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TITLE: Genetic evolution among unicornfishes of the genus *Naso* (Acanthuridae)

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There is no species-level phylogeny within any family of the suborder Acanthuroidei. The present study uses allozymic data to generate a phylogeny for five species of the genus *Naso* (unicornfishes): *Naso caesius*, *N. hexacanthus*, *N. lituratus*, *N. unicornis*, and *N. thynnoides*. For the purpose of outgroup comparisons, *Acanthurus lineatus* and *Zanclus cornutus* were included. The allozyme data set was analyzed phenetically and cladistically. The results of the phenetic (i.e. genetic distances) and cladistic (i.e. three uniquely shared derived character states) analyses both indicate that the sibling species pair, *N. caesius* and *N. hexacanthus* are the most closely related taxa among the species of *Naso* investigated in this study. The relationship between the two benthic algal grazers, *N. lituratus* and *N. unicornis*, is not resolved in this study. Members of the subgenus *Naso* (*Naso caesius*, *N. hexacanthus*, *N. lituratus*, and *N. unicornis*) are characterized by two uniquely shared derived character states or synapomorphies. Many autapomorphies were detected in a member of the subgenus *Axinurus*, *N. thynnoides*. The present study provides evidence to support the two subgenera in the genus *Naso*.

Genetic Evolution Among Unicornfishes of the Genus *Naso* (Acanthuridae)

BY

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INTRODUCTION

Members of the genus *Naso* (unicornfishes), are common inhabitants of shallow waters surrounding coral reefs. Several members of this genus (e.g. *Naso hexacanthus*, *N. thynnoides*, and *N. unicornis*) have been examined in phylogenetic studies on the evolution of the families comprising of the suborder Acanthuroidei (Luvaridae, Siganidae, Zanclidae, Acanthuridae) (Winterbottom 1993; Winterbottom and McLennan 1993; Guiasu and Winterbottom 1993; Tyler 1989; 1970; and Leis and Richards 1984). These studies on the evolution of the Acanthuroidei used morphological characters and screened selected species to characterize the various genera within the acanthuroid assemblage (Winterbottom 1993; Winterbottom and McLennan 1993; Guiasu and Winterbottom 1993; Tyler 1989; 1970; Leis and Richards 1984; and Jones 1968). However, no species-level phylogenies within any family of the Acanthuroidei have been resolved.

Allozyme electrophoresis is a powerful taxonomic tool that uses a data base independent of morphological character data sets (Lacson and Bassler 1993; Hillis 1985; and Shaklee et. al 1984). This method can be used to distinguish between closely related species and to evaluate evolutionary relationships among such taxa (Dayton et al. 1994; Lacson and Nelson 1993; Lacson and Bassler 1993; Randall and Bell 1992; Vainola 1992; Hillis 1985; and Shaklee et al. 1982). The present study uses allozymic data to generate a phylogeny for selected members of the Nasinae (unicornfishes).

The genus *Naso* consists of morphologically and ecologically diverse fishes. For example, the subfamily includes both zooplanktivores (ca. 10 spp., e.g. *Naso caesius, N. hexacanthus,* and *N. thynnoides*) and benthic algal grazers (ca. 7 spp., e.g. *N. lituratus* and

N. unicornis). Morphological divergence is evident in osteology, musculature, dentition, number and shape of caudal spines, and the presence or absence of a rostral protuberance (the source of the name "unicornfish") (Randall 1955; Winterbottom 1992; 1993; Randall and Bell 1992; Tyler 1970, 1989; Guiasu and Winterbottom 1993; and Winterbottom and McLennan 1993). Variation in these characters accounts for the many controversies surrounding the taxonomy of these fishes (Winterbottom 1993; Randall 1955; 1986; 1990; 1994; and Smith 1951; 1956; 1966). Randall (1955) recognized only the genus *Naso* within the Nasinae but did believe that it was reasonable to place species of *Naso* exhibiting one spine on the caudal peduncle into a subgenus, *Axinurus*. Smith (1966) raised *Axinurus* to generic rank based on a single instead of two fixed peduncular plates in *N. thynnoides* and *N. minor*. Winterbottom's (1992) and Tyler's (1970; 1989) morphological studies revealed several apomorphies (derived characters) that characterized species of the subgenus *Axinurus* (then including only *N. thynnoides* and *N. minor*). Tyler's (1970) and Winterbottom's (1993) studies of acanthurids also detected a high number of specialized morphological features (synapomorphies) within the genus *Naso* that are not shared by other acanthurids. These morphological synapomorphies support the monophyly of the genus *Naso* but do not necessitate that only a single subgenus be recognized.

The primary goal of the present study is to examine phylogenetic relationships among selected species of the genus *Naso* by using allozyme electrophoresis. I examined five species: *N. caesius*, *N. hexacanthus*, *N. lituratus*, *N. thynnoides* and *N. unicornis*. *N. thynnoides*, together with two other species of *Naso*, *N. caeruleacauda* and *N. minor*, has been placed in the subgenus *Axinurus* (Randall 1994). A secondary objective of this study

was to use genetic methods to examine the validity of the two recognized subgenera (i.e. *Naso* and *Axinurus*). Recently *N. caesius* and *N. hexacanthus* were confirmed to be separate biological species by a fixed genetic difference at the protein-coding locus creatine kinase (Dayton et al. 1994). Therefore, this study was able to quantify the genetic divergence of this species-pair with respect to other species of *Naso* included in this study.

For outgroup comparisons, I included *Acanthurus lineatus* (Acanthuridae; Acanthurinae) and *Zanclus cornutus*, the sole member of the related family Zanclidae. Several authors (Winterbottom 1993; Guiasu and Winterbottom 1993; Winterbottom and McLlennan 1993; Tyler 1970; 1989) have postulated that the Nasinae is the sister group of the Acanthurinae based on morphological similarities in osteology and musculature. Differences in meristic characters (such as, for example the number and type (i.e. moveable or fixed) of caudal spines, dentition, and scales) separate the subfamilies. The osteology and musculature of *Z. cornutus*, indicate that the family Zanclidae is the sister taxon to the Acanthuridae (Winterbottom 1993, Guiasu and Winterbottom 1993, Tyler 1989). The specialized prejuvenile pelagic stage indicates the close evolutionary relationship among the Zanclidae, Acanthurinae, and Nasinae, along with the rest of the members of the suborder Acanthuroidei (Leis and Richards 1984).

MATERIALS AND METHODS

Collection, Storage, and Dissection of Specimens

Fish were speared at depths between 6 and 25m at Cocos Lagoon, Bile Bay, Blue Hole, Gab Gab II, Glass Breakwater, and Gun Beach on Guam. Individuals were placed on ice and brought to the UOG Marine Laboratory (maximum exposure time on ice was 1-2hrs) then stored at -70° C until used in electrophoretic analysis. The standard length of each fish was measured, and individuals were photographed prior to dissection. Two voucher specimens of *Naso thynnoides*, which is a species not previously recorded from Guam, have been deposited in the UOG Systematic Collection.

Preparation of Samples for Electrophoresis

Samples of brain, eye, gonad (if mature), liver, and muscle tissue were taken from each specimen. Tissue samples were placed in 1.5-ml polypropylene microcentrifuge tubes and stored at -70° C until all specimens were collected. Prior to electrophoresis, tissue samples were centrifuged (14,000 rpm at room temperature for three minutes); then a homogenization buffer (0.01 M Tris-HCL, 0.001 M EDTA, 0.001 M 2-mercaptoethanol) was added, and samples were recentrifuged (14,000 rpm at room temperature for three minutes), then refrozen for future analysis.

Starch Gel Preparation

Starch gels (12.5%) were prepared with hydrolyzed starch (Starch Art, Smithville, Texas) and either Tris-citrate (TC, pH 6.7, 7.0 or 8.0) or Tris-EDTA-Borate (TEB, pH 8.0) gel buffers. Gels were cooked in a 2-l Erlenmeyer flask on a hot plate with a stirring blade driven by a stirring motor. Gels were degassed for about 20 seconds after addition of two

drops of β-mercaptoethanol and/or NADP. Gels were then cooled for one hour at room temperature and refrigerated for 45 minutes.

Sample Loading, Electrophoresis, Gel Slicing, and Staining

Homogenized tissue samples were centrifuged for 3 minutes at 14,000 rpm. Between 30 to 33 µl of tissue homogenate supernatant were loaded into each sample well (15 wells/gel). Tissues and gel buffer systems used to give optimal resolution for detection of maximum heterozygosity for each enzyme are given in Table 1. Electrophoresis was performed at constant current (15-25 milliamps) for 24 hours at 4°C. Gels were then removed from the molds, transferred to a formica table top, and sliced horizontally to 1.5 mm thickness. Each of 5-6 slices were then placed in a glass tray and stained (see Appendix I). The gel trays were covered with plastic wrap and incubated at 37°C for 0.5-6 hours, depending on the stain, then photographed.

Gel Scoring and Data Recording

Allozyme mobility was determined by measuring the distance between the loading well and the center of bands representing allozymic activity. Genotypes were scored if banding patterns were consistent with the known subunit structure of the enzyme and simple models of Mendelian inheritance. Electromorphs were designated with numerical values signifying their mobility relative to the most common allozyme in the array of taxa studied, the most common allozyme being designated "100". Loci are generally considered polymorphic if the frequency of the most common allele is ≤ 0.99 in either of the species. In view of the limited number of individuals assayed, all detected alleles were taken into account in this electrophoretic study. Multiple loci (i.e. isozymes) were assigned numerical values, with the

Table 1. Enzyme systems with Enzyme Commission (EC) numbers, optimal tissues for resolution (M = muscle, L = liver), and buffers used in *Naso spp.* electrophoretic analysis. TEB = Tris EDTA Borate, TC = Tris Citrate.

Locus	Symbol	EC	Tissue	Buffer	pH
Aconitase	ACON	4.2.1.3	L	TC	6.7
Adenosine deaminase	ADA	3.5.4.4	M	TC	7.0
Adenylate kinase	AK	2.7.4.3	M	TEB	8.0
Asparate amino acid	AAT-1, AAT-2	2.7.4.3	M	TEB	8.0
Creatine kinase	CK-A	2.7.3.2	M	TEB	8.0
Glucose-6-phosphate dehydrogenase	G6PD	1.1.1.49	M	TEB	8.0
Glucosephosphate isomerase	GPI-A, GPI-B	5.3.1.9	M	TC	7.0
6-Phosphogluconate dehydrogenase	6PGD	1.1.1.44	M	TC	7.0
Isocitrate dehydrogenase	IDH	1.1.1.42	L	TC	6.7
Lactate dehydrogenase	LDH-A	1.1.1.27	M	TC	7.0
Mannosephosphate isomerase	MPI	5.3.1.8	M	TC	7.0
Peptidase (leu-gly-gly)	PEPLGG	3.4.11.-	M	TC	8.0
Peptidase (leu-tyr)	PEPLT	3.4.11.-	M	TC	8.0
Phosphoglucomtase	PGM	2.7.5.1	M	TC	7.0

isozyme that migrated fastest toward the anode being designated as Isozyme A or 1 (e.g. GPI-A* and GPI-B*). The stained gels were photographed and illustrated in a laboratory notebook.

Data Analysis

There are two contending schools of contempory systematics: phenetics and cladistics. Electrophoretic data sets are usually analyzed by phenetic methods (Buth 1984), however this is just one procedure of several that may be pursued. Phenetic methods are based on overall similarity, and may be misleading about the evolutionary patterns of a group, if rates of evolution in different lineages are unequal. However, a phenetic analysis incorporates all data generated from the study (i.e. all loci are informative). A cladistic analysis, is based only on synapomorphic or "shared derived" character states, scoring of which is not affected by variations in evolutionary rate. Thus a cladistic analysis is more likely to reflect the true phylogeny, i.e., the patterns of evolutionary divergence within the selected group. For these reasons and others explained by Buth (1984), the data were analyzed both phenetically and cladistically to compare these two classifications of the species of *Naso* included in the present study.

Phenetic Analysis

Phenetic analysis was performed by the BIOSYS-1 Program (Swofford and Selander 1981) and NTSYS-PC (Rohlf 1990). Allele frequencies for the 16 loci screened in each taxon were calculated. These frequencies of electromorphs were used to estimate Nei's (1972) genetic distance between the species surveyed.

Nei's (1972) genetic distance values for the 7 species studied were used to construct dendrograms with the UPGMA (unweighted pair-group method of arithmetic averages) and the neighbor-joining (Saitou and Nei 1987) algorithms. Hillis (1992) examined five methods used to reconstruct branching patterns of a known phylogeny. The results of his study were that the neighbor-joining method was significantly better than the UPGMA method in predicting branch lengths. Both methods were used in this study. The UPGMA dendrogram was constructed by the BIOSYS-1 Program and the neighbor-joining tree was generated by the NTSYS-PC program.

Cladistic Analysis

Cladistic analysis was performed by the PAUP Program (Phylogenetic Analysis Using Parsimony) version 3.1.1 (Swofford 1993). Only evolutionarily informative characters (i.e. characters whose polarity could be determined by outgroup comparison and characters that had synapomorphic character states) were used in the cladistic analysis. Characters (i.e loci) that exhibited multiple states were labeled "polymorphic" in PAUP analysis. Both a hypothetical ancestor and *Acanthurus lineatus* were used for outgroup comparisons. Including an outgroup in a cladistic analysis of electrophoretic data is necessary to allow the determination of character state polarity. The hypothetical ancestor was composed of presumed ancestral character states derived from alleles shared by *A. lineatus* and at least one member of the genus *Naso*. Alternately, plus, for characters in which *A. lineatus* and members of the genus *Naso* did not share character states, character states shared between *Zanclus cornutus* and at least one species of the genus *Naso* were used. This was done to

optimal information from the allozymic data matrix, since some alleles at certain loci were shared by the Nasinae and *Z. cornutus* but not by the Nasinae and *A. lineatus*.

The rescaled consistency index (RC), which excludes autapomorphies (single apomorphies in a taxon) and totally homoplastic characters (character states shared by two or more taxa that were derived from convergence, i.e., parallelism or reversals) was calculated for each minimal length tree. Most of the characters (i.e. loci) in this electrophoretic study are multistate "polymorphic" (exhibiting variability within the terminal taxon). The PAUP program deals with this polymorphism by treating the terminal taxon as a heterogeneous group. Thus, the homoplasy index has a different meaning than its general definition of one minus the consistency index. When individuals are labeled as "polymorphic", PAUP assumes that one state is derived and if the other state is present in the outgroup, then PAUP assumes the primitive state is a reversal. Thus the consistency index of a tree may be one and the homoplasy index nevertheless may be greater than zero.

RESULTS

Phenetic Analysis

The allele frequencies for each of the 16 presumptive loci screened in *Naso spp.*, *Acanthurus lineatus* and *Zanclus cornutus* are presented in Table 2. Genetic distance matrices with estimates of Nei's (D_n) (1972) genetic distances are given in Table 3. The lowest amount of genetic divergence was observed between the sibling species *Naso caesius* and *N. hexacanthus*. The genetic distances between *Naso (Axinurus) thynnoides* and each of the other species of *Naso* in the present study are larger than any other genetic distance among the other *Naso spp.* examined.

The resulting UPGMA and neighbor-joining phenograms are presented in Figures 1 and 2. In both topologies, the sibling species *Naso caesius* and *N. hexacanthus* are the most genetically similar species of *Naso* surveyed . The UPGMA phenogram (Figure 1) indicates the two ingroup benthic algal grazers of this study, *N. lituratus* and *N. unicornis* are more genetically similar to each other than to the other species of *Naso*, all of which are zooplanktivores. The results of the neighbor-joining phenogram (which calculates a tree based on the branch lengths) indicates that *N. lituratus* is more similar genetically to the *N. caesius* and *N. hexacanthus* complex.

Acanthurus lineatus and *Naso thynnoides* formed a complex in both phenograms (Figures 1 and 2). The genetic distance between *Zanclus cornutus* and all other species of *Naso* was greater in all but one case, (*N. unicornis*) than the distance between the other outgroup, *A. lineatus*, and species of *Naso*.

Cladistic Analysis

The character-state data sets for the cladistic analysis are presented in Tables 4 and 5. The results of an exhaustive search (i.e one in which all possible trees were evaluated) generated one tree when *Acanthurus lineatus* was used as the outgroup (Figure 3). The single tree had a length of 24, along with a rescaled consistency index of 1.00 and homoplasy index of 0.375. Two equally parsimonius trees (i.e. two trees with equal minimum lengths) were generated when the hypothetical ancestor was used as the outgroup (Figures 4a and 4b). The rescaled consistency index for those trees was 0.881 and the homoplasy index 0.385. The strict consensus tree calculated from the two trees is presented in Figure 5.

Four salient results of all the cladograms are the following: (1) one synapomorphy at the ADA* locus supports the monophyly of the genus *Naso*; (2) synapomorphies at loci AAT-2* and GPI-A* characterize the members of the subgenus *Naso* (excluding *Naso thynnoides*); (3) synapomorphies at loci AAT-1*, GPD*, and PGDH* link the sibling species *N. caesius* and *N. hexacanthus*; and (4) *N. thynnoides* (subgenus *Axinurus*) is the sister taxon to all other species of *Naso* included in the present study.

The relationship of the two ingroup benthic algal grazers, *N. lituratus* and *N.unicornis* is inconsistent among the trees. Figures 3 and 4a, along with the UPGMA phenogram (Figure 1), support the conclusion that *Naso lituratus* and *N. unicornis* are sister taxa. This relationship is illustrated by the detection of one synapomorphy at the GPI-B* locus in the cladogram. In contrast, the cladogram in Figure 4b, suggests that *Naso unicornis* is the sister taxon to the sibling species *N. caesius* and *N. hexacanthus*, which is also supported with a synapomorphy at the PGM* locus. One synapomorphy also unites *N. lituratus* to the sibling

species branch. The strict consensus tree illustrated in Figure 5 provides no resolution of the relationship between *Naso lituratus* and *N. unicornis*.

Table 2. Frequencies of presumptive alleles at 16 loci resolved in the 7 species surveyed. NC= *Naso caesius*, NH= *N. hexacanthus*, NL= *N. lituratus*, NU= *N. unicornis*, NT= *N. thynnoides*, AL= *Acanthurus lineatus*, and ZC= *Zanclus cornutus*. Enzyme abbreviations are given in Table 1.

Species	NC	NH	NL	NU	NT	AL	ZC
Locus, allele							
AAT-1*							
*206	0.00	0.00	0.00	0.00	1.00	0.90	0.00
*152	0.20	0.26	0.00	0.00	0.00	0.00	0.00
*120	0.00	0.00	0.00	0.00	0.00	0.10	0.00
*114	0.00	0.00	0.00	1.00	0.00	0.00	1.00
*100	0.78	0.74	1.00	0.00	0.00	0.00	0.00
*18	0.02	0.00	0.00	0.00	0.00	0.00	0.00
AAT-2*							
*100	0.07	0.70	0.20	0.00	0.33	0.00	1.00
*34	0.93	0.30	0.80	1.00	0.00	0.00	0.00
*-58	0.00	0.00	0.00	0.00	0.67	1.00	0.00
ACON*							
*164	0.00	0.00	0.00	0.00	0.00	0.00	0.50
*153	0.00	0.00	0.00	0.00	0.00	0.00	0.40
*140	0.00	0.00	0.00	0.00	0.00	0.00	0.10
*125	0.00	0.00	0.00	0.00	0.00	0.20	0.00
*110	0.00	0.00	0.00	0.00	0.00	0.80	0.00
*100	1.00	1.00	1.00	1.00	1.00	0.00	0.00
ADA*							
*142	0.00	0.00	0.00	0.00	0.00	0.80	0.00
*117	0.00	0.00	0.00	0.30	0.00	0.20	0.00
*112	0.05	0.03	0.00	0.00	0.00	0.00	1.00
*100	0.93	0.92	1.00	0.70	1.00	0.00	0.00
*92	0.02	0.05	0.00	0.00	0.00	0.00	0.00
AK*							
*100	1.00	1.00	1.00	1.00	0.00	1.00	1.00
*95	0.00	0.00	0.00	0.00	1.00	0.00	0.00

continued.

Table 2. *continued.*

Species	NC	NH	NL	NU	NT	AL	ZC
Locus, allele							
CK-A*							
*138	0.98	0.00	0.10	0.00	0.00	0.00	0.00
*100	0.02	0.00	0.90	1.00	0.00	1.00	1.00
*88	0.00	1.00	0.00	0.00	0.00	0.00	0.00
*75	0.00	0.00	0.00	0.00	1.00	0.00	0.00
GPD*							
*110	0.00	0.00	0.00	0.00	0.00	0.00	1.00
*105	0.00	0.00	0.00	0.00	0.00	0.38	0.00
*100	0.00	0.00	1.00	1.00	1.00	0.62	0.00
*97	1.00	1.00	0.00	0.00	0.00	0.00	0.00
GPI-A*							
*103	0.00	0.00	0.00	0.00	0.00	0.00	1.00
*100	1.00	1.00	1.00	1.00	0.00	0.00	0.00
*80	0.00	0.00	0.00	0.00	1.00	1.00	0.00
GPI-B*							
*135	0.00	0.00	0.00	0.00	0.00	0.00	0.33
*118	0.00	0.00	0.00	0.00	0.00	0.00	0.67
*116	0.00	0.00	1.00	1.00	0.00	0.00	0.00
*100	1.00	1.00	0.00	0.00	0.00	1.00	0.00
*77	0.00	0.00	0.00	0.00	1.00	0.00	0.00
IDH*							
*183	0.00	0.00	0.00	0.00	0.00	0.00	1.00
*136	0.00	0.00	0.00	0.00	0.00	0.83	0.00
*125	0.00	0.00	0.00	0.00	1.00	0.00	0.00
*100	1.00	1.00	1.00	1.00	0.00	0.17	0.00

continued.

Table 2. *continued.*

Species	NC	NH	NL	NU	NT	AL	ZC
Locus, allele							
LDH-A*							
*100	0.36	0.75	1.00	0.00	0.00	1.00	0.00
*82	0.00	0.00	0.00	0.00	0.00	0.00	1.00
*78	0.00	0.00	0.00	1.00	0.00	0.00	0.00
*42	0.64	0.00	0.00	0.00	0.00	0.00	0.00
*28	0.00	0.25	0.00	0.00	0.00	0.00	0.00
*-13	0.00	0.00	0.00	0.00	1.00	0.00	0.00
MPI*							
*126	0.00	0.00	0.00	0.00	0.00	1.00	0.00
*122	0.00	0.00	0.00	0.00	1.00	0.00	0.00
*104	0.02	0.00	0.00	0.00	0.00	0.00	0.00
*100	0.98	1.00	1.00	1.00	0.00	0.00	1.00
PEPA*							
*108	0.00	0.00	0.00	0.40	0.00	0.00	0.00
*105	0.00	0.00	0.00	0.00	0.00	0.60	1.00
*100	1.00	1.00	1.00	0.60	0.33	0.40	0.00
*98	0.00	0.00	0.00	0.00	0.67	0.00	0.00
PEPB*							
*118	0.00	0.00	0.00	0.00	0.33	0.00	1.00
*111	0.00	0.00	0.00	0.00	0.67	0.00	0.00
*106	0.00	0.00	0.00	0.00	0.00	0.50	0.00
*105	0.00	0.00	1.00	0.00	0.00	0.00	0.00
*100	0.88	1.00	0.00	0.00	0.00	0.40	0.00
*94	0.00	0.00	0.00	0.00	0.00	0.10	0.00
*89	0.00	0.00	0.00	0.90	0.00	0.00	0.00
*74	0.12	0.00	0.00	0.10	0.00	0.00	0.00
PGDH*							
*106	0.00	0.00	0.00	0.00	0.00	0.17	1.00
*100	0.00	0.00	1.00	1.00	1.00	0.83	0.00
*95	1.00	0.98	0.00	0.00	0.00	0.00	0.00
*78	0.00	0.02	0.00	0.00	0.00	0.00	0.00

continued.

Table 2. *continued.*

Species	NC	NH	NL	NU	NT	AL	ZC
Locus, allele							
PGM*							
*172	0.00	0.00	0.00	0.00	0.00	1.00	0.00
*120	0.00	0.00	0.10	0.00	0.00	0.00	0.00
*100	0.00	0.00	0.90	0.00	1.00	0.00	1.00
*36	0.18	0.91	0.00	0.70	0.00	0.00	0.00
*15	0.82	0.09	0.00	0.30	0.00	0.00	0.00

Table 3. Estimates of Nei's (1972) genetic distances among species surveyed. NC= *Naso caesius*, NH= *N. hexancanthus*, NL= *N. lituratus*, NU= *N. unicornis*, NT= *N. thynnoides*, AL= *Acanthurus lineatus*, and ZC= *Zanclus cornutus*.

Species	*NC*	*NH*	*NL*	*NU*	*NT*	*AL*	*ZC*
NC	***	0.169	0.508	0.646	1.847	1.482	1.940
NH		***	0.527	0.698	1.770	1.359	1.68
NL			***	0.304	1.040	1.095	1.33
NU				***	1.319	1.303	1.06
NT					***	1.208	2.18
AL						***	1.62
ZC							***

Table 4. Characters and character-states of *Acanthurus lineatus* and *Naso spp.* Numbers indicate different alleles; 1 = allele A, 2 = allele B, 3 = allele C, 4 = allele D.

	Characters								
	AAT-1*	AAT-2*	ADA*	CK*	GPD*	GPI-A*	GPI-B*	PEP-B*	PGDH*
Species									
Acanthurus lineatus	1	1	1	1	1	1	1	1	1
Naso thynnoides	1	1,3	2	2	1	1	3	2	1
N. unicornis	2	2	1,2	1	1	2	2	3	1
N. lituratus	3	2,3	2	1,3	1	2	2	4	1
N. hexacanthus	3,4	2,3	2	4	2	2	1	1	2
N. caesius	3,4	2,3	2	3	2	2	1	1,3	2

Table 5. Characters and character-states of the hypothetical ancestor (character states from both outgroups, *Acanthurus lineatus* and *Zanclus cornutus*) and *Naso spp.*. Numbers indicate different alleles; 1 = allele A, 2 = allele B, 3 = allele C, 4 = allele D.

	Characters									
	AAT-1*	AAT-2*	ADA*	CK*	GPD*	GPI-A*	GPI-B*	PEP-B*	PGDH*	PGM*
Species										
hypothetical ancestor	1	1	1	1	1	1	1	1	1	1
Naso thynnoides	1	1,3	2	2	1	1	3	2	1	1
N. unicornis	2	2	1,2	1	1	2	2	3	1	2
N. lituratus	3	2,3	2	1,3	1	2	2	4	1	1
N. hexacanthus	3,4	2,3	2	4	2	2	1	1	2	2
N. caesius	3,4	2,3	2	3	2	2	1	1,3	2	2

Table 6. Morphological character states used to distinguish between *Naso caesius* and *N. hexacanthus* (Randall and Bell 1992).

Species	Character states (adults)				Character states (juveniles)
	shape of caudal spines	color of tongue	color of lower lip	body color	body color
Naso caesius	rounded	white	bluish	bluish gray	bluish gray
Naso hexacanthus	pointed anteriorly	black	white	yellowish ventrally	yellowish ventrally

Table 7. Morphological characters of species examined in the present study (Randall 1994, 1990, Myers 1989). NC= *N. caesius*, NH = *N. hexacanthus*, NL = *N. lituratus*, NT = *N. thynnoides*, NU = *N. unicornis*, AL = *Acanthurus lineatus*, and ZC = *Zanclus cornutus*.

	SPECIES						
	NC	NH	NL	NT	NU	AL	ZC
CHARACTER							
# of dorsal spines	VI-VII	VI	VI	IV	VI	IX	VI -VII
# of anal spines	II	II	II	II	II	III	III
shape of tail	trun	trun	trun	trun	trun	lun	trun
# of caudal spines	2	2	2	1	2	1	0
shape of caudal spines	retro	antro	antro	semi-cir	cir	oval	--
foraging mode	Z	Z	H	Z	H	H	S
type of teeth	lanc	lanc	incis	hast	lanc	incis	bris
rostral prominence	no	no	no	no	yes	no	no

bris = bristle-like, hast = hastate, H = herbivore, incis = incisiform, lanc = lanceolate, lun = lunate, S = spongivore, trun = truncated, Z = zooplanktivore, retro = retrose, antro = antrose, semi-cir = semi-circular, cir = circular, and oval = oval shaped.

Figure 1. The UPGMA (unweighted pair group method using arithmetic mean) phenogram of *Naso spp.*, *Acanthurus lineatus*, and *Zanclus cornutus*. Estimates of Nei's (1972) genetic distance among the study species were used to construct this phenogram.

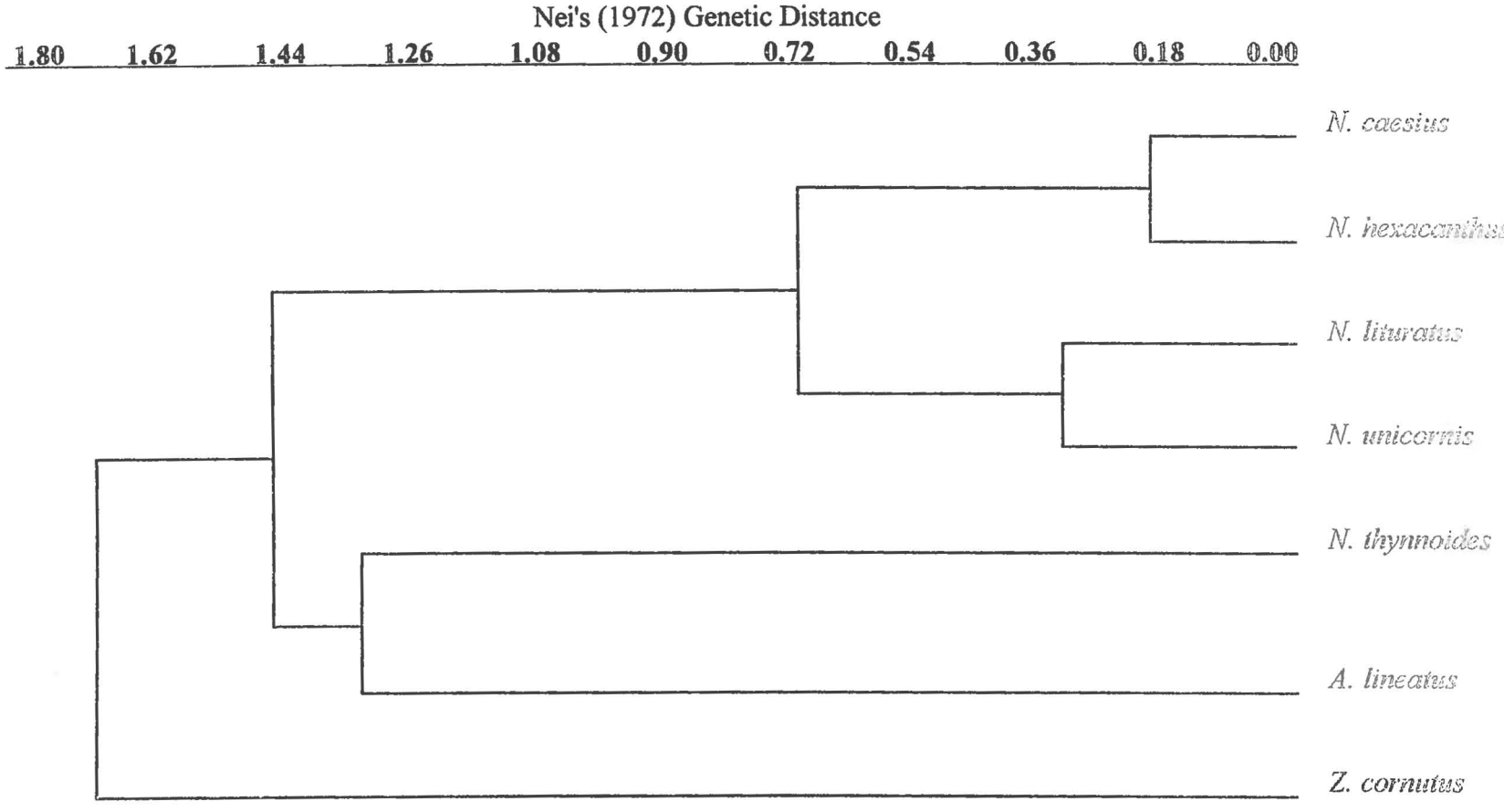


Figure 2. Neighbor-joining phenogram of *Naso spp.*, *Acanthurus lineatus*, and *Zanclus cornutus*. The neighbor-joining phenogram was constructed by estimates of Nei's (1972) genetic distance among the study species.

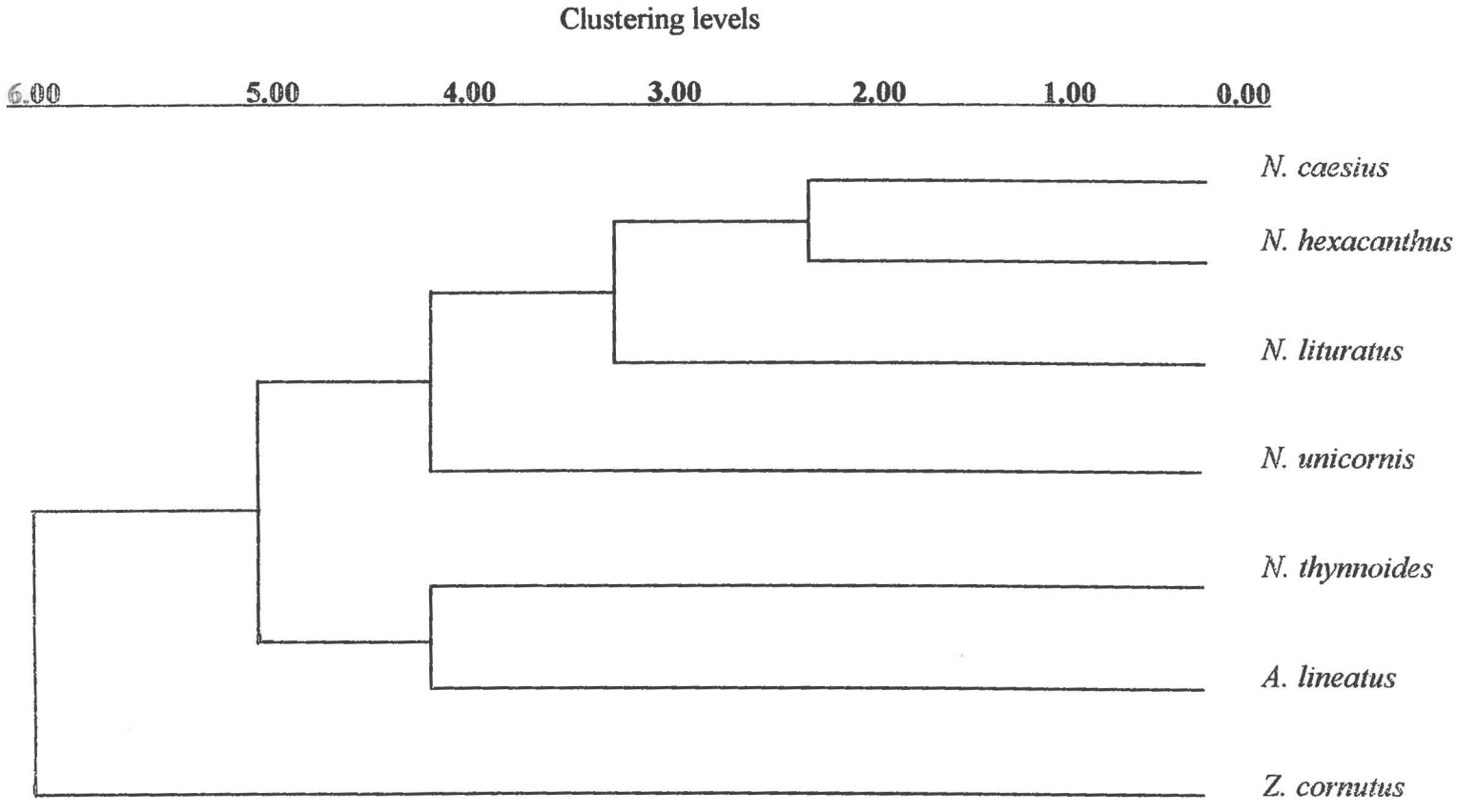


Figure 3. The cladogram generated with *Naso spp.* and *Acanthurus lineatus*. Enzyme abbreviations "synapomorphies", are given in Table 1.

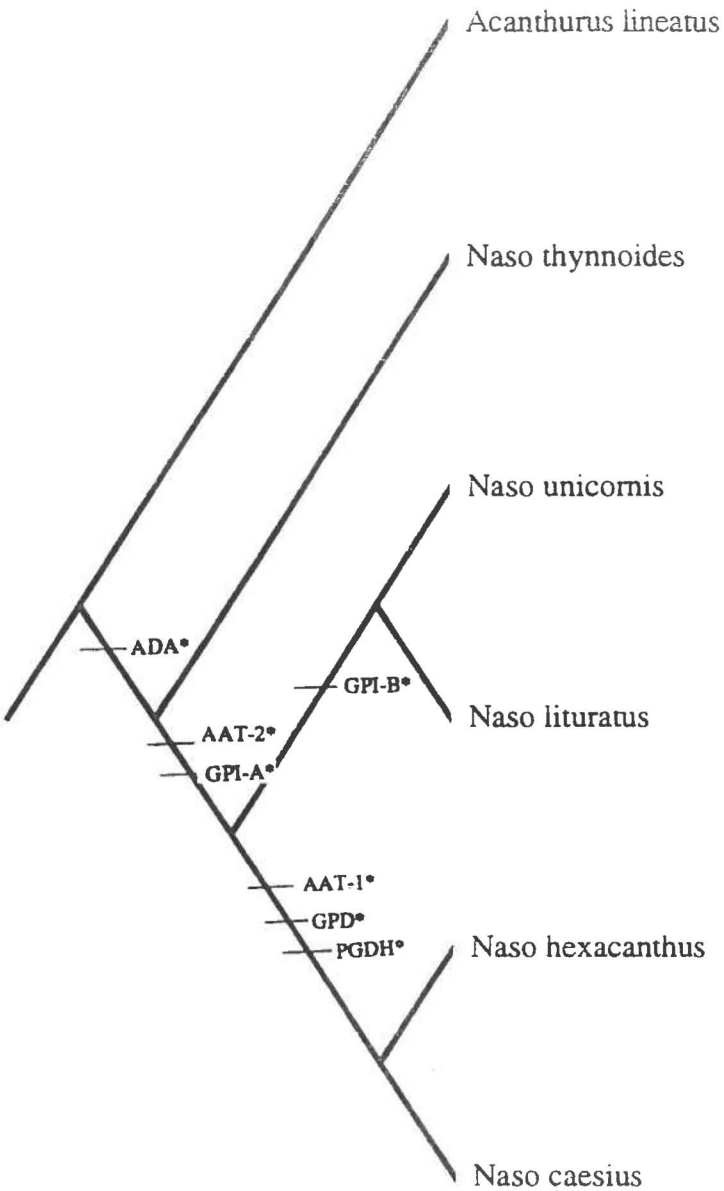


Figure 4a. A caldogram generated with *Naso spp.* and the hypothetical ancestor (see Results for details). Enzyme abbreviations "synapomorphies", are given in Table 1.

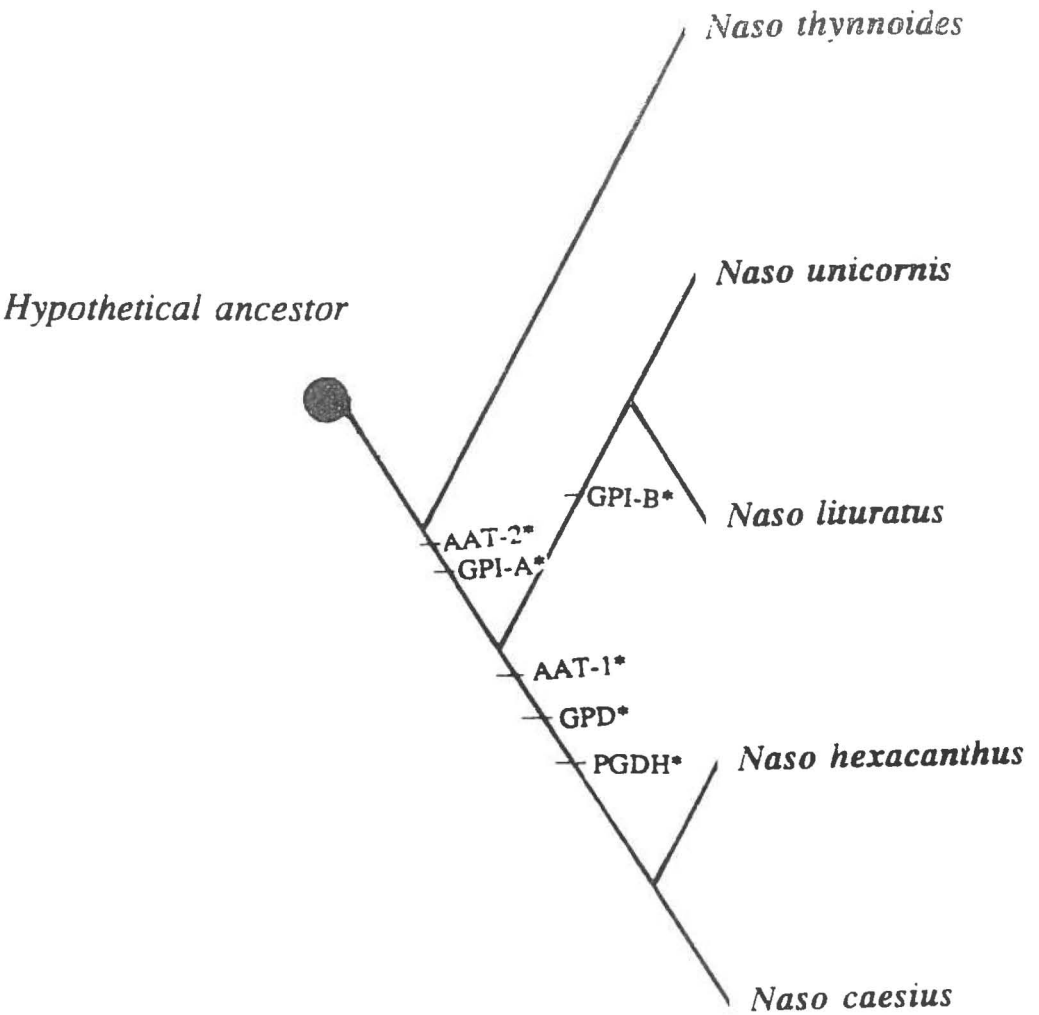


Figure 4b. A caldogram generated with *Naso spp.* and the hypothetical ancestor (see Results for details). Enzyme abbreviations "synapomorphies", are given in Table 1.

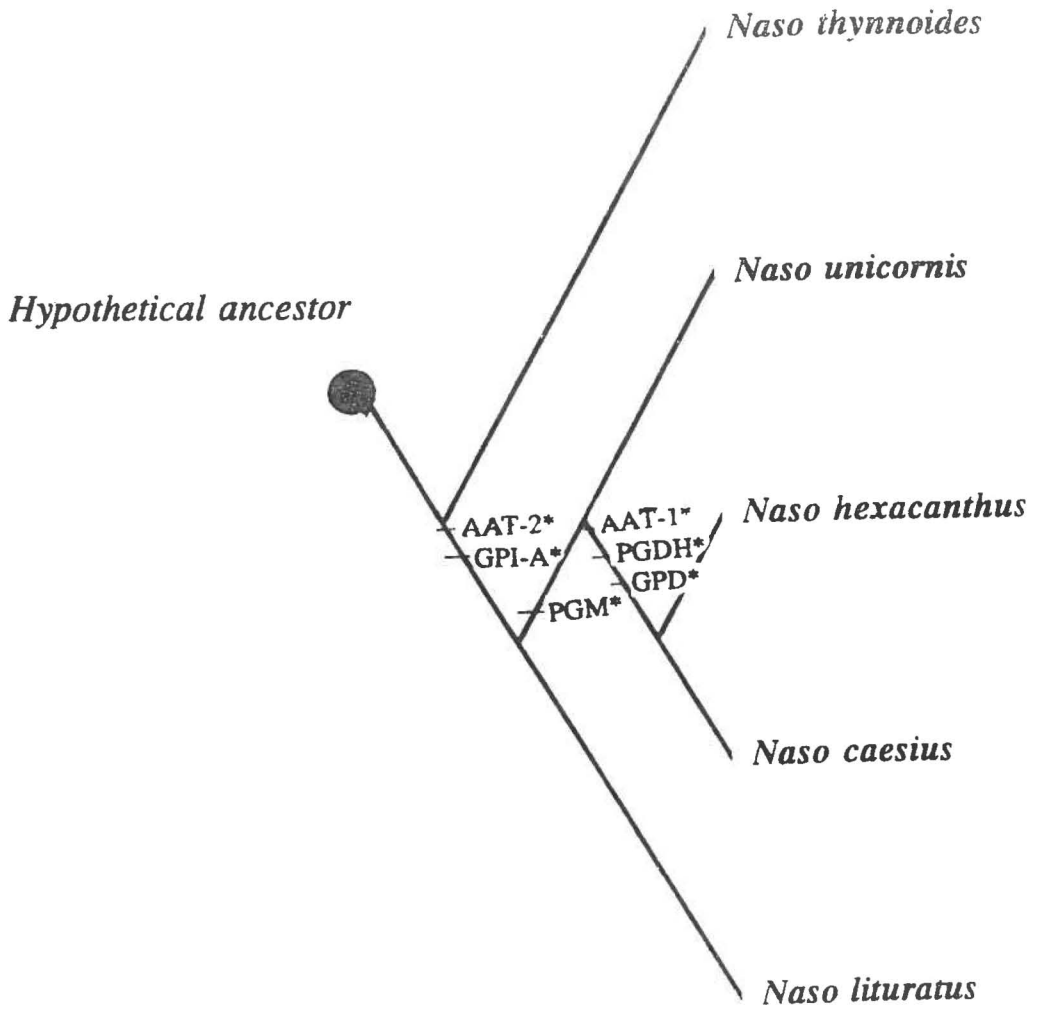
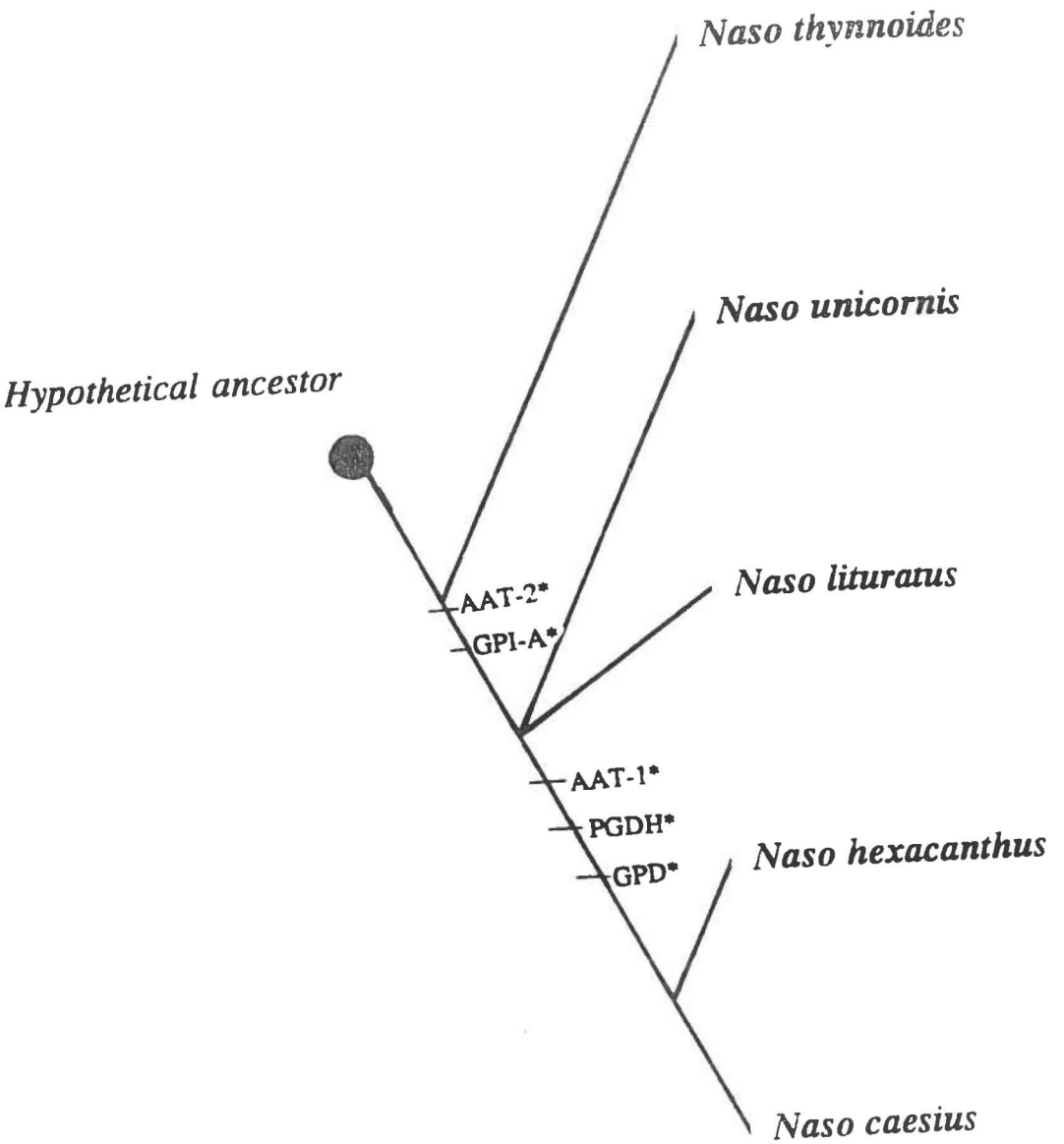


Figure 5. The strict consensus tree of Figures 4a and 4b *Naso spp.* and hypothetical ancestor (see Results for details). Enzyme abbreviations "synapomorphies", are given in Table 1.



DISCUSSION

The sibling species *Naso caesius* and *N. hexacanthus* are genetically the most closely related taxa among the species of *Naso* investigated in this study. This species-pair branch is supported by three synapomorphies, AAT-1*, GPD* and PGDH*, in all cladograms. Besides being the most genetically similar species of *Naso* in this study, *N. caesius* and *N. hexacanthus* are morphologically similar as well (Tables 6 and 7). Thus the degree of morphological as well as genetic evolution within the *N. caesius* and *N. hexacanthus* branch is comparably low.

The evolutionarily relationship between *Naso lituratus* and *N. unicornis* is not resolved in the present study as all 3 possible relationships between *N. unicornis*, *N. lituratus*, and *N. caesius* - *N. hexacanthus* species pair are shown by the various phenograms and cladograms (Figures 1-5). However, two possible, equally parsimonious evolutionary pathways are proposed from this study. One possible pathway, which is supported by the UPGMA phenogram, is that these two species are sister taxa (Figures 1, 3, and 4). One synapomorphy at the protein coding locus GPI-B* supports this branch. In contrast, one synapomorphy at the PGM* locus supports *N. unicornis* as the sister taxon to the sibling species pair of *N. caesius* and *N. hexacanthus*.

Naso thynnoides, a member of the recently recognized subgenus *Axinurus* (Randall 1994), is characterized by several allelic autapomorphies (Table 5) and is the sister taxon to all the other species of *Naso* in the present study. Recently, Randall (1994) concluded that the unique morphological characters detected in *Naso thynnoides* and *N. minor* by Winterbottom (1992) and Tyler (1970, 1989) appeared adequate to characterize *Axinurus* as a subgenus. Winterbottom (1992) suggested that the members of the subgenus *Axinurus*,

(then including only *N. thynnoides* and *N. minor*) comprise the sister group to all other species of *Naso* based on three morphological synapomorphies. The present study supports Winterbottom's conclusion. Furthermore, some of the allelic autapomorphies detected in *N. thynnoides* may turn out to be synapomorphies that unite the members of the proposed subgenus *Axinurus*, (*N. thynnoides*, *N. minor* and *N. caeruleacauda*) when the latter two species are studied.

The phenograms (Figures 1 and 2) indicate that *Naso thynnoides* is genetically more similar to *Acanthurus lineatus* than to the other *Naso* species. Comparison with the cladogram and allele table (Figure 5 and Table 2) explains this similarity: *N. thynnoides* and *A. lineatus* group together because they share many primitive or plesiomorphic character states.

Two synapomorphies at the AAT-2* and GPI-A* loci support the monophyletic assemblage of the members of the subgenus *Naso* (excluding *Naso thynnoides*) sampled in this study (Figure 5). Only one synapomorphic allele at the polymorphic locus ADA* supports the monophyly of members of the genus *Naso* surveyed. Randall (1994) defines the genus *Naso* by six morphological character states. Therefore, stronger morphological data than genetic evidence supports monophyly of all the species of *Naso*. However, screening of additional loci may detect more synapomorphic alleles that unite the genus *Naso* and thus might eventually provide as much evidence as morphological data.

The cladogram can be used to indicate how the feeding mode (i.e. herbivory vs. zooplanktivory) changed within the genus *Naso*. Winterbottom and McLennan (1993) examined the foraging modes of families within the order Acanthuroidei and suggested that

the common ancestor of the Nasinae was a benthic algal grazer based on the assumption that the most common equals the most primitive. Whether the common ancestor of the Nasinae was a benthic algal grazer or a zooplanktivore, the feeding mode switched.

A possible evolutionary pathway would be to assume the common ancestor of the Nasinae was a zooplanktivore because this assumes the least amount of evolution (i.e. the least number of evolutionary steps). I do not mean to imply by this statement that the common ancestor of the Nasinae and Acanthurinae was a zooplankton feeder, but rather that the basal species of genus *Naso* may have been a zooplanktivore. However, if I apply the assumption that the most common equals the most primitive, then it would seem logical that the basal species of the Nasinae was a zooplanktivore, because most species within the subfamily are zooplanktivores (e.g. all members of the subgenus *Axinurus*, *Naso caesius*, *N. hexacanthus*, *N. annulatus* and *N. brevirostris*). The inclusion of more species of *Naso*, may help determine the number of times the feeding mode switched and, more importantly, the ecological behavior of the common ancestor of the Nasinae.

In summary, the data from this study supports the two subgenera (*Naso* and *Axinurus*) presently recognized within the genus *Naso*. It also provides suggestions for continuing research to resolve relationships within the Nasinae. For example, including *Naso minor* and *N. caeruleacauda* in a future study may determine whether all species of *Axinu*rus form a sister group to all other species of *Naso*. Through continuing sampling efforts, this present partial phylogeny may become part of a future well-corroborated phylogeny of all species included in the Nasinae.

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APPENDIX I

Histochemical Stains (Morizot and Schmidt1990).

Aconitase (ACON) E.C. 4.2.1.3

0.1 M cis-aconitic acid pH 8.0	4ml
NADP	7.5mg
NBT	15mg
PMS	1ml
$MgCl_2$	1ml
0.2M Tris/HCL pH 8.0	2ml
Isocitrate dehydrogenase	72ul
1% agar	8ml

Adenosine deaminase (ADA) E.C. 3.5.4.4

Adenosine	40mg
Sodium arsenate	50mg
NBT	15mg
PMS	1mg
0.2M Tris/HCl pH 8.0	4ml
Xanthine oxidase	1.6u
Nucleoside phosphorlase	5u
1% agar	11ml

Adenylate kinase (AK) E.C. 2.7.4.3

ADP	20mg
Glucose	30mg
NADP	4.5mg
NBT	5mg
PMS	0.5mg
$MgCl_2$	20mg
0.2M Tris/HCL pH 8.0	4ml
Hexokinase	70ul
G6PD	35ul
1% agar	10ml

Asparate amino transferase (AAT) E.C. 2.6.1.1

substrate	50ml
Pyridoxal-5-phosphate	1mg
Fast blue BB salt	150mg

Creatine kinase (CK) E.C. 2.7.3.2

Phosphocreatine	15mg
ADP	28mg
Glucose	36mg
NADP	4.5mg
PMS	0.5mg
MTT	5mg
$MgCl_2$	40mg
0.1M Tris/HCl pH 7.0	10ml
Hexokinase	70u
G6PD	35u
1% agar	10ml

Glucose-6-phosphate dehyrogenase (G6PD) E.C. 1.1.149

Glucose-6-phosphate (Na_2 salt)	200mg
NADP	9mg
PMS	1mg
MTT	10mg
$MgCl_2$	40mg
0.2M Tris/HCl pH 8.0	10ml
H_2O	40ml
G6PD	80u

Glucosephosphate isomerase-1 (GPI) E.C. 5.3.1.9

Fructose-6-phosphate (Na_2 salt)	50mg
NADP	9mg
PMS	1mg
MTT	10mg
$MgCl_2$	40mg
0.2M Tris/HCl pH 8.0	10ml
H_2O	40ml
G6PD	80u

6-Phosphogluconate dehydrogenase (6PGD) E.C. 1.1.1.44

6-phosphogluconic acid (Na_2 salt)	100mg
NADP	15mg
NBT	15mg
PMS	1mg
$MgCl_2$	50mg
0.2M Tris/HCl pH 8.0	10ml
H_2O	40ml

Isocitrate dehydrogenase (IDH) E.C. 1.1.1.42

0.1M isocitrate (Na_2 salt) pH 7.0	8ml
NADP	15mg
NBT	15mg
PMS	1mg
$MgCl_2$	50mg
0.2M Tris/HCl pH 8.0	10ml
H_2O	32ml

Lactate dehydrogenase (LDH) E.C. 1.1.1.27

LDH substrate	5ml
NAD	15mg
NBT	15mg
PMS	1mg
0.2M Tris/HCl pH 8.0	10mg
H_2O	35ml

Mannosephosphate isomerase (MPI) E.C. 5.3.1.8

Mannose-6-phosphate (Na_2 salt)	50mg
NADP	15mg
NBT	15mg
PMS	1mg
$MgCl_2$	50mg
0.2M Tris/HCl pH 8.0	10ml
Phosphoglucose isomerase	100u
G6PD	80u
1% agar	10ml

Peptidase (PEP) E.C. 3.4.11-
-(leu-gly-gly)
-(leu-tyr)

snake venom (L-amino acid oxidase)	10mg
peroxidase	1500u
O-dianisidine	50mg
Di- or tripeptide substrate	40-60mg
$MgCl_2$	50mg
H_2O	5ml
0.2M Tris/HCl pH 8.0	5ml
1% agar	10ml

Phosphoglucomutase (PGM) E.C. 1.15.1.1

Glucose-1-phosphate (Na_2 salt)	300mg
NADP	15mg
PMS	1mg
MTT	20mg
$MgCl_2$	50mg
0.2M Tris/HCl pH 8.0	10ml
G6PD	80u
1% agar	10ml