CHAPTER 4

Temporal patterns of lineage diversification in the genus *Naso* (Section A)

4.1 Introduction

The previous chapter (3) resolved the species-level relationships of the genus *Naso*. Having inferred a well-resolved phylogeny for the genus, questions about the dates of putative lineage diversification events in this genus can be examined. Estimating the temporal pattern of divergence of lineages of a group allows for evolutionary hypotheses to be formulated to explain what may have driven cladogenesis. The factors driving diversification may be ecological and/or geographical coupled with historical events, such as continental drift, sea level and temperature changes.

Here, I use fossil records of related fossil acanthurid ancestors to estimate ages and timing of divergence for the genus *Naso*. The temporal pattern of lineage diversification of the genus can then be determined by applying this rate. Lineage diversification can then be related to biogeographic events in order to ascertain if diversification has been associated with major tectonic, climatic and oceanographic events.

The critical issue for a proper molecular clock calibration is to have a comprehensive phylogeny of all known species of the genus and to be able to date lineage diversification appropriately (using related fossils and/or tectonic events) for the group in question.

Molecular techniques are increasingly being used to date diversification of lineages by assuming that substitution rates (especially for protein coding gene regions) are roughly constant over evolutionary time (Hillis et al. 1996; Nei and Kumar 2000). However, there is mounting evidence that rate constancy is not maintained across different gene regions or at codon positions within these, for example 3rd codon positions of protein coding genes mutate more rapidly than 1st and 2nd codon positions (Martin 1995b). Likewise, stem and loop regions in ribosomal RNA evolve at different rates (Martin 1995b), as do the same gene regions between different organisms (Nei and Kumar 2000). A study of sharks (cytochrome *b*, protein coding) suggested a much slower mutation rate (5 – 7 times) for poikilotherms (e.g. amphibians, fish, sharks) than for homeotherms (e.g. birds, mammals, primates) (Martin et al. 1992; Martin and Palumbi 1993b; Martin 1995a). Martin and Palumbi (Martin and Palumbi 1993b; Martin and Palumbi 1993a) attributed the large differences in mutation rates between poikilotherms and homeotherms to a combination of factors influencing the rate of nucleotide substitution, including metabolic rates, generation times and, to a lesser extent, body size. Despite this, a more general mtDNA mutation rate of between 1.2% and 2.0% per MY, calibrated for terrestrial vertebrates (e.g. primates) (see comments by Avise et al. 1998; Avise 2000), has been used for a number of fish studies (e.g. Shulman and Bermingham 1995; Stepien and Faber 1998; Bowen et al. 2001; Muss et al. 2001; Stepien et al. 2001). Using such a mutation rate, which is too fast, may result in estimated divergence dates for fish lineages that are about one-fifth the age that they should be. It is therefore advisable, to calibrate a molecular clock so that the rate of divergence for the group of interest can be more accurately estimated, rather than accepting a rate established for other taxa. Once the age of a lineage has been ascertained, it is possible to determine which historic events are correlated with lineage diversification.

Fossil dates are useful to calibrate molecular clocks. However, for a large number of taxa fossil records are lacking. Major geological events associated with lineage diversification may also be used to calibrate molecular clocks (e.g. Bermingham et al. 1997; Knowlton and Weigt 1998; Lessios et al. 2001). A large number of studies, which have used fossil records and/or major geological events have calibrated molecular clocks of several mt markers for several fish species (e.g. Orti et al. 1994; Bermingham et al. 1997; Briolay et al. 1998; Alvez-Gomes 1999; Zardoya and Doadrio 1999; Kumazawa and Nishida 2000; Machordom and Doadrio 2001) (see Appendix 1). The calibrated rates obtained for fish species differed between mt gene regions, an indication that they evolve at different rates, which were also on average lower than the values of 1.2 – 2.0% per MY estimated for terrestrial vertebrates (Appendix 1).

A study by Glazko and Nei (2003) showed that by concatenating several gene fragments a more robust estimate of divergence time can be obtained, especially if unlinked genes are included. The authors argued that by using only one individual gene, a biased estimate towards divergence times was recorded (due to variance and covariance values using branch length see equations in Glazko and Nei 2003, p.427). By adding or concatenating gene fragments, (including nuclear genes) the variance and covariance of the same equation was reduced, and as a consequence, the estimation bias of divergence times (Glazko and Nei 2003).

In this study, appropriate fossils from the extensive acanthurid fossil record are used in combination with a comprehensive molecular phylogeny to estimate the rate of divergence

of *Naso* lineages. Although none of the extant genera of acanthurids occur as fossils, the family has an informative fossil record, especially from the early Tertiary, a period when critical events in acanthurid lineage diversification must have occurred (e.g. Bannikov and Tyler 1992; Sorbini and Tyler 1998a; Tyler 2000; Clements et al. 2003). Extensive fossil records which date back to the Eocene, Oligocene and Miocene have been described for surgeonfishes (Bannikov and Tyler 1995; Tyler 1997; Sorbini and Tyler 1998b; Tyler and Sorbini 1998; Tyler 1999a; Tyler 1999b; Tyler 2000; Tyler and Bannikov 2000). Indeed, according to Bellwood and Wainwright (2002) most of the fossils found in Tethyan deposits of Monte Bolca, Italy can be referred to the Acanthuridae than to any other family. Nearly all genera of this family have one or more related species recorded from the Eocene. Fossil acanthurid fishes including pelagic species were examined by Tyler & Sorbini (1998) and Tyler (1999b; 2000). Tyler (2000) established a phylogeny of the Nasinae demonstrating that the extant *Naso* (Indo-Pacific Ocean) and its sister taxon *Eonaso* (Caribbean, possibly Oligocene) were a sister group to *Arambourgthurus* (Oligocene, Tethys), *Sorbinithurus* (Eocene, Tethys) and *Marosichthys* (Miocene, Celebes, West Pacific) (Figure 4.1). *Arambourgthurus* is a distinctive genus of nasines displaying a scombriform morphology and caudal propulsive unit with extensive hypurostegy (see Chapter 3). Such a modified caudal fin structure is also seen in *Naso* and *Eonaso* although, in these genera hypurostegy is rarely strongly developed (Tyler 2000). This caudal fin occurs throughout the Nasinae including species with a benthic reef-associated foraging mode. A capacity for pelagic foraging appears to be widespread within the subfamily.

The temporal distribution of acanthurid fossils (Blot and Tyler 1991; Sorbini and Tyler 1998b; Tyler 1999a; Tyler 2000; Tyler and Bannikov 2000) strongly suggests that initial

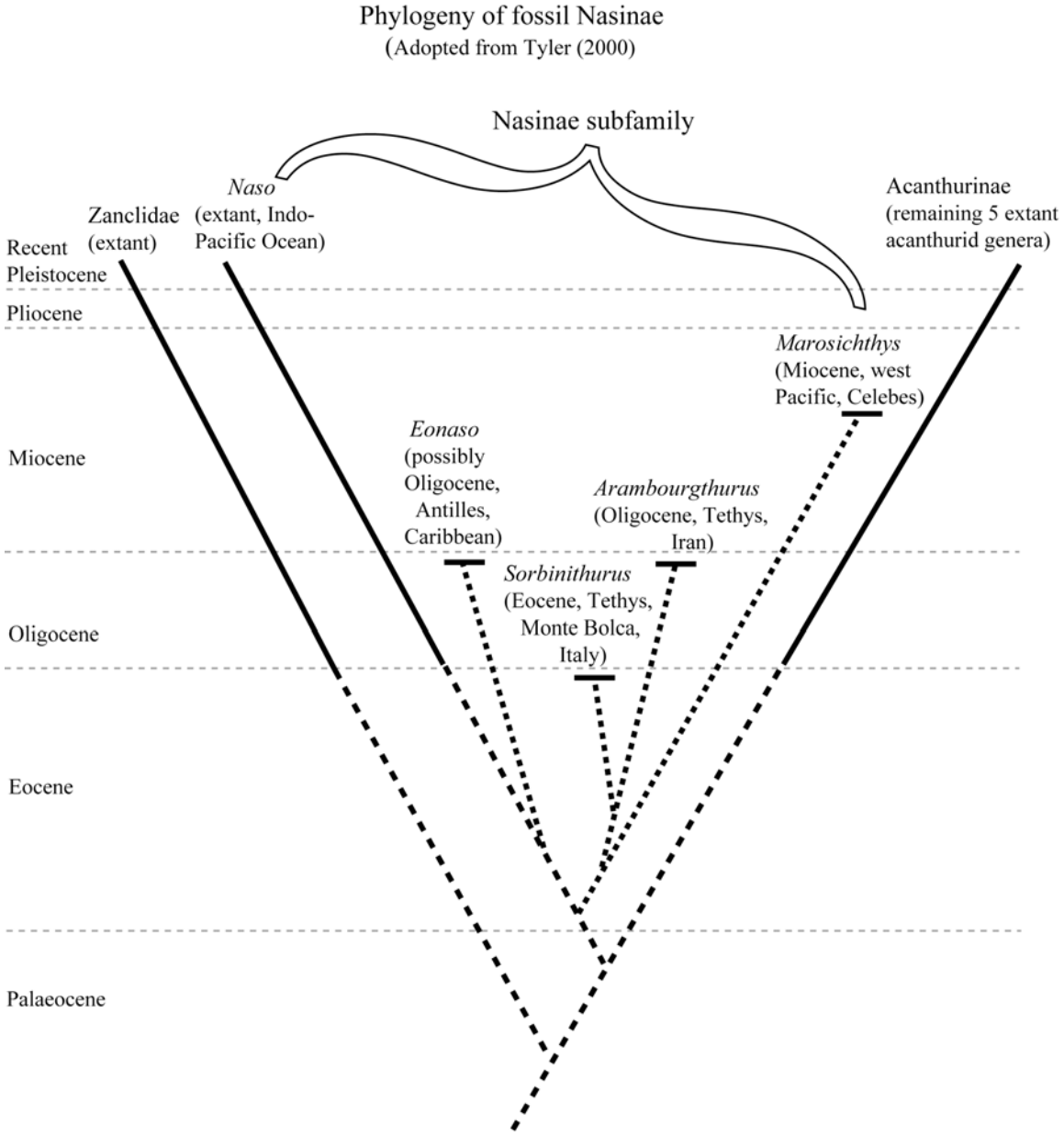


Figure 4.1: This Nasinae phylogeny illustrates relationships between the extant genus *Naso* and fossil relatives of Nasinae based on 14 characters (osteological) from fossilized specimens. Bars at the end of a lineage indicate extinction events. See Tyler (2000) for details.

diversification within the Acanthuridae occurred very early in the Tertiary (Clements et al. 2003). *Proacanthurus* (subfamily Acanthurinae) (Sorbini and Tyler 1998b) the

morphologically primitive sister taxon of the recent genera *Paracanthurus, Zebrasoma, Acanthurus* and *Ctenochaetus* is of early-Eocene age, as is the Nasinae genus *Sorbinithurus* (52MY according to Tyler 2000), demonstrating that the Nasinae existed at this time.

4.2 Materials and Methods

The inferred phylogenetic trees of chapter 3 are used to estimate dates of divergence.

To obtain estimates of the timing of lineage diversification in *Naso*, a two-step protocol was followed. The first step was to obtain a range of dates (in MY) for the MRCA (most recent common ancestor) of the genus *Naso*. For this, the genus-level phylogeny inferred using ETS2 sequences (chapter 3) was used. Two nodes, one basal to *Luvarus* and the other to the family Acanthuridae were fixed (following Sanderson 1997; Sanderson 2003) with ages of 55- and 52MY respectively. These dates are based on ages of related fossil fish (*Proluvarus necopinatus*, 55MY, *Proacanthurus tenuis* and *Sorbinithurus sorbinii*, both 52MY) (see Bannikov and Tyler 1995; Sorbini and Tyler 1998b; Tyler 1999a; Tyler 2000). To establish if sequences accumulate substitutions in a clock-like manner, they were analysed with the Langley-Fitch (LF) (Langley and Fitch 1974) method, which uses maximum likelihood under the assumption of a molecular clock as implemented in r8s version 1.60 (Sanderson 2003). This method generates a chi-square value, which if accepted ($p>0.05$), indicates that sequences act in a clock-like manner. If however this hypothesis is rejected and sequences do not conform to a strict molecular clock, a nonparametric rate smoothing (NPRS) approach can be used (Sanderson 1997) implemented in r8s. The latter approach relaxes the stringent assumptions of a molecular clock (Sanderson 1997), while still estimating ages for each node, local rate estimates for

each branch and a mean value of these divergence rates (substitution/site/unit time). Once a range of age estimates was obtained for the MRCA of *Naso*, the second step involved generating estimates of divergence times for each node in the tree of *Naso* species. For this, the same methodology as described above was followed using the tree inferred from combined sequence data (ETS2, 16S & cyt *b*) (chapter 3). This allowed a curve, illustrating the accumulation of extant lineages over evolutionary time, to be constructed for the genus *Naso* for each of the age estimates (MY). The curve was generated following a similar methodology developed by Barraclough and Nee (2001). The accumulation of lineages giving rise to all extant *Naso* species from the MRCA node to the tip of the tree was plotted against time (MY) (both axes used an arithmetic scale).

4.3 Results

ETS2 sequences did not behave in a clock-like manner (χ^2 =164.98, d.f. 37, $p<0.001$). Consequently, estimates of ages leading to different genera were obtained by nonparametric rate smoothing (NPRS). The average divergence rate (%/MY) was 0.21% (with S.D. 0.55%) and a range of local rates: min. 0.0 and max. 3.3%. The MRCA of *Naso* had a max. age of 52MY and a min. of 43.3MY. The MRCA of the genus *Prionurus* had an estimated age range between max. 49.7 and min. 45.9MY and that of *Zebrasoma* ranged between max. 36.9 and min. 31.6MY. The *Acanthurus-Ctenochaetus* clade was in-between the latter two genera, but younger than *Naso* with age estimates ranging between max. 47.8 and min. 31.1MY (Figure 4.2).

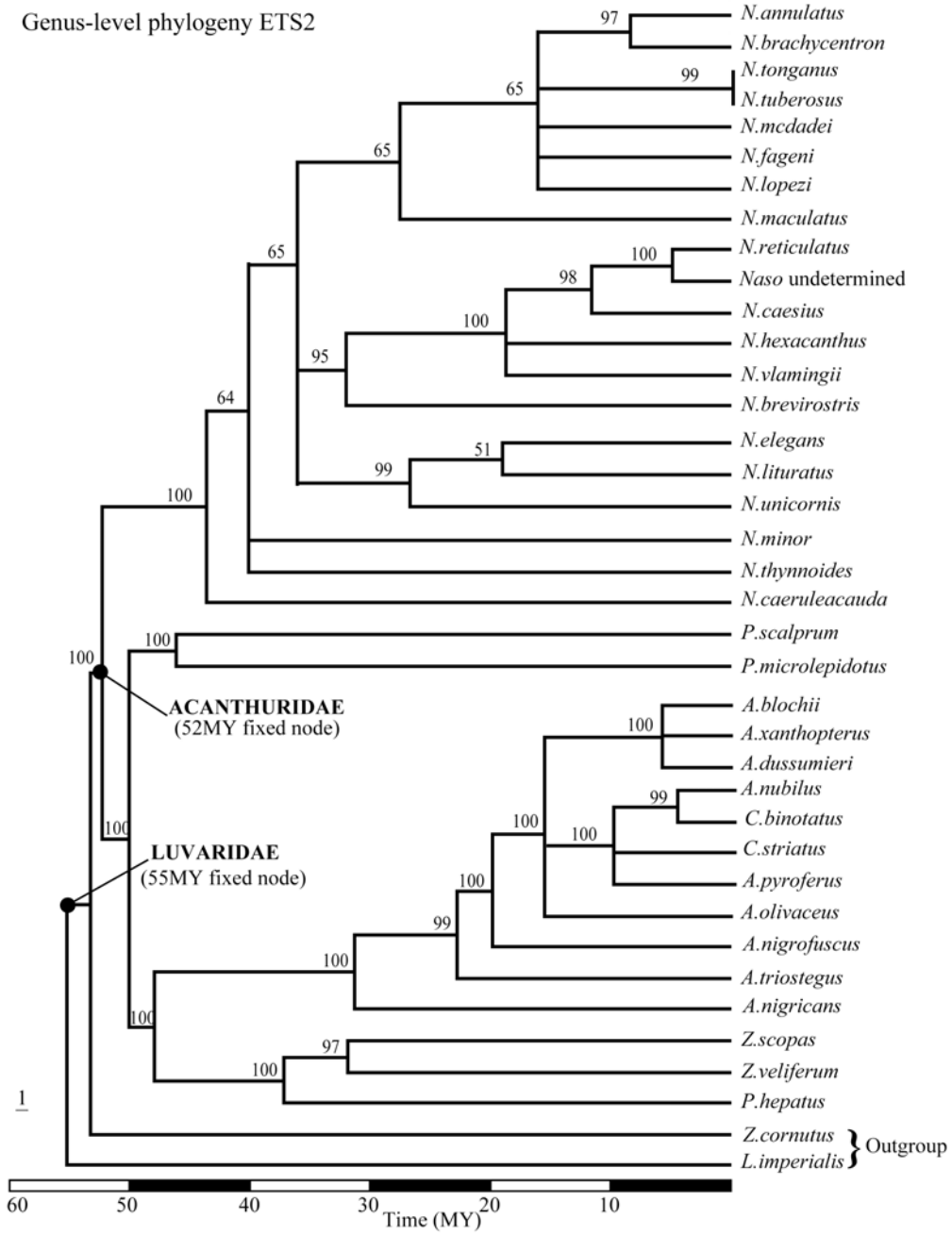


Figure 4.2: Ultrametric tree inferred from ETS2 sequences (ML topology, see Chapter 3). Branch length are proportional to absolute time (generated by NPRS method in r8s, see text for information). Two nodes were fixed using fossil records indicated by black circles. Bayesian posterior probabilities (in %) are indicated above branches.

Age estimates for the MRCA of *Naso* were obtained from the nuclear marker (range 52 to 43.3: thus the average is 47.6MY, Table 4.1). The combined sequences did not act in a clock-like manner (χ^2 =59.18, d.f. 19, p<0.001).

A NPRS method was therefore used and divergence times were estimated for each node assuming each of 3 different ages in turn (Figure 4.3B). The mean % divergence rates were: 0.063% (S.D. 0.027%) for a 52MY MRCA; 0.068% (S.D. 0.03%) for a 47.6MY MRCA and 0.075% (S.D. 0.033%) for the youngest, 43.3MY, estimated age of the MRCA. Different curves were generated for each age estimate, which displayed similar divergence patterns over evolutionary time (Figure 4.3B).

Table 4.1: Estimated ages of the most recent common ancestor (MRCA) giving rise to *Naso* lineages as generated by nonparametric rate smoothing implemented in r8s (Sanderson 2003).

Nodes (Fig. 4.3)	Max. age (52MY)	Mean age (47.6MY)	Min. age (43.3MY)	Giving rise to extant species
a	52	47.6	43.3	Root
b	46.1	42.2	38.39	*N. minor*
c	43.8	40.1	36.47	
d	37.05	33.92	30.85	*N.caeruleacauda, N. thynnoides*
e	35.83	32.8	29.84	
f	34.21	31.31	28.49	
g	24.24	22.71	20.66	*N. lopezi*
h	23.75	21.74	19.78	*N. brevirostris*
j	23.01	21.57	19.62	
k	21.83	19.98	18.18	*N. maculatus, N. reticulatus*
l	20.19	18.48	16.81	*N. unicornis*
m	19.86	18.18	16.54	*N. vlamingii*
n	18.48	16.91	15.38	
o	16.8	15.37	13.99	*N. annulatus, N. brachycentron*
p	14.19	12.99	11.81	*N. fageni, N. mcdadei*
q	7.18	6.57	5.98	*N. tonganus, N. tuberosus*
r	5.62	5.15	4.68	*N. elegans. N. lituratus*
s	3.24	2.97	2.7	*N. hexacanthus*
t	1.71	1.56	1.42	*N. caesius, Naso* undetermined

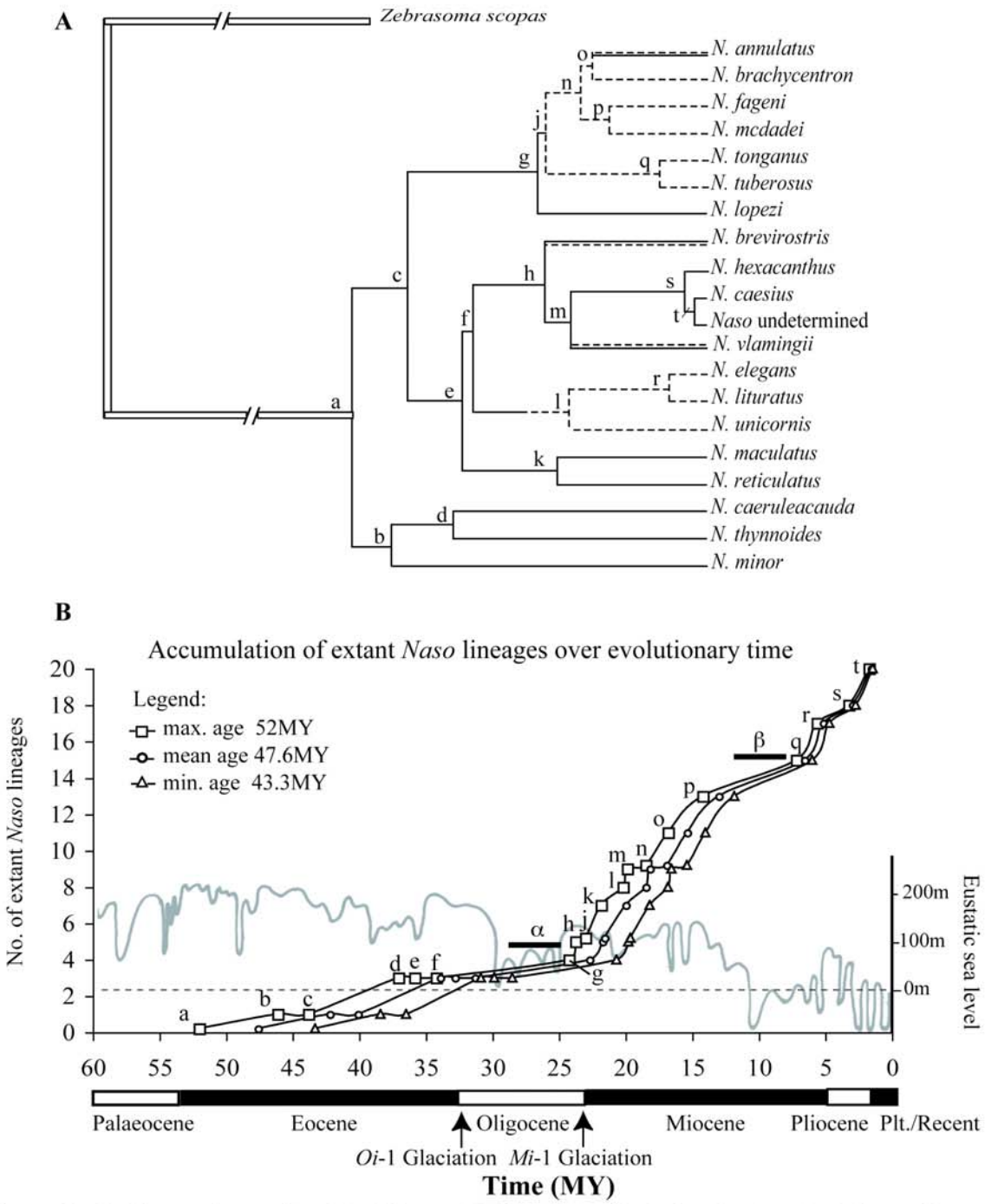


Figure 4.3: **A)** Ultrametric tree of combined data set (ETS2, 16S, cyt *b*) obtained by Bayesian inference (see Chapter 3). Foraging modes are indicated, pelagic as black and benthic as dotted lines. Small letters (a-t) refer to nodes giving rise to *Naso* species. **B)** Curves illustrating the diversification of *Naso* lineages through evolutionary time. The x-axis depicts relative time since the ancestral root node, values in MY. The y-axis illustrates the number of *Naso* lineages. Eustatic sea levels with present day level (dotted line), *Oi*-1 and *Mi*-1 glaciation times (arrow) modified from Haq et al. (1987), Pickering (2000) and Zachos et al (2001) are shown. Thick bars, α and β, indicate periods of no diversification. Small letters (a-t) as above (**A**) indicated on the curves are the same across the horizontal plane for all 3 curves.

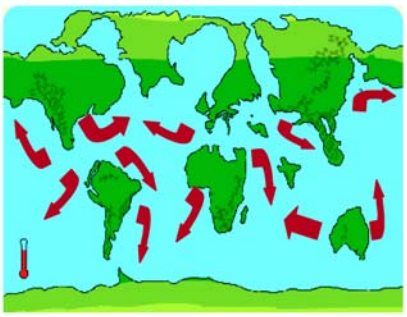
Basal lineages in *Naso* (especially members of *Axinurus*), all exhibiting a pelagic foraging mode (plesiomorphic state), arose from the early Eocene to early Oligocene (nodes a→d, all 3 curves, Figure 4.3A,B). The majority of the MRCAs to extant lineages however, arose throughout the Miocene (nodes g/h→r, Figure 4.3B), subsequent to the *Mi*-1 glaciation.

For the first time, during the early Miocene, ancestors of extant species with a benthic foraging mode arose (e.g. node j, *N. annulatus* sub-clade). Sea levels were overall high (~ 100m) in the early Miocene (landmasses of some continents were submerged, e.g. South America, see Figure 4.4) and cold ocean currents were established (Figure 4.4).
Because of repeatedly fluctuating sea levels, during the Miocene (Figure 4.3B), a number of *Naso* lineages arose (nodes k→p, Figure 4.3B). These lineages gave rise to pelagic foraging species (node k, *N. maculatus* sub-clade), generalist species (node m, *N. vlamingii* and node o, *N. annulatus*) as well as exclusively benthic foraging species (node l, *N. elegans* sub-clade).

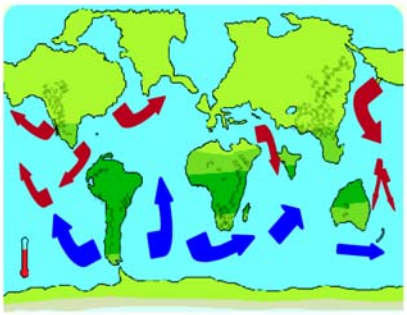
All 3 curves suggest that there were two extended periods during which no extant lineages arose (α& β□□□ure□.3□). The first of these was in the mid – late Oligocene and the second in mid – late Miocene (Figure 4.3B).
The most recent diversification of extant lineages occurred during the Plio-Pleistocene (nodes s & t) giving rise to extant species displaying a pelagic foraging mode (Figure 4.3A,B).

Cenozoic maps depicting continental drift, overall temperatures and ocean circulation

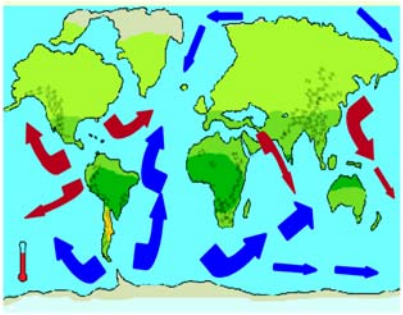
Eocene (54.8 - 33.7MY, nodes a-d)



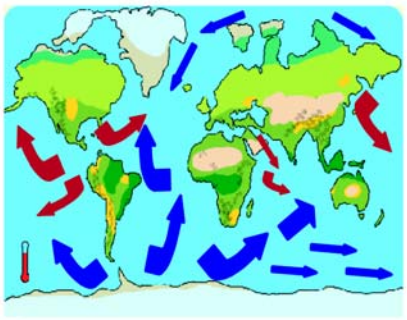
Oligocene (33.7 - 23.8MY, nodes d-g)



Miocene (23.8 - 5.3MY, nodes g-r)



Pliocene (5.3 - 1.8MY, node r-t)



Maps adopted from the BBC webside
www.bbc.co.uk

Figure 4.4: World maps depicting continental drift which relate to the different periods. Major ocean currents are indicated, warm currents (red arrows) and cold ocean currents (blue arrows).
The continents are coloured according to vegetation. For example, during the Eocene the continents are mainly green coloured, indicating a "greenhouse" period on land, but also in the oceans, with overall warm (red) currents. Cold (blue) ocean currents occur for the first time during the Oligocene.
Major glaciation events (*Oi*- and *Mi*-glaciation) during the Oligocene and Miocene resulted in fluctuating sea levels. During the Miocene modern coral reefs and ocean currents became established.
Nodes are indicated in brackets (accounting for age estimates of all 3 curves).

4.4 Discussion

The primary issue arising from the estimation of divergence times of the genus *Naso* is the relationship between major plate tectonic, glaciations, palaeoclimatic events and the evolutionary diversification of the genus.

4.4.1 Estimation of divergence rates

The average rate estimate obtained for the combined *Naso* sequences is quite low (~ 0.065% divergence/MY) compared to other fish studies (e.g. between 0.05% to 1.32% divergence/MY Bermingham et al. 1997; Alvez-Gomes 1999; Kumazawa and Nishida 2000; Machordom and Doadrio 2001, see also Appendix 1), which used at least 2 mt genes and also used either fossil calibrations and/or geological events. However, those studies based their calibrations exclusively on mtDNA sequences. This study concatenated 3 gene regions (including one nuclear) for the calibration, because a more robust estimate of divergence time can be obtained in this manner, especially when unlinked genes are included (Glazko and Nei 2003).

Other studies estimated rates ranging between 0.22% and 0.76% divergence/MY (Martin and Palumbi 1993b; Cantantore et al. 1994; Briolay et al. 1998; Zardoya and Doadrio 1999), but based their calibrations on only one mt gene region. All these studies apart from Cantantore et al. (1994), used fossil dates and/or geological events to calibrate the molecular clocks. The implication of the slower rates suggest, for example, that an average substitution rate of 1.5 – 2% for mammals/birds would correspond to a substitution rate of around 0.3 – 0.4% for fish species. Cantantore et al. (1994) developed a software program

based on a stationary Markov clock to calculate the substitution rates at the 3 different codon positions (cyt *b*) for several marine fish species (1^{st} 0.045%, 2^{nd} 0.015%, 3^{rd} 0.75%, resulting in an overall average of 0.27% divergence/MY).

Another two of the studies (Briolay et al. 1998; Zardoya and Doadrio 1999) were on freshwater fish (Cyprinidae in Europe) and used fossil dates as well as tectonic events to calibrate their molecular clocks. Martin and Palumbi (1993b) compared nucleotide substitutions (1^{st} and 2^{nd} codon positions) of shark cyt *b* calibrated with fossil dates to mammalian cyt *b* substitution rates (resulting in 0.22%/MY and 1.4%/MY respectively). Such comparisons between rates of divergence for different species groups or taxa indicate that it is better to use a taxon specific calibrated molecular clock to date cladogenetic events in a group, if possible, than simply to apply rates obtained from the literature.

4.4.2 Timing of divergence and biogeographic history of **Naso**

Complex perciform lineages that constitute the modern reef fish fauna proliferated in the early to mid Eocene, although basal perciform lineages must have been present earlier (Tyler et al. 1989; Bellwood 1996; Bellwood and Wainwright 2002). Among the morphologically specialized Eocene acanthuroid fishes were 11 genera of acanthurids, 2 genera of luvarids, 3 genera of siganids and a zanclid (Bannikov and Tyler 1995; Tyler 1997; Tyler 1999a; Tyler 2000). The tropical shallow-water reef environment of this period was particularly favourable to reef fishes. Sea levels were substantially higher (150 – 200m) than at present (Hallam 1984; Haq et al. 1987) and sea-surface temperatures in low latitude habitats were uniformly high (also called greenhouse period Pickering 2000; Stanley 2001) in association with the 55 MY early Eocene climatic optimum (Zachos et al.

2001a). Biological reef systems including both algae and coral dominated reefs achieved their greatest latitudinal spread of the Tertiary at this time (Kiessling 2001a). This period of rapid proliferation of reef fish lineages was followed by relative stasis of the fauna (Bellwood 1996; Bellwood and Wainwright 2002) for much of the remainder of the Tertiary. More recent episodes of diversification of reef fishes occurred during the period of fluctuating sea levels throughout the Plio-Pleistocene (McMillan and Palumbi 1995; Palumbi 1997; Clements et al. 2003).

The basal *Naso* species, which arose during the Eocene, were pelagic foragers. The subsequent diversification leading to the variety of morphological and foraging modes that characterizes the present fauna occurred post-Eocene/Oligocene. The critical episode of lineage diversification of *Naso* commenced in the very late Oligocene/early Miocene. This coincides with the brief, but intense *Mi*-1 glaciation (Zachos et al. 2001b), a period of profound modification to shallow-water marine habitats associated with increased glaciations, falling temperatures, fluctuating sea levels and substantial increase in ocean productivity (Zachos et al. 2001a; Zachos et al. 2001b). Algal reefs may have dominated during such glacial periods (Kiessling 2001a), however the mid Miocene saw a rise in sea levels and an increase in coral reef systems covering a range similar to that of present day tropical coral reefs (Kiessling 2001a).

Ancestors to generalist species such as *N. brevirostris* (node h, Figure 4.3), which display both benthic and pelagic foraging modes, arose for the first time. The MRCA leading to the *N. annulatus* clade (node j), the majority of which are benthic feeding, diverged during the early Miocene, probably an example of ecological diversification in the new algal-reef

dominated environment. Subsequent lineages arising during the remainder of the Miocene include species that forage exclusively on algae (*N. elegans* clade), species that are pelagic foraging (*N. maculatus* clade) and generalist species (*N. vlamingii* and *N. annulatus*).

All fossil representatives of the subfamily Nasinae with the exception of *Eonaso* (sister to *Naso*) occur in the Tethyan, *Sorbinithurus* (Eocene), *Arambourgthurus* (Oligocene) or western Pacific deposits *Marosichthys* (Miocene) (Tyler 2000).

The fossil acanthurid, *Eonaso*, closely related to *Naso* (Tyler 2000) was recorded from the tropical Atlantic Ocean (Antilles, Caribbean) (Tyler and Sorbini 1998; Tyler 2000). The age of this important fossil remains unresolved. As with many groups of reef fishes, early diversification of the Nasinae occurred in the Tethys (Tyler 2000), with most of the subsequent taxa colonizing eastwards into the Indo-Pacific Ocean. The most parsimonious explanation accounting for the location of *Eonaso*, is westward colonization into the tropical Atlantic and subsequent extinction. It seems likely that the extinction of *Eonaso* as the only Nasinae species in the tropical Atlantic occurred during events associated with the *Mi*-1 glaciation, a period when widespread extinctions of corals occurred in the Caribbean (Zachos et al. 2001a). It is of interest to note that the well-preserved fossil of *Eonaso* shows clear evidence of scombriform caudal structure, but an external morphology consistent with benthic foraging (Tyler and Sorbini 1998).

4.4.3 Comparison to other acanthurids

The pattern of diversification of lineages within the genus *Naso* contrasts with the evolutionary pattern of reef fishes in general and other acanthurids in particular. Evolution

in the Acanthuridae was characterised by two episodes of rapid diversification. Firstly, in the early to mid-Eocene a complex of benthic browsing taxa arose (Tyler et al. 1989; Blot and Tyler 1991) and secondly, there was a much more recent proliferation of grazing species within the genera *Acanthurus* and *Ctenochaetus* (Tang et al. 1999; Clements et al. 2003). In contrast to the pattern of rapid Eocene diversification seen in other acanthuroids, the curve for the genus *Naso* suggests a slow accumulation of lineages during the Eocene, none during the Oligocene, followed by an increase during the Miocene. Unlike *Acanthurus* and *Ctenochaetus* there is little evidence of rapid and recent speciation of *Naso*. With the exception of *Sorbinithurus* (Tyler 1999a; Tyler 2000), the subfamily Nasinae was not represented in the Eocene Monte Bolca beds. This may reflect the pelagic foraging mode of the basal lineages. Similar conclusions were reached concerning the early evolution of the luvarid acanthuroids (Bannikov and Tyler 1995). The main episode of diversification of *Naso* occurred during the Miocene, represented by a period when shallow reef systems at all latitudes were subject to major disturbances and faunal turnover (Prothero 1994; Sheehan 2001).

Although many of the present day species of *Naso* are consistently associated with coral reefs, with the exception of the smaller pelagic species, they are rarely abundant compared to *Acanthurus* and *Ctenochaetus*, and many taxa (e.g. *N. fageni, N. mcdadei*) occur in deeper non-reef environments, unlike *Acanthurus* and *Ctenochaetus* that are closely associated with reef environments.

In summary, the temporal pattern of lineage diversification in the genus *Naso* demonstrates a long evolutionary history. We now know when all extant species arose and which species are most closely related (sister species).

Interestingly, questions arise from this knowledge (phylogeny & timing of divergence, chapters 3 & 4) particularly (a) are these widespread species, genetically differentiated across their distribution range or is there evidence of cryptic speciation, (b) what process has driven speciation in the genus, and (c) does a long evolutionary history influence the population structure of a species?

These issues will be examined in the following 2 data chapters (6 & 7).

Chapter 5 reviews the phylogeographic literature with a focus on marine species. Chapter 6 examines the population structure of the widely distributed species, *N. vlamingii* and chapter 7 examines the genetic interactions between the members of each of two pairs of sister species (*N. lituratus – N. elegans* and *N. tuberosus – N. tonganus*).