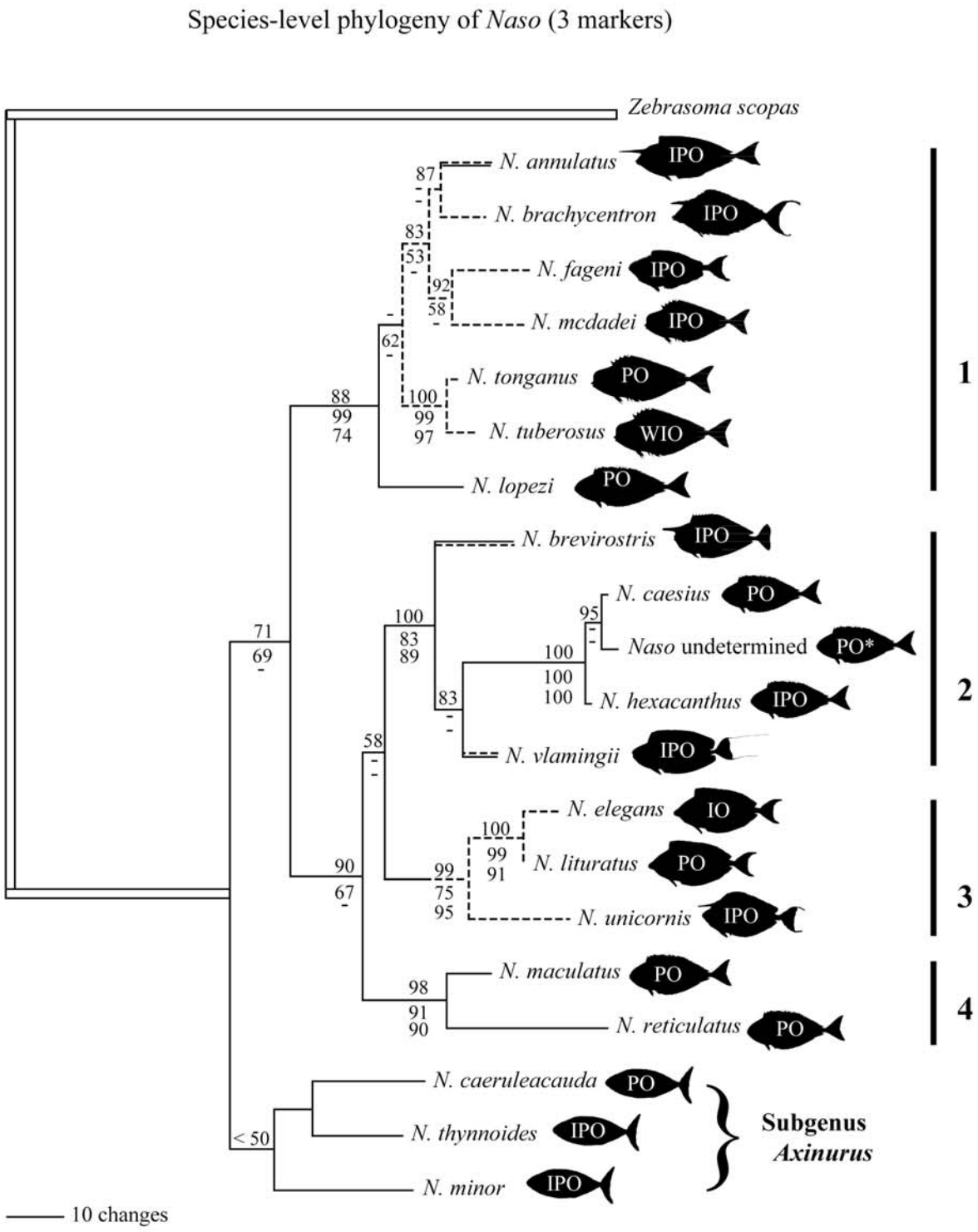

Figure 3.2: Topology of combined data (ETS2, 16S, cyt *b*) from Bayesian inference for 20 *Naso* species and *Z. scopas* as the outgroup. Bayesian posterior probablilities (in %) are indicated above branches, bootstrap values (> 50) for MP and ML below branches (upper and lower values respectively). Numbers refer to sub-clades: **1** *N.annulatus* sub-clade, **2** *N. brevirostris* sub-clade, **3** *N. elegans* and **4** *N. maculatus* sub-clades. Foraging modes are indicated, pelagic as black and benthic as dotted lines. Body shapes are silhouetted and distribution ranges indicated. IPO: Indo-Pacific Ocean, PO: Pacific O., IO: Indian O. and WIO: West Indian O. * Two specimens collected from One Tree Island, Great Barrier Reef.

the genus and segregated all *Naso* species as follows (Figure 3.2). The subgenus *Axinurus* was basal but paraphyletic for MP and ML (not shown). BI however, grouped members of *Axinurus* in a monophyletic clade with little support (< 50) (Figure 3.2).

The remaining species segregated into 4 distinct sub-clades, which were identified as: *N. annulatus* sub-clade (1), *N. brevirostris* sub-clade (2), *N. elegans* sub-clade (3) and *N. maculatus* sub-clade (4). Bootstrap values were high for all 4 sub-clades by all 3 methods (Figure 3.2). Sub-clades 2 – 4 were collectively sister to the *N. annulatus* sub-clade (Figure 3.2). For the MP and ML topologies, these three sub-clades (2 – 4) essentially arose from a polytomy.

The *N. annulatus* sub-clade (1) consists of 7 species, 5 of which are exclusively benthic browsing species (*N. brachycentron*, *N. tonganus*, *N. tuberosus*, *N. mcdadei*, *N. fageni*), one is a pelagic foraging species (*N. lopezi*) and the large horned species, *N. annulatus*, forages on the benthos as a juvenile and in the pelagic zone as an adult (Figure 3.2, also Table 3.2). The *N. brevirostris* sub-clade (2) consists of 5 species. *N. brevirostris* is similar in morphology (horned) and feeding ecology to *N. annulatus*, whilst *N. vlamingii* is a largely benthic-foraging species that also consumes fish faeces (Table 3.2). The remaining 3 species in this clade are pelagic foraging species (*N. hexacanthus*, *N. caesius* and *Naso* undetermined). The *N. elegans* sub-clade (3) is an exclusively benthic foraging clade and includes both horned (*N. unicornis*) and non-horned (*N. lituratus*, *N. elegans*) species. Members of the *N. maculatus* sub-clade (4) are probably pelagic foraging species although ecological details are unclear (Figure 3.2, Table 3.2).

The majority of *Naso* species occur throughout the Indo-Pacific Ocean (Figure 3.2). For a few sister species such as *N. tuberosus – N. tonganus* (sub-clade 1) and *N. elegans – N. lituratus* (sub-clade 3), distinct distribution ranges (by ocean basins) have been recorded

(Johnson 2002; Randall 2002). For other sister pairs (e.g. *N. mcdadei – N. fageni* and *N. reticulatus – N. maculatus*) only limited information about their distribution ranges is available (see also Kuiter and Debelius 2001; Randall 2002).

Having established a well-resolved molecular phylogeny it is now possible to test various hypotheses about the evolution of morphology and foraging modes.

3.3.3 Tests of hypotheses

Hypothesis 1: *The morphological tree of Borden (1998) is congruent with the molecular tree generated in this study.* The forced tree topology was 147 steps longer, when evaluated against the best topology inferred from molecular sequences. It was thus significantly worse according to both KH and nonparametric tests (Wilcoxon signed-ranks and sign test) (Table 3.3).

Borden's (1998) morphological phylogeny formed the basis for the argument that benthic foraging is plesiomorphic (Figure 3A) and pelagic foraging is derived at one point in the phylogeny. This contrasts with the molecular phylogeny where pelagic foraging appears to be plesiomorphic (Figure 3.2 & 3.3B). Therefore, alternative scenarios were tested statistically. These hypotheses were tested against the most parsimonious MP tree (tree length = 519) obtained from the combined molecular sequences including all 20 *Naso* species with *Z. scopas* as outgroup.

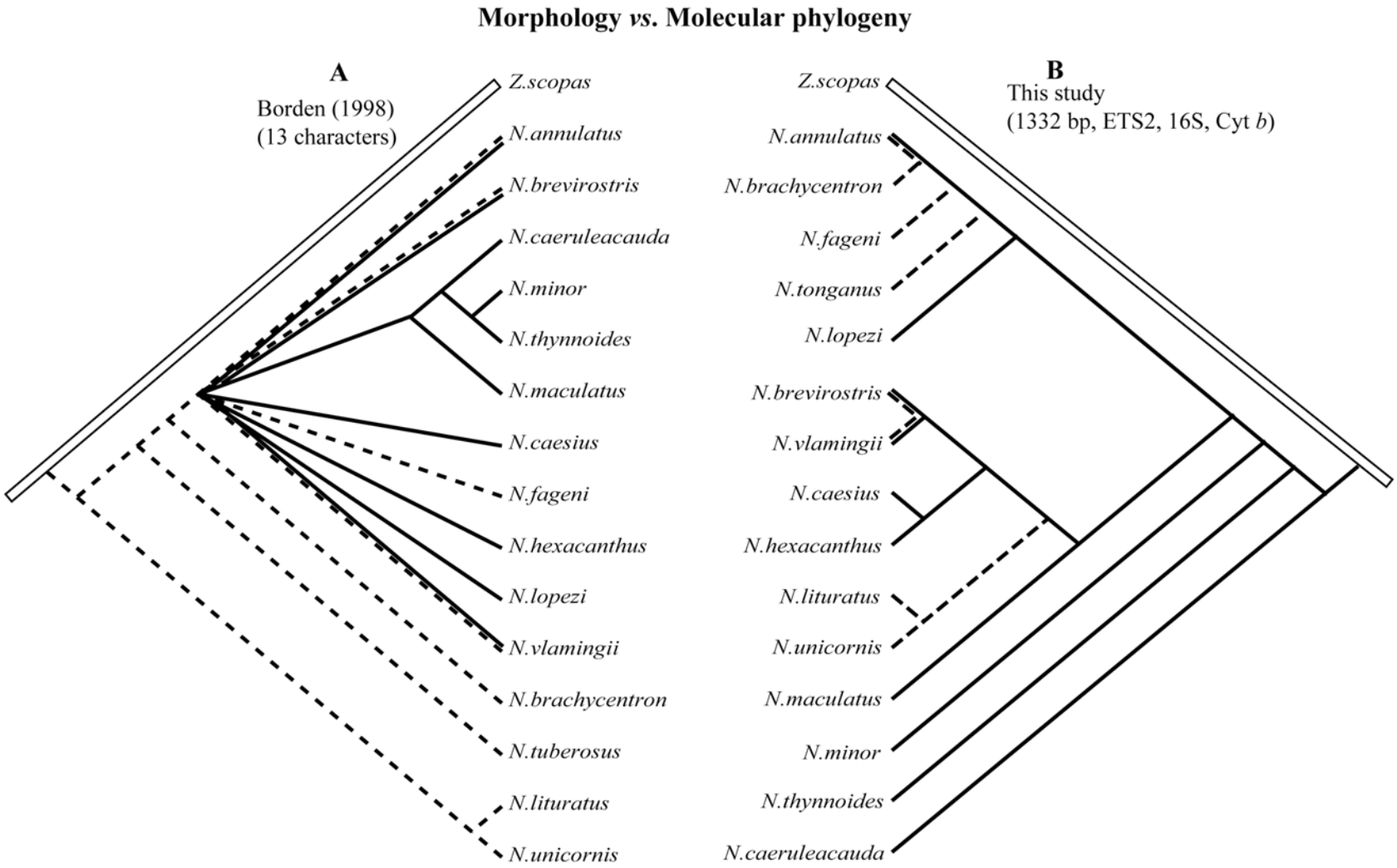

Figure 3.3: Borden's (1998) morphological phylogeny compared to the best molecular MP tree for combined ETS2, 16S and cyt *b* data set. **A**) Borden's constrained morphological topology, tree length 604. **B**) Unconstrained best parsimony tree (length 457) for the equivalent species of *Naso*. At the time of Borden's analysis *N. tonganus* was identified as a single widespread species *N. tuberosus*. Foraging modes are indicated by dotted (benthic) and solid (pelagic) lines.

Hypothesis 2: *Benthic foraging on macroscopic algae is plesiomorphic within the genus Naso (pelagic foraging is derived)* (Winterbottom 1993; Winterbottom and McLennan 1993; Borden 1998). This hypothesis was rejected for both cases, when the constrained topologies (either for a herbivorous or a pelagic clade) were compared to the MP molecular tree (Table 3.3, Appendix 1).

Hypothesis 3: *The presence of frontal horns and other cephalic structures does not reflect evolutionary relationships within the genus Naso*, *i*.e., this trait occurs independently in different clades (Borden 1998). The existence of a cephalic horn clade was rejected (Table 3.3, Appendix 1), thereby confirming Borden's (1998) hypothesis.

Hypothesis 4: *All members of the subgenus Axinurus constitute a single morphologically defined group within the genus* (Smith 1966; Randall 2001) *and are basal to other Naso species* (Dayton 2001). This hypothetical tree topology was the only one not rejected by all 3 tests (Table 3.3, Appendix 1).

Table 3.3: Tests of 4 hypotheses, including constrained tree length with most parsimonious molecular MP tree length of this study in brackets, the difference to the most parsimonious molecular tree (number of extra steps), p-values for KH and nonparametric tests (Wilcoxon signed-ranks, winning-sites (sign) test).

Hypotheses	Constrained tree length	No. of extra steps	KH-test (2 tailed)	Wilcoxon signed-rank test	Winning-sites (sign) test
1. Morphology	604 (457)	147	<0.0001*	<0.0001*	<0.0001*
2. Foraging mode:					
Benthic clade	549 (519)	30	0.0001*	0.0001*	0.0002*
Pelagic clade	553 (519)	34	<0.0001*	0.0001*	<0.0001*
3. Horned clade	565 (519)	46	<0.0001*	<0.0001*	<0.0001*
4. *Axinurus* monophyly	522 (519)	3	0.57	0.56	0.70

* significance-level p<0.001

3.4 Discussion

The primary issue arising from the phylogenetic reconstruction and history of the genus *Naso* is the relationship between structure, external morphologies, foraging modes and lineage diversification.

The pattern of generic relationships within the family Acanthuridae, as retrieved by the nuclear marker ETS2, confirmed those of previous studies (Winterbottom and McLennan 1993; Tang et al. 1999; Clements et al. 2003). The genus *Naso* is monophyletic and forms a sister clade to the remaining five extant acanthurid genera. The pattern retrieved by a new marker (ETS2) emphasizes the underlying agreement of generic relationships that is reflected by both morphological and molecular data (Clements et al. 2003). However, in marked contrast, the sequence data provided strong evidence against the species relationships within *Naso* as proposed by Borden (1998) on the basis of morphology. The difference between morphological and molecular topologies was most clearly seen in the relationship between benthic and pelagic foraging species. The primary morphology based hypothesis of species relationships within *Naso* (benthic foraging is plesiomorphic) was clearly rejected by the sequence data. This does not however, refute a hypothesis that benthic foraging may be plesiomorphic in other acanthurid genera (Winterbottom and McLennan 1993), such as *Acanthurus*. Secondly, the sequence data supported the hypothesis that frontal horns do not reflect evolutionary relationships within the genus (Borden 1998). The subgenus *Axinurus* was basal in all trees examined (as suggested by Dayton 2001), moreover we could not reject the hypothesis of monophyly of *Axinurus* on the basis of available sequence data (Smith 1966; Randall 1994).

Distinctive structural features, such as cephalic horns, tuberosities and scombriform morphology were distributed amongst the 4 sub-clades in a manner suggesting independent origins of or, in the case of scombriform morphology, retention of these features. Significantly, occurrence of species with streamlined scombriform morphologies within 3 of all the *Naso* clades, including the basal subgenus *Axinurus*, suggests that this plesiomorphic morphology has been retained and other morphologies have arisen repeatedly and independently. The distinctive morphological features that characterise *Naso* appear to be prone to convergence. They do not provide a reliable basis for classification within the genus.

Since Borden's study in 1998, four more species have been described (Randall 2001; Johnson 2002), all of which are included in this study. These are *N. tonganus*, *N. mcdadei* (sub-clade 1), *N. elegans* (sub-clade 3) and *N. reticulatus* (sub-clade 4).
Johnson (Johnson 2002) suggested that *N. tonganus – tuberosus – mcdadei* are closely related and considered them a complex of 3 species, which is true for *N. tonganus – N. tuberosus*, but not *N. mcdadei*, which appears to be sister to *N. fageni*, rather than *N. tuberosus*. *N. tuberosus* is restricted to the west Indian Ocean (e.g. recorded from Mauritius, Seychelles), and *N. tonganus* has a range from the Indian (photographic evidence only, no voucher specimens) - to the central Pacific Ocean (Randall 2002). However, all three species group in the *N. annulatus* sub-clade and are benthic foraging species.
N. elegans was redescribed by Randall (Randall 2001) and was suggested to be closely related to *N. lituratus*, as was also evident from this study. The sister relationship of *N. lituratus – elegans* was always well supported forming the 3rd sub-clade with *N. unicornis*

basal. *N. lituratus* and *N. elegans* have distinct distribution ranges (former in the Pacific -, and latter in the Indian Ocean) with some overlap in the east Indian Ocean (Andaman Sea, West Indonesia) (Randall 2002). Both species are benthic foragers and appear to be more reef-associated.

Naso reticulatus, recently described by Randall (2001), and *N. maculatus* formed a separate (4^{th}) sub-clade. *N. maculatus* appears around Hawaii, Japan, Taiwan and off the southern-east coast of Australia (antitropical distribution) whilst *N. reticulatus* is recorded from the tropical west Pacific Ocean (Philippines) (Randall 2002). It is suggested that this sub-clade (4) consists of pelagic feeding species, they also occur at depth > 30m.

In addition to clarifying the evolutionary history of the genus *Naso*, this molecular phylogeny forms the basis to investigate the temporal pattern of lineage diversification in the genus. In the next chapter, timing of divergence and age estimates for *Naso* lineages are obtained using fossil records.

Having this information, will allow for an examination which historic events may have driven cladogenesis in this genus.