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Cladistic and phenetic analysis of allozyme data for nine species of sea anemones of the family Actiniidae (Cnidaria: Anthozoa)

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The aim of this study was to infer from allozyme data the phylogenetic relationships of nine species of actiniid sea anemones, and also use these data to assess the various methods (phenetic and cladistic) available for phylogenetic analysis. Starch gel electrophoresis was used to obtain genetic data from 13 gene loci. The anemone *Metridium senile*, from the family Metridiidae, was used as an outgroup. For the phenetic analysis a matrix of pairwise unbiased genetic distances was computed and, from this, dendrograms were produced both by the Wagner distance and the UPGMA methods. For the cladistic analyses three different approaches were used: the first was to treat the allele as a binary character; this was investigated using a Wagner parsimony algorithm. Another approach used was to consider the locus as an unordered character, using the alleles as states. Finally, we used the locus as an ordered multistate character, where mutation, fixation and elimination of each allele were treated as evolutionary novelties, and the heterozygotes were used as cues for the construction of transformation series. The trees produced by the phenetic and cladistic methods were highly congruent. This result suggests that allozymes can be used to produce phylogenetic hypotheses at higher taxonomic levels than those at which they are more usually employed. The sole difference between the various trees was the relative positions of *Bunodosoma caissarum* and *Bunodactis verrucosa* in relation to the two species of *Urticina*. This difference was probably due to a high rate of anagenic change in *B. verrucosa*, which distorted the UPGMA dendrogram. The genera *Actinia* and *Urticina* appeared monophyletic in all of the trees produced. Also, the sea anemones with specialized column structures such as verrucae and vesicles (*U. felina, U. eques, B. verrucosa, B. caissarum*) formed a monophyletic cluster, a result compatible with the suggestion that these structures may have appeared only once in the evolutionary history of the Actiniidae.

ADDITIONAL KEY WORDS:—*Actinia – Urticina – Bunodosoma – Bunodactis – Metridium – Anemonia* – phylogenetic analysis – allozymes – genetic distance – cladistic methods.

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INTRODUCTION

Sea anemones are a very diverse, ecologically important group of organisms (Schick, 1991). However, their systematics are still very confused, largely due to their structural simplicity and the consequent lack of taxonomically useful morphological characters. Recent work has demonstrated the utility of allozymes for the identification of species of sea anemones (McCommas & Lester, 1980; Carter & Thorpe, 1981; Bucklin & Hedgecock, 1982; Haylor *et al.*, 1984; Solé-Cava *et al.*, 1985; Shaw *et al.*, 1987; Solé-Cava & Thorpe, 1987, 1992), but very little is known about the phylogenetic relationships even between the most common members of the largest family, the Actiniidae (McCommas, 1991). In this work we formulate phylogenetic hypotheses for the relationships of four species of *Actinia* and five species from four other related genera (family Actiniidae), using one species of the related family Metridiidae as an outgroup.

The analytical approach classically used in molecular systematics has been the conversion of gene frequency data into single pairwise genetic identity or distance values (Nei, 1978; Thorpe, 1979), and their use to produce phenetic trees (Ferguson, 1980; Richardson *et al.*, 1986). This approach has been criticized on the grounds that homoplasies may severely distort phylogenies based on overall similarities (Buth, 1984; Mindell *et al.*, 1989, 1990). An additional problem is that only given numerous assumptions can dendrograms constructed using overall distances clustered through phenetic algorithms be expected to reflect the evolutionary history of the group. One assumption, for example, is the stochastic constancy of rates of divergence between different evolutionary lines (molecular clock hypothesis), although it is known that such estimates can have very large associated errors (Thorpe, 1982; Hillis & Moritz, 1990).

An alternative that has received increasing attention in recent years is the cladistic analysis of allozyme data, the aim of which is to discriminate between phyllogenetically informative and uninformative allozymes, and which uses a methodology which depends on fewer evolutionary assumptions (see Baverstock *et al.*, 1979; Patton & Avise, 1983; Kettler *et al.*, 1986). There is still some debate about what may be considered to constitute a character for the phylogenetic analysis of allozymes (Swofford & Olsen, 1990). Some authors use the allele as a binary character, whereas others prefer the biologically more realistic, but, in practice, more difficult, use of the locus as the character (see Buth, 1984; Swofford & Olsen, 1990 for discussions of the advantages and pitfalls of each approach). In this paper we used both approaches for the analysis of electromorph data, together with some classical phenetic methods, for comparison.

MATERIAL AND METHODS

Samples of a number of sea anemone species were collected intertidally or by scuba diving and frozen until required for electrophoretic analysis. *Actinia equina* (L.), *Actinia prasina* Gosse, *Urticina felina* (L.), *Urticina eques* (Gosse), *Anemonia viridis* (Forskal), and *Metridium senile* (L.) were collected from the Isle of Man (Northern Irish Sea); *Actinia fragacea* Tugwell and *Bunodactis verrucosa* (Pennant) were collected from the English Channel coast of Dorset and Devon, respectively; and *Actinia bermudensis* (McMurrich) and *Bunodosoma caissarum* Correa were collected from Rio de Janeiro (Brazil). Since there is still much

controversy about the use of the colour of the column and the pedal discs for the systematics of *A. equina* and *A. bermudensis* (Solé-Cava & Thorpe, 1992; Russo *et al.*, 1994), for this study only individuals with pink column and pink pedal disc were collected. In the laboratory, tissue samples from the oral disc and tentacles were homogenized with a glass rod on an acrylic plate and analysed by horizontal 12.5% starch gel electrophoresis using methods previously described (Solé-Cava *et al.*, 1985). The buffer system used throughout was Tris-Citrate, pH 8.0 (Ward & Beardmore, 1977). Gels were stained for 20 different enzymes, of which 11 (coded by 13 loci) gave interpretable and reproducible results for all the species studied. Enzyme staining methods and nomenclature follow standard procedures (Harris & Hopkinson, 1978; Richardson *et al.*, 1986). The enzymes studied and their abbreviations were: Glutamate dehydrogenase, GDH, E.C.1.1.1.47; Hexokinase, HK, E.C.2.7.1.1; Isocitrate dehydrogenase, IDH, E.C.1.1.1.42; Malate dehydrogenase, MDH, E.C.1.1.1.37; Malic enzyme, ME, E.C.1.1.1.40; Mannose 6-Phosphate Isomerase, MPI, E.C.5.3.1.8; Pro-Phe Peptidase, PEP, E.C.3.4.11.1; Phosphoglucose isomerase, PGI, E.C.5.3.1.9; Phosphoglucomutase, PGM, E.C.5.4.2.2; Superoxide dismutase, SOD, E.C.1.15.1.1; and Xanthine oxidase, XOD, E.C.1.2.3.2. Genotype frequencies were used to estimate gene frequencies, from which levels of genetic variation and pairwise genetic identities (Nei, 1978) and distances (Prevosti's D; Wright, 1978) were calculated. Genetic identities were then used to build UPGMA dendrograms (Sneath & Sokal, 1973) and genetic distances were used to produce Distance-Wagner trees (Farris, 1970), using the program BIOSYS-1 (Swofford & Selander, 1981).

Qualitative (presence/absence) allozyme analysis is very dependent on sample size (Buth, 1984; Swofford & Olsen, 1990). To avoid this problem, particularly in view of the large variation in sample sizes studied, alleles with frequency lower than 0.05 were omitted from the phylogenetic analysis (as suggested by Buth, 1984). Binary matrices thus constructed were used for cladistic analysis by three different methods: the first was to use the allele as a binary character, from which Wagner parsimony (Farris, 1970) cladograms were constructed. For these the implicit enumeration option of the program HENNIG 86 (Felsenstein, 1985) and the exhaustive search option of the PAUP (Swofford, 1990) program were used. The second method was to use the locus as an unordered, multi-state, polymorphic character. For that we used the exhaustive search and the majority-consensus options of the PAUP program. Finally, the locus was used as a multistate character, which was ordered using the heterozygotes as intermediate steps between homozygote states, and was polarized, whenever possible, by the outgroup criterion.

In this approach, the appearance of a new allele and the elimination of the more plesiomorphic (ancestral) one were treated as two independent evolutionary events (called, respectively, syntrepty and synapousy in the terminology of Amorim *et al.*, 1993), which were counted as individual steps in the transformation series.

It is very important that in any phylogenetic study, the procedures used to establish polarization should be made explicit (Patton & Avise, 1983). In this study the following criteria were used:

(1) Alleles present both in the ingroup and the outgroup were considered to be plesiomorphic (Group I of Patton & Avise, 1983);

(2) The emergence of a new allele by mutation from a plesiomorphic one was

preferably evidenced by the presence of heterozygous individuals, and was considered to be an intermediate state on a transformation series ('syntrepty' of Amorim *et al.*, 1993);

(3) When heterozygous individuals (as above) could not be found, the transformation series had to be based on the congruence of other isoenzymatic characters and followed the group subordination (part of group III of Patton & Avise, 1983);

(4) The fixation of the new allele also means the elimination of the more plesiomorphic one, and hence was considered the most apomorphic (derived) state of the sequence ('synapousy' of Amorim *et al.*, 1993);

(5) In some cases the transformation series could be recursively built from a derived allele to other alleles, to derive a putative sequence of stepwise mutations. Such series were the most informative, and defined clades in the tree (part of group III of Patton & Avise, 1983; and TSA informative characters of Mickevich & Mitter, 1983);

(6) In other cases, when an evolutionary step was observed for a single group, the character was treated as an autapomorphy for the group (group II of Patton & Avise, 1983);

(7) When a single allele gave rise in different moments to different alleles, each mutation started an independent transformation series;

(8) When a derived allele characterized a monophyletic group, but one of the groups subordinated to that one possessed a more plesiomorphic allele for the same locus, this step was treated as a reversal (group IV of Patton & Avise, 1983). It should be noted that reversal does not mean the (extremely unlikely) back mutation to the more plesiomorphic allele, but rather the increase in frequency of an allele that remained in very low frequencies at the ancestral nodes. It can also be the consequence of (non-selective) convergence in electrophoretic mobility in the gel, since the number of different alleles that can be resolved by conventional electrophoretic analysis is limited and empirical data suggest that any new electromorph may have about a 5% chance of having a similar electrophoretic mobility (Thorpe, 1982). This phenomenon can lead to a saturation effect if the technique is used for comparisons of distantly related species (Thorpe, 1983, 1989). Note that this probability increases with the amount of genetic diversity in the species studied, so that homoplasies are likely to be a bigger problem in highly polymorphic species (such as those studied here);

(9) The most plesiomorphic characters in the transformation series were marked in the cladogram, on the assumption that these might subsequently be found to be synapomorphies at the basal levels. This can occur because the evaluation of phylogenetic status can only be clarified through the analysis of a more distant outgroup;

(10) When it was not possible to determine the most plesiomorphic state in a transformation series, the autapomorphic characters were used to define the monophyletic groups, and were marked in the cladogram built through the use of the other, unambiguous characters (group V of Patton & Avise, 1983).

RESULTS

The populations studied had high levels of genetic variation (Table 1). Genetic distance levels were small between congeneric species, but relatively

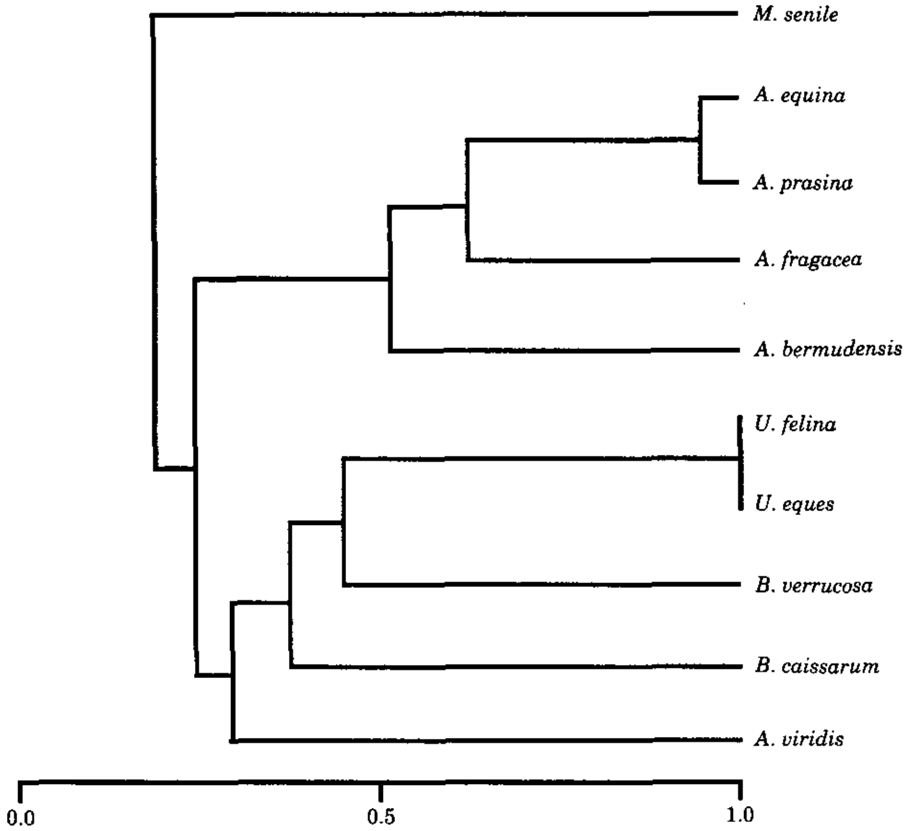

Figure 1. UPGMA phenetic dendrogram, using pairwise Nei's (1978) unbiased Genetic Identities.

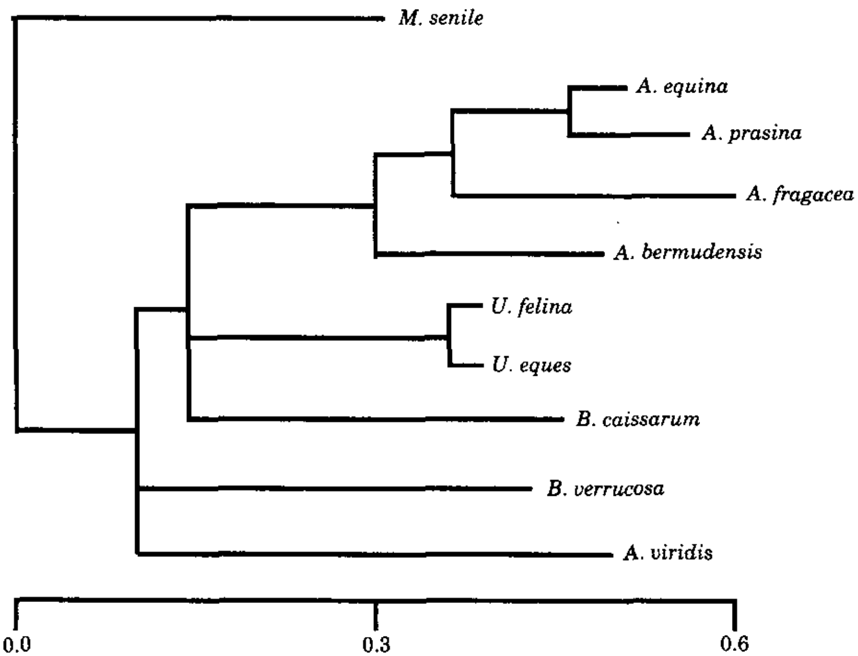

Figure 2. Wagner distance tree (Farris, 1970), using Prevosti's (Wright, 1978) genetic distances.

TABLE 1. Allele frequencies for 13 loci in the 10 sea anemone species studied. Sample size per locus are given in italics. Mean levels of unbiased Hardy-Weinberg expected heterozygosity (Het) for each species are given at the end of the table. Alleles marked in **bold** were not considered for cladistic analysis. Species names are: *Metridium senile* (*Msen*); *Actinia equina* (*Aequ*); *A. fragacea* (*Afra*); *A. bermudensis* (*Aber*); *A. prasina* (*Apra*); *Urticina felina* (*Ufel*); *Bunodosoma caissarum* (*Bcai*); *Anemonia viridis* (*Avir*); *Bunodactis verrucosa* (*Bver*); *Urticina eques* (*Uequ*)

Locus	*Msen*	*Aequ*	*Afra*	*Aber*	*Apra*	*Ufel*	*Bcai*	*Avir*	*Bver*	*Uequ*
Gdh	*13*	*29*	*1*	*27*	*6*	*5*	*41*	*22*	*5*	*5*
A	0	0	0	0	0	1.00	0	0	0	1.00
B	1.00	0	0	0	0	0	0	0	0	0
C	0	0	0	0.17	0	0	0	0	0	0
D	0	0	0	0	0	0	0.11	0	0	0
E	0	0.67	1.00	0.68	1.00	0	0.89	0	0	0
F	0	0	0	0	0	0	0	0	1.00	0
G	0	0	0	0.15	0	0	0	0	0	0
H	0	0	0	0	0	0	0	1.00	0	0
I	0	0.38	0	0	0	0	0	0	0	0
Hk-2	*2*	*22*	*1*	*29*	*16*	*3*	*5*	*20*	*5*	*5*
A	0	0	0	0	0	0	0	0	0.20	0
B	0.75	0.66	0	1.00	0.59	1.00	0.70	0.10	0.80	1.00
C	0.25	0.34	0	0	0.41	0	0.30	0.90	0	0
D	0	0	1.00	0	0	0	0	0	0	0
Idh	*4*	*4*	*1*	*4*	*5*	*6*	*6*	*4*	*3*	*5*
A	0	0.25	0	0	0.80	0	0	0	0	0
B	0	0.75	1.00	1.00	0.20	0.75	0.17	0.25	1.00	0.70
C	0	0	0	0	0	0.25	0.83	0.75	0	0.20
D	1.00	0	0	0	0	0	0	0	0	0
Mdh-2	*2*	*5*	*1*	*5*	*4*	*4*	*2*	*2*	*2*	*5*
A	1.00	0	0	0	0	0	0	0	1.00	0
B	0	0	0	0	0	0	0	1.00	0	0
C	0	0	0	0	0	0	1.00	0	0	0
D	0	0.20	0	0	0	0	0	0	0	0
E	0	0.80	1.00	1.00	1.00	1.00	0	0	0	1.00
Me	*4*	*2*	*1*	*8*	*6*	*7*	*8*	*3*	*5*	*5*
A	0.13	0	0	0	0	0	0	0	0	0
B	0.87	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	0.86	0	0.67	1.00	0.80
D	0	0	0	0	0	0.14	0	0.33	0	0.20
E	0	0	1.00	0	0	0	0	0	0	0
F	0	0.75	0	0.75	1.00	0	1.00	0	0	0
G	0	0.25	0	0.25	0	0	0	0	0	0
Mpi	*4*	*31*	*1*	*33*	*21*	*6*	*34*	*4*	*5*	*5*
A	0	0	0	0	0	0.17	0	1.00	0	0.20
B	0	0	0	0	0	0.25	0.34	0	0	0.30
C	1.00	0.31	0	0.11	0	0.58	0.40	0	0.20	0.50
D	0	0	0	0	0	0	0.09	0	0.80	0
E	0	0.69	0.50	0.73	0.71	0	0.17	0	0	0
F	0	0	0.50	0	0.29	0	0	0	0	0
G	0	0	0	0.16	0	0	0	0	0	0
Pep-1	*13*	*29*	*1*	*8*	*28*	*5*	*7*	*22*	*5*	*5*
A	0	0	0	0.13	0	0	0	0	0	0
B	0	1.00	1.00	0.87	1.00	0	0	0	0	0
C	1.00	0	0	0	0	1.00	1.00	1.00	1.00	1.00
Pep-2	*13*	*8*	*1*	*8*	*6*	*5*	*7*	*22*	*5*	*5*
A	1.00	0.75	0.50	0.19	0.50	0	0	1.00	0	0
B	0	0	0.50	0.81	0	0.30	0	0	0	0.30
C	0	0.25	0	0	0.50	0.50	0.64	0	1.00	0.50
D	0	0	0	0	0	0.20	0.36	0	0	0.20

TABLE 1. *Cont.*

Locus	*Msen*	*Aequ*	*Afra*	*Aber*	*Apra*	*Ufel*	*Bcai*	*Avir*	*Bver*	*Uequ*
Pgi-1	*11*	*29*	*1*	*32*	*28*	*39*	*36*	*22*	*5*	*20*
A	0	0.05	1.00	0	0.11	0	**0.03**	0	0	0
B	0	0	0	**0.02**	0	0	0	0	0	0
C	0	0.88	0	0	0.89	0	0	0	0	0
D	0	0	0	0	0	0.91	0.53	0	1.00	0.65
E	0	0.07	0	0.98	0	0	0.44	0	0	0
F	0	0	0	0	0	0.09	0	0	0	0.35
G	0	0	0	0	0	0	0	1.00	0	0
H	0.73	0	0	0	0	0	0	0	0	0
I	0.27	0	0	0	0	0	0	0	0	0
Pgi-2	*13*	*30*	*1*	*28*	*28*	*41*	*37*	*22*	*5*	*20*
A	0	0.02	0.50	0	0.13	0	0	0	0	0
B	0	0.98	0.50	0.14	0.87	0	0	0	0	0
C	0	0	0	0.86	0	0.83	0.62	0	0	0.75
D	0	0	0	0	0	0	0	1.00	1.00	0
E	1.00	0	0	0	0	0	0	0	0	0
F	0	0	0	0	0	0.17	0	0	0	0.25
G	0	0	0	0	0	0	0.38	0	0	0
Pgm	*13*	*26*	*1*	*8*	*28*	*39*	*28*	*22*	*5*	*22*
A	0.50	0	0	0	0	0.56	0	0	0	0.34
B	0.50	0.54	1.00	1.00	0.12	0.44	0.64	0.18	0	0.66
C	0	0.03	0	0	0	0	0.36	0.82	0.60	0
D	0	0.43	0	0	0.82	0	0	0	0.40	0
E	0	0	0	0	**0.04**	0	0	0	0	0
F	0	0	0	0	**0.02**	0	0	0	0	0
Sod	*13*	*27*	*1*	*1*	*24*	*43*	*26*	*20*	*5*	*20*
A	0	0	0	0	0	0	1.00	0	0	0
B	0	0	0	0	0	1.00	0	1.00	0	1.00
C	0	1.00	1.00	0	1.00	0	0	0	0	0
D	1.00	0	0	0	0	0	0	0	0	0
E	0	0	0	1.00	0	0	0	0	1.00	0
Xod	*11*	*25*	*1*	*8*	*23*	*17*	*5*	*20*	*5*	*25*
A	0	0.78	1.00	0	0.76	0	0	1.00	0	0
B	0	0	0	0	0	0	0	0	1.00	0
C	0	0.22	0	0.94	0.24	0.91	0	0	0	1.00
D	0	0	0	0.06	0	0.09	0	0	0	0
E	0.96	0	0	0	0	0	0	0	0	0
F	0	0	0	0	0	0	1.00	0	0	0
G	**0.04**	0	0	0	0	0	0	0	0	0
Het.	0.31	0.23	0.18	0.23	0.24	0.28	0.11	0.10	0.14	0.27

large between species from different genera (Table 2, Figs 1 and 2). *Metridium senile*, which belongs to a different family from the other species studied, was also the species which was most genetically divergent. The 77 electromorphs used as characters in a Wagner parsimony analysis produced only one cladogram (Consistency index, CI = 0.68) by the implicit enumeration (HENNIG 86) or the exhaustive search (PAUP) methods. The consistency of the cladogram is very much comparable to that obtained by other authors using cladistic methods for the analysis of allozymes (Patton & Avise, 1983; Hudon & Guderley, 1984; Murphy *et al.*, 1990). The maximum parsimony tree (Fig. 3) only differed from the UPGMA dendrogram (Fig. 1) in the relative positions of *Bunodosoma caissarum* and *Bunodactis verrucosa*, and was topologically congruent with the Wagner Distance tree (Fig. 2). The use of the locus as an unordered character,

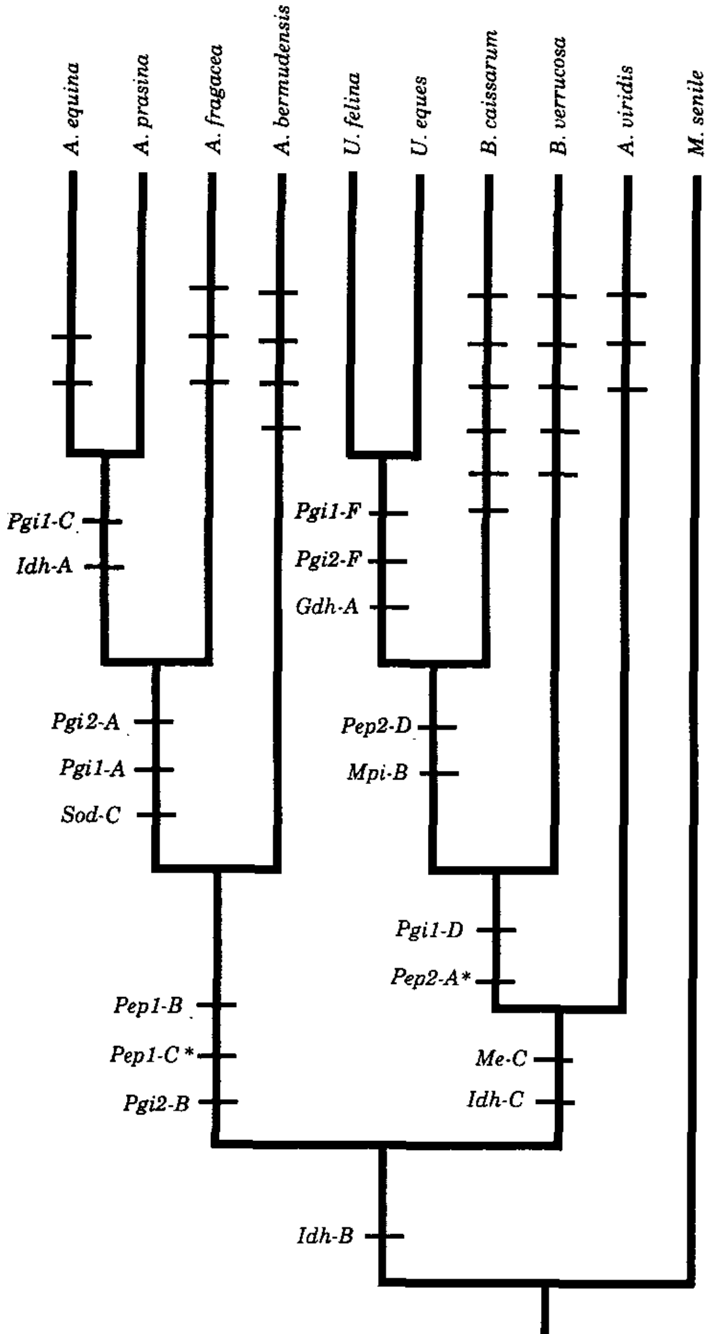

Figure 3. Maximum parsimony cladogram, using the allele as a binary character. Autapomorphies are only indicated as steps in the tree; *indicates the disappearance of the more plesiomorphic character ('synapousy' *sensu* Amorim et al., 1993). Consistency index = 0.61.

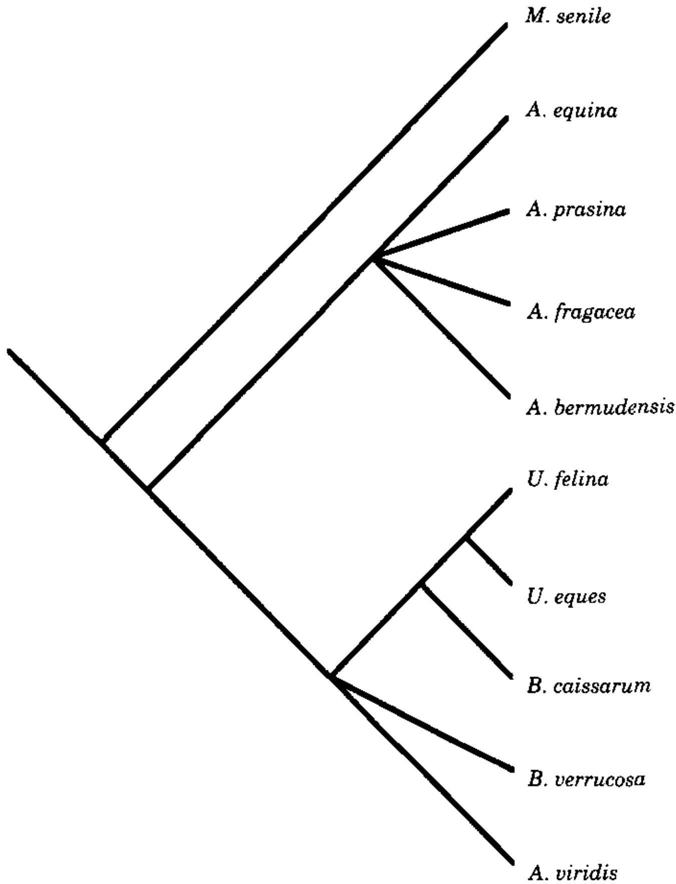

Figure 4. Majority-rule consensus tree, from 20 trees obtained through maximum parsimony using the locus as an unordered multi-state character (alleles as states). Consistency index = 0.83.

having the alleles as states, produced 29 trees in the exhaustive search. This result occurred principally because of the relatively weak resolution of the clade of *Actinia* species, a conclusion which is confirmed by the majority-rule consensus tree (Fig. 4).

TABLE 2. Unbiased genetic Identities (Nei, 1978, below the diagonal) and Prevosti's distances (Wright, 1978, above the diagonal) for pairwise comparisons between 10 species of sea anemones. Abbreviations as in Table 1

	Msen	*Aequ*	*Afra*	*Aber*	*Apra*	*Ufel*	*Bcai*	*Avir*	*Bver*	*Uequ*
M. senile	***	0.82	0.92	0.88	0.89	0.75	0.78	0.81	0.77	0.76
A. equina	0.19	***	0.46	0.49	0.19	0.74	0.70	0.82	0.83	0.73
A. fragacea	0.09	0.67	***	0.55	0.45	0.81	0.85	0.85	0.92	0.79
A. bermudensis	0.14	0.59	0.50	***	0.58	0.59	0.67	0.95	0.78	0.58
A. prasina	0.10	0.94	0.60	0.50	***	0.80	0.71	0.84	0.87	0.79
U. felina	0.27	0.29	0.22	0.49	0.23	***	0.61	0.71	0.61	0.07
B. caissarum	0.23	0.31	0.18	0.40	0.31	0.42	***	0.78	0.72	0.59
A. viridis	0.21	0.22	0.17	0.07	0.16	0.31	0.23	***	0.72	0.70
B. verrucosa	0.24	0.18	0.09	0.25	0.14	0.46	0.31	0.30	***	0.64
U. eques	0.27	0.31	0.24	0.52	0.24	1.00	0.42	0.32	0.43	***

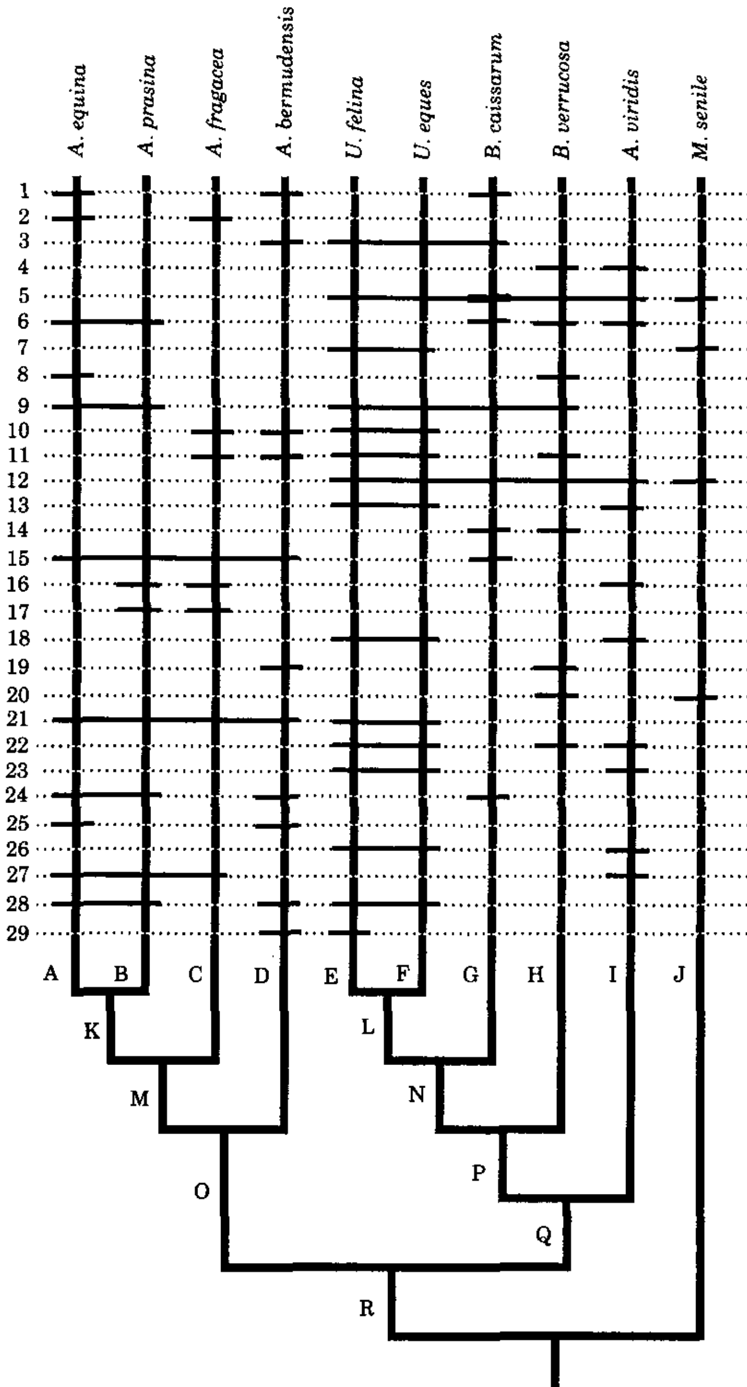

Figure 5. Maximum parsimony cladogram, using the locus as an ordered multi-state character (alleles as states, using heterozygotes as intermediate steps within transformation series). Ambiguous characters (marked numerically) and synapomorphies (marked alphabetically) are given in Table 3. Consistency index = 0.51.

TABLE 3. Ambiguous characters, indicated by numbers below the species names in the cladogram in Fig. 5; → indicates a transformation along the series; *indicates the disappearance of the more plesiomorphic character ('synapousy' *sensu* Amorim *et al.*, 1992)

1. *Pgi-1*(E)	11. *Hk-2*(C*)	21. *Mdh-2*(E)
2. *Pgi-2*(B → A)	12. *Pep-1*(C)	22. *Me*(C)
3. *Pgi-2*(C)	13. *Mpi*(C → A)	23. *Me*(C → D)
4. *Pgi-2*(D)	14. *Mpi*(C → D)	24. *Me*(F)
5. Gdh(E*)	15. *Mpi*(C → E)	25. *Me*(F → G)
6. *Pgm*(B → C)	16. *Mpi*(C*)	26. *Idh*(B → C)
7. *Pgm*(B → A)	17. *Mpi*(E → F)	27. *Xod*(A)
8. *Pgm*(C → D)	18. *Sod*(B)	28. *Xod*(C)
9. *Pep-2*(A → C)	19. *Sod*(E)	29. *Xod*(C → D)
10. *Pep-2*(A → B)	20. *Mdh-2*(A)	

As an inevitable consequence of the difficulty of finding and collecting anemones, sample sizes used were variable (one to 41 individuals per locus). Smaller samples will lead to sampling errors in estimates of allele frequencies as some, particularly less common, alleles will not be represented in the data set (for problems related to sample size in qualitative Hennigan analysis see Rogers, 1986). However, it is generally considered that, for interspecific molecular comparisons, the major component of the variance of the genetic identities is a function of the number of loci analysed (Nei, 1978), and even sample sizes as small as one individual can produce robust (within 90% confidence limits) estimates of genetic identity between species, when the number of loci analysed is not small (Gorman & Renzi, 1979).

The levels of gene identity for the pairwise comparisons between all the species studied (Table 2) were within the range expected for intra and suprageneric classification (Thorpe, 1983).

TABLE 4. Unambiguous derived steps, indicated by capital letters in the cladogram in Fig. 5 (Locus as an ordered multistate character). Symbols as in Table 3. States in step "J" can be ancestral to the ingroup and the outgroup, and are only indicated for comparison

A	*Gdh*(E → I), *Mdh-2*(E → D)
B	—
C	*Hk-2*(B*), *Hk-2*(D), *Me*(E)
D	*Gdh*(E → C), *Gdh*(E → G), *Pep-1*(B → A), *Mpi*(E → G)
E	—
F	*Pgi-1*(I)
G	*Gdh*(E → D), *Pgi-1*(C → G), *Sod*(A), *Mdh-2*(C), *Xod*(F)
H	*Hk-2*(B → A), *Pgm*(B*), *Xod*(B)
I	*Pgi-1*(G),*Gdh*(H), *Mdh-2*(B)
J	*Pgi-1*(I), *Pgi-1*(H), *Pgi-2*(E), *Gdh*(B), *Sod*(D), *Me*(A), *Me*(B), *Idh*(D), *Xod*(E)
K	*Idh*(B → A), *Pgi-1*(C)
L	*Pgi-1*(D → F), *Pgi-2*(C → F), *Gdh*(A)
M	*Sod*(C), *Pgi-1*(E → A)
N	*Pep-2*(C → D), *Mpi*(C → B)
O	*Pep-1*(C → B), *Pgi-2*(B), *Pep-1*(C*)
P	*Pep-2*(A*)
Q	*Pgi-2*(D)
R	*Idh*(D → B)

The electromorphs defining branches of the Hennigian tree in Fig. 5 were letter-coded and are given in Tables 3 and 4. Also in those tables can be found the number-coded homoplasious characters from the same figure.

DISCUSSION

The salient feature of the overall results is that phenetic and cladistic interpretations of the isozyme data discriminated unequivocally between the several anemone species studied (Figs 1–5). Even where no *a priori* information as to its phylogenetic position is given, *Metridium senile* appeared in the phenetic analysis, as the most divergent species of the group for which data were available (Figs 1, 2). Also, the species of *Actinia* formed a monophyletic group, and so did the species of *Urticina* studied. This result indicates that, contrary to what is usually assumed (Ferguson, 1980; Solé-Cava & Thorpe, 1987; Murphy *et al.*, 1990), allozymes can be used, at least in some cases, to estimate phylogenetic relationships at the genus and family levels (see Araujo & Solé-Cava, 1993, for a discussion on the application of allozymes to family level phylogeny). With increasing divergence times there is a concomitant increase in the number of homoplasies due to the 'saturation' of the technique (Thorpe, 1989). There is also an exacerbation of the problem of different divergence rates between lineages. This was probably responsible for the difference between the UPGMA dendrogram and the other trees (transposition of *Bunodactis verrucosa* and *Bunodosoma caissarum*), since the UPGMA analysis, unlike the other analyses, depends on the assumption of similar divergence rates between branches (Buth, 1984; Rogers, 1986). From the data it appears that *Bunodosoma caissarum* may have had an accelerated rate of allozyme evolution when compared with *Bunodactis verrucosa* (five autapomorphies in *Bunodosoma caissarum* against three in *Bunodactis verrucosa*; Fig. 5 and Table 4). Also, *Urticina* spp. shared a higher number of homoplasies with *Bunodactis verrucosa* than they did with *Bunodosoma caissarum* (Fig. 5; Table 3). These two factors increased the phenetic similarity between *Bunodactis verrucosa* and *Urticina* spp. The Wagner distance (Fig. 2) and the cladistical analysis (Figs 3, 4, 5), however, accounted for those effects, and indicated that *Bunodosoma caissarum* had a closer relationship with the two *Urticina* species.

The use of transformation series analysis produced well-resolved trees for the sea anemone data (Fig. 5). As is common in sea anemones species (Shick, 1991; Solé-Cava & Thorpe, 1991), very high levels of genetic variation were found within the species studied (Table 1). It could be argued that highly polymorphic loci pose potential problems for phylogenetical analysis because they are likely to show enhanced rates of evolutionary change (Skibinski & Ward, 1981), and therefore more likely to produce parallelisms and convergences. Indeed, the large number of alleles caused some difficulties in the ordering of the loci from the sea anemones species studied, since many different orders were equally possible (e.g. *Pep-2* had four and *Pgm* had 12 possible transformation series). Such problems were not found in the earlier study of Araujo & Solé-Cava (1993) (on flatfish), and probably resulted from the very high levels of heterozygosity at most loci in the sea anemones studied. Nevertheless, some of the polymorphic loci were highly informative (the most informative being *Pgi-1*, *Pgi-2* and *Gdh*, each of which produced clear ordered series) since the presence of transient

heterozygote apomorphies (syntrepties; Amorim *et al.*, 1993) permitted ordering of the alleles in the transformation series. For some loci the ordering of alleles was not feasible because informative heterozygote states were not found (possibly because of the small sample sizes used.) In such cases, the lack of definitive information as to the possible directions of the mutational changes hampered the formulation of evolutionary hypotheses *a priori*. This difficulty was somewhat mitigated by the superposition of other characters and the formulation of polarization hypotheses *a posteriori*, using group subordination (as in Patton & Avise, 1983).

The overall results indicate that the species of *Actinia* studied constitute, in relation to the other species studied, a monophyletic group. It also shows that the South Atlantic species (*A. bermudensis*) is the most divergent of the group. This is to be expected since the fauna from the tropical west Atlantic and the British Isles have been separated since the separation of the Tethys (mid-Miocene), so it is unlikely that the separation of the British *Actinia* species is more recent than that of *A. bermudensis*. Also, the asexually reproducing (Orr *et al.*, 1982) *A. equina* and *A. prasina* seem to have diverged the most recently since, as observed previously, genetic identity levels between them were high (Haylor *et al.*, 1984; Solé-Cava & Thorpe, 1987). The very high level of identity observed between *U. felina* and *U. eques* (only one synapomorphy, in *U. eques*), also confirms earlier results for these species (Solé-Cava *et al.*, 1985).

Although the number of species studied is rather small, in all the trees produced the clustering of the species indicates that those with vesicles or verrucae (*Urticina eques*, *U. felina*, *Bunodactis verrucosa*, *Bunodosoma caissarum*) form a monophyletic cluster, and are distinct from the other species that have smooth columns. Verrucae and vesicles are considered to be specialized structures evolved as an adaptation for defence and for the control of desiccation (Shick, 1991). This result is compatible with the suggestion that these structures may have appeared only once in the evolutionary history of the Actiniidae, although a study of a larger number of species would be necessary for confirmation of this hypothesis. Our conclusion is at odds with that of McCommas (1991), who concluded that species with and without verrucae clustered together in a cladogram produced from a less detailed analysis (using alleles as binary characters only) of 12 allozyme loci.

From the present work it seems that the use of allozymes could be potentially very useful for phylogenetic reconstruction in sea anemones. At the level of genetic divergence studied, it appears that cladistics are superior to phenetic analyses, since the former are less sensitive to differences in divergence rates. Particularly in species with moderate to high amounts of gene variation, the use of transient polymorphic states as links in the analysis of transformation series must be considered as a valuable additional use of allozymes for the generation of phylogenetic hypotheses.

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