

# Molecular evidence for the hybrid origin of species in the soft coral genus *Alcyonium* (Cnidaria: Anthozoa: Octocorallia)

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## Abstract

Several recent studies have suggested that hybridization may play a previously unrecognized and important role in the evolution of corals. Our observations of polymorphic and recombinant sequences in the multicopy ribosomal internal transcribed spacer (ITS) region suggested the possible hybrid origin of two European soft coral species, *Alcyonium hibernicum* and *Bellonella bocagei*. To examine this possibility further we cloned and sequenced *ITS-1* from multiple individuals and populations of these two species as well as two sympatric congeners, *A. coralloides* and *A. sp. M2*. Phylogenetic analyses separated the observed sequence variants into two distinct clades. All *A. coralloides* sequences belonged to clade A, while *A. sp. M2* had only clade B sequences. A majority of *A. hibernicum* individuals, however, contained both clade A and B sequences that were identical to the predominant sequence variants found in *A. coralloides* and *A. sp. M2*, respectively. This pattern of additivity suggests that *A. hibernicum* originated from a hybrid cross between *A. coralloides* and *A. sp. M2*, a hypothesis that is supported by its unusual mode of reproduction (meiotic parthenogenesis). The predominant sequence variant found in *B. bocagei* was a unique, derived clade B sequence; in addition, however, most individuals of this species also had copies of a sequence identified as a recombinant between clade A and clade B sequence types. The presence of this recombinant sequence in the *B. bocagei* genome suggests that this species may also be the product of past hybridization events within the clade. Reticulate evolution may explain the failure of several previous studies to resolve the phylogeny of these four species.

**Keywords:** concerted evolution, hybrid speciation, *ITS-1*, octocoral, recombination, reticulate evolution

Received 6 November 2003; revision received 4 February 2004; accepted 4 February 2004

## Introduction

The importance of hybridization in the evolution of terrestrial plants and some terrestrial animals has long been recognized (reviewed by Arnold 1997). In contrast, the role this process may play in the evolution of marine invertebrate groups has begun to be appreciated only recently (Gardner 1997). The increasing use of molecular markers in phylogenetic studies has led to the realization that hybridization may be particularly widespread within several of the major groups of reef-building scleractinian corals (Veron 1995). Shared

polymorphisms in multicopy nuclear ribosomal DNA and in some single-copy nuclear intron genes, incongruence between nuclear and mitochondrial gene phylogenies, and successful interspecies crosses conducted in the laboratory have provided evidence for hybridization in well-known scleractinian genera such as *Acropora*, *Montastraea* and *Madracis* (Wallace & Willis 1994; Odorico & Miller 1997; Szmant *et al.* 1997; Hatta *et al.* 1999; van Oppen *et al.* 2000, 2001, 2002; Diekmann *et al.* 2001; Vollmer & Palumbi 2002; Fukami *et al.* 2003; Wolstenholme *et al.* 2003).

Soft corals of the anthozoan subclass Octocorallia share many ecological and reproductive traits with scleractinians, including those that may predispose them to hybridization. Reproduction occurs either by broadcast spawning of both male and female gametes into the water column, or, in brooding species, by release of sperm that subsequently

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enter the gastrovascular cavities of neighbouring female colonies to fertilize oocytes that are retained internally. Closely related, morphologically similar species frequently live in close proximity to one another and have overlapping reproductive periods, providing opportunities for interspecific encounters among gametes (McFadden 1999). Although most studies of scleractinian corals have focused on species that broadcast spawn synchronously in response to a common environmental cue (Wallace & Willis 1994; Babcock 1995; Veron 1995), recently it has been suggested that species that brood their embryos may also have a high potential for hybridization (Diekmann *et al.* 2001). Brooders typically have longer reproductive periods than broadcast spawners, increasing the opportunity for interspecific hybridization temporally. Moreover, the ability of some brooding species to self-fertilize (Carlon 1999) could ensure the subsequent reproductive success of hybrid individuals. Here we examine the potential occurrence of hybridization among a group of sympatric, brooding species in the temperate soft coral genus *Alcyonium*.

Four closely related species of *Alcyonium* have overlapping distributions along the Mediterranean and north-eastern Atlantic coasts of Europe (McFadden 1999) (Fig. 1). The most geographically widespread species, *Alcyonium coralloides* Pallas 1766, occurs throughout the western basin of the Mediterranean and along the Atlantic coast from Portugal to northern France. Within this broad range it is sympatric with each of the other three species, whose ranges do not overlap one another. *Alcyonium hibernicum* Renouf 1931 occurs throughout the western British Isles and northern France; it is sympatric with *A. coralloides* within a narrow range in northwestern France. *Bellonella bocagei* Saville Kent 1870 is found along the Atlantic coast from southern Portugal to West Africa and in the Azores (Verseveldt & Bayer 1988) and is sympatric with *A. coralloides* in southwestern Portugal [recent genetic studies suggest that *B. bocagei* should be reassigned to the genus *Alcyonium* (McFadden 1999)]. Finally, an undescribed species, previously designated *Alcyonium* sp. M2 (McFadden 1999), is sympatric with *A. coralloides* throughout the western basin of the Mediterranean.

Although these four species were at one time synonymized under the name *Alcyonium coralloides* (van Soest & Weinberg 1980; Groot & Weinberg 1982), recent genetic studies suggest that they are reproductively isolated and belong to two distinct clades (McFadden 1999; McFadden *et al.* 2001). Populations of *A. coralloides* comprise one clade; the second clade includes *A. hibernicum*, *B. bocagei* and *A. sp. M2*. Where *A. coralloides* occurs sympatrically with each of these three 'A. hibernicum clade' species, fixed allozyme differences indicate that they do not interbreed (McFadden 1999). Although all four species have similar sclerite morphology (van Soest & Weinberg 1980; Groot & Weinberg 1982; C.S. McFadden unpublished data), they are easily

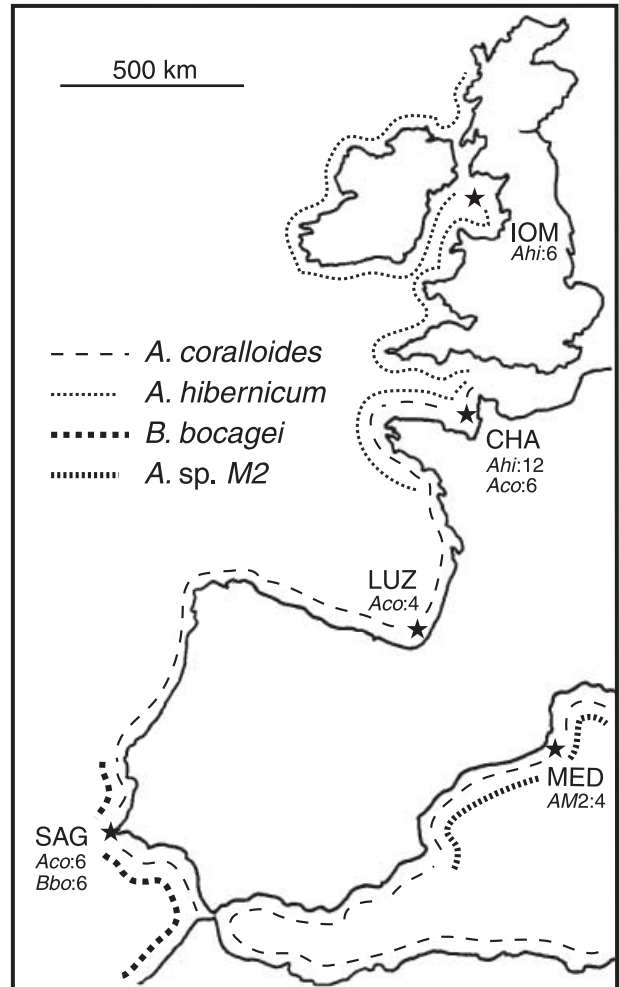


Fig. 1 Map of western Europe showing sites at which soft coral species were collected. Dashed lines indicate the known geographical range of each species. Numbers of individuals of each species sampled per site are shown below site labels. Collection sites indicated by stars: IOM = Isle of Man; CHA = Iles Chausey, France; LUZ = St Jean-de-Luz, France; SAG = Sagres, Portugal; MED = Illes Medes, Spain.

distinguishable by differences in colony growth form, colour, and substrate specificity (McFadden 1999).

During the course of molecular phylogenetic studies (McFadden *et al.* 2001), several sources of evidence suggested to us that hybridization may have occurred in the past or be ongoing between *A. coralloides* and members of the *A. hibernicum* clade. At two sites in France where *A. hibernicum* and *A. coralloides* co-occur a few individuals of each species were found that had allozyme alleles characteristic of the other species (McFadden 1999). Moreover, routine cloning of the multicopy internal transcribed spacer region *ITS-1* revealed the occasional presence of sequences characteristic of *A. coralloides* in individuals of *A. hibernicum*. In addition, an *ITS-1* sequence variant cloned from an individual of *B. bocagei* appeared to be a



Variant	Length (bp)	% G + C	Frequency among sequenced clones			
			<i>Ahi</i> ( <i>n</i> = 80)	<i>Aco</i> ( <i>n</i> = 42)	<i>Bbo</i> ( <i>n</i> = 52)	<i>AM2</i> ( <i>n</i> = 20)
<i>Ahi1</i>	209	39.2	0.44	0.00	0.00	0.75
<i>Ahi2</i>	193	40.9	0.10	0.00	0.00	0.00
<i>Aco1</i>	196	44.4	0.26*	0.60	0.00	0.00
<i>Aco3</i>	192	43.2	0.00	0.31	0.00	0.00
<i>Bbo1</i>	207	40.6	0.00	0.00	0.48	0.00
<i>Bbo2</i>	200	44.0	0.00	0.00	0.40*	0.00
Other	183–219	38.5–44.3	0.20	0.09	0.12	0.25

'Other' includes 20 variants that were present at low frequencies; *Ahi* = *Alcyonium hibernicum*; *Aco* = *A. coralloides*; *Bbo* = *Bellonella bocagei*; *AM2* = *A. sp. M2*; *n* = number of clones sequenced.

\**Aco1* and *Bbo2* sequences are over-represented in *Alcyonium hibernicum* and *Bellonella bocagei*, respectively, because clones with those variants were preselected for sequencing based on results of restriction digests (see text for actual frequencies).

analyses were run with the program MRBAYES vs. 3.0b4 (Huelsenbeck & Ronquist 2001) using the best-fit model of evolution (HK80 + G) estimated by MODELTEST vs. 3.06 (Posada & Crandall 1998). *Alcyonium glomeratum* was used as the outgroup for all phylogenetic analyses.

Sequences were tested for signatures of recombination using the Phylogenetic Profile method (PHYLPRO (BETA) vs. 0.8, Weiller 1998), with linear correlation and a sliding window of 10 differences (nearly identical results were obtained with a window of unlimited size). Only those nucleotide positions at which there was parsimony-informative variation were analysed, with gaps included as variable sites. Sequences with regions of low phylogenetic correlation were identified as putative recombinants and were removed from subsequent phylogenetic analyses. Recombinant sequences were examined further using the programs GENECONV (Sawyer 1999) and MAXIMUM CHI-SQUARED (Maynard Smith 1992). Of the many tests currently available, these two substitution methods have been demonstrated to be among the most powerful for detecting recombination (Posada & Crandall 2001; Posada 2002), and, unlike the Phylogenetic Profile method, can be used to identify potential parental sequences. Several of the recombinant variants were distinguished by indels, which can be utilized informatively by GENECONV but not by MAXIMUM CHI-SQUARED. For the MAXIMUM CHI-SQUARED analysis therefore each gap was recoded as a nucleotide using a base not present at the homologous position in any of the sequences being compared.

## Results

### Sequencing

A total of 194 clones were sequenced from 44 individuals, and 77 distinct *ITS-1* sequences were identified. Sixty-

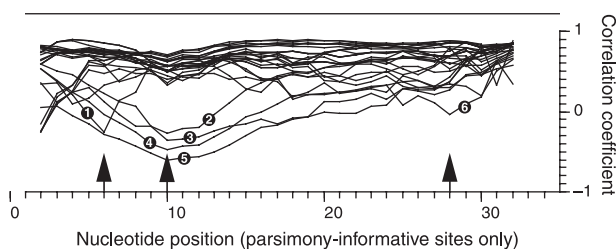
**Table 1** Properties of the six most common *ITS-1* sequence variants and the frequencies at which they were found among sequenced clones from four soft coral species

seven sequences were represented by only a single clone, while the most common sequence was shared by 38 clones. Sequences that differed from one another at three or more nucleotide positions (i.e. > 1.0%) were classified as distinct variants; sequences that differed by no more than two single base-pair substitutions or a one-base-pair indel (differences that could have resulted from PCR error) were considered to belong to the same variant. By these criteria, 26 distinct *ITS-1* variants were identified among the 77 sequences. The lengths of the variants ranged from 183 to 219 base pairs and GC content ranged from 38.5 to 44.4% (Table 1). A representative clone of each of these 26 variants was sequenced in both directions and deposited in GenBank (Accession numbers AY243538–AY243564).

A majority (85%) of the 194 clones sequenced belonged to just six common variants (Table 1); most of the other 20 variants were represented by only a single clone. All but two of the variants were unique to the species in which they were found: 11 variants were found only in *Alcyonium hibernicum*, four only in *A. coralloides*, six only in *Bellonella bocagei* and three only in *A. sp. M2*. Variant *Ahi1*, however, was shared by *A. hibernicum* and *A. sp. M2*, and was the predominant sequence type found in both species (Table 1). *Aco1*, the predominant variant found in *A. coralloides*, was also shared by 26% of the clones from *A. hibernicum*. None of the variants found in *B. bocagei* were shared with another species.

### Recombinant sequences

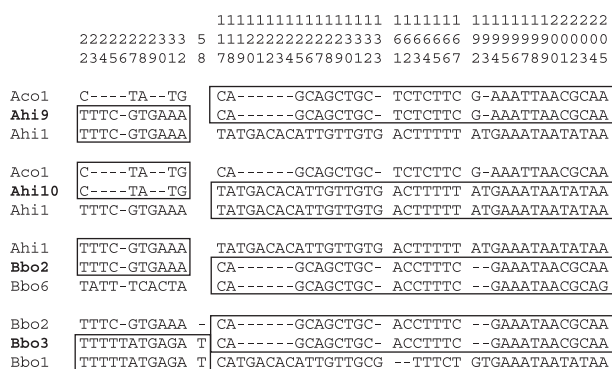
Although many of the 26 variant sequences showed some regions of low correlation coefficients suggestive of past recombination events, six variants had particularly strong recombination signals (Fig. 3). These included the *Bbo2* variant that was found at high frequency in *B. bocagei*



**Fig. 3** Phylogenetic profile of 26 distinct *ITS-1* sequence variants. Six sequences with particularly strong recombination signals (low correlation coefficients) are identified with dark circles and numbers: 1 = *AM3*; 2 = *Bbo3*; 3 = *Ahi10*; 4 = *Bbo2*; 5 = *Ahi9*; 6 = *Ahi8*. Arrows indicate the probable recombination sites. Only parsimony-informative variable sites were included in the analysis.

(Table 1), as well as one unique variant found in a single clone of *B. bocagei* (*Bbo3*), three variants unique to *A. hibernicum* (*Ahi8*, *Ahi9*, *Ahi10*), and a single variant found in one clone of *A. sp. M2* (*AM3*).

Four of these six recombinant variants (*Bbo2*, *Bbo3*, *Ahi9* and *Ahi10*) had correlation coefficient minima at parsimony-informative site 9 (Fig. 3), indicative of a recombination event having occurred in the relatively invariant region between nucleotide positions 32 and 117 of the sequence alignment (Fig. 2). Individual tests of each recombinant and its potential parents using the programs MAXIMUM CHI-SQUARED and GENECONV strongly supported *Ahi1* and *Aco1* as parent sequences of both *Ahi9* ( $P < 0.001$ ) and *Ahi10* ( $P < 0.001$ ), *Ahi1* and *Bbo6* as parents of *Bbo2* ( $P < 0.001$ ), and *Bbo1* and either *Bbo6* or *Bbo2* as parents of *Bbo3* ( $P < 0.001$ ). Alignments of the recombinants with their most likely parental sequences support these interpretations visually (Fig. 4), and reveal that *Ahi9* and *Ahi10* are the reciprocal



**Fig. 4** Alignments of four recombinant *ITS-1* variants with the sequences identified as their most likely parents from the results of analyses using the programs GENECONV and MAXIMUM CHI-SQUARED. Recombinants are shown in the middle of each sequence trio. Boxes enclose regions of the sequence where the recombinant is identical to one of the parents. Only variable regions of the alignment are shown.

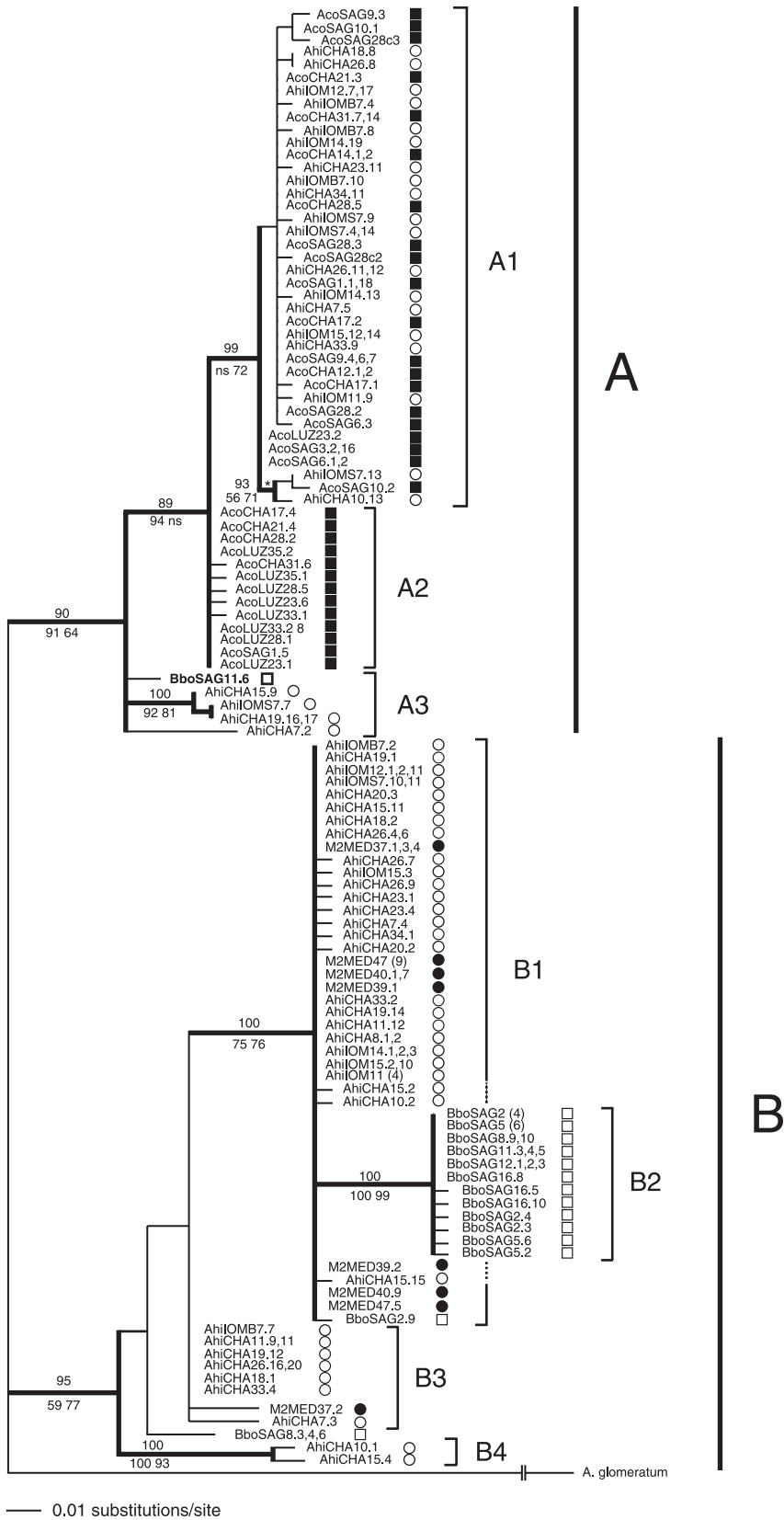
products of a single recombination event. Putative recombinants *Ahi8* and *AM3* were both sufficiently divergent from all other variants that it was not possible to identify candidate parental sequences.

*Phylogenetic analysis*

All clones with the *Bbo2* recombinant sequence ( $n = 21$ ) as well as any belonging to the other five recombinant variants identified above were removed from the dataset prior to phylogenetic analysis. The nonrecombinant sequences ( $n = 166$  clones) fell into two distinct clades (Fig. 5). Clade A included all the clones from *A. coralloides*, clones representing a majority of the *A. hibernicum* individuals, and a single clone from *B. bocagei* (variant *Bbo6*). Clade B included clones from every individual of *A. hibernicum*, *A. sp. M2*, and *B. bocagei*. No *A. coralloides* clones were found in clade B and no clones of *A. sp. M2* were found in clade A. Maximum parsimony (with gaps coded as fifth characters), Bayesian-likelihood and neighbour-joining analyses all recovered these same major clades, although relative support for each of the subclades within clades varied among analyses (Fig. 5). When the recombinant sequences were included in the analysis, they grouped together at the base of the tree and reduced support for the two major clades (tree not shown).

Clade A could be divided into three moderately supported subclades. Subclade A1 was composed predominantly of the *Aco1* sequence variant shared by both *A. coralloides* and *A. hibernicum*. Subclade A2 comprised the *Aco3* sequence variant found only in *A. coralloides*. This variant was the majority sequence type found at the LUZ site (nine of 10 clones) but was not common at CHA or SAG (five of 32 clones), suggesting some geographic structuring of *A. coralloides* populations. A third small subclade (A3) included several divergent sequence variants found in *A. hibernicum* as well as the *Bbo6* variant from *B. bocagei* (putative parent of the *Bbo2* recombinant). All of the individuals of *A. hibernicum* represented in clade A also appeared in clade B.

Clade B encompassed four subclades. Subclade B1 included the *Ahi1* sequence variant found in all individuals of *A. hibernicum* and *A. sp. M2*; one clone from *B. bocagei* also fell into this subclade. Subclade B2 represented the *Bbo1* sequence variant found in all individuals of *B. bocagei* only. Subclade B3 included the *Ahi2* sequence variant found at moderate frequency in *A. hibernicum* (Table 1), and subclade B4 consisted of two *A. hibernicum* clones with divergent sequence types. There was some conflict among phylogenetic methods regarding the relationships of subclades B1, B2 and B3. In both Bayesian-likelihood and maximum parsimony trees subclade B1 was paraphyletic with B2 (Fig. 5), while in the neighbour-joining tree subclade B3 formed a paraphyletic group with both B1 and B2 (tree not shown).



**Fig. 5** Bayesian-likelihood tree of phylogenetic relationships among ITS-1 clones isolated from *Alcyonium coralloides* (■), *A. hibernicum* (○), *A. sp. M2* (●) and *Bellonella bocagei* (□). Sequences identified *a priori* as recombinants are not included in the tree. Numbers above nodes are Bayesian probabilities (generations = 10<sup>6</sup>, burn-in = 2000); below nodes are bootstrap percentages for maximum parsimony (left, 100 replicates) and neighbour-joining (right, 1000 replicates) analyses; ns indicates that a branch was not supported (bootstrap value < 50). Branches shown in bold were supported by at least two of three phylogenetic methods (bootstrap value > 70, Bayesian probability > 85). Tree is rooted with *A. glomeratum* as the outgroup (full branch length not shown). Clone abbreviations indicate species, collection site and individual number; number(s) following dot are clone number(s); numbers in parentheses indicate number of clones with same sequence for values > 3. Variant *Bbo6*, the only *B. bocagei* sequence found in clade A, is indicated in bold.

### Restriction digests

*XmnI* or *AluI* restriction digests were obtained for 15–20 clones from each of four to 12 individuals of each species from each site (total number of individuals = 44; Fig. 1). Exceptions were two individuals of *A. coralloides* from SAG and the four individuals of *A. sp. M2* from which only four to 10 clones were screened. All clones from *A. coralloides* ( $n = 270$ ) had the expected *XmnI*- restriction pattern, consistent with membership of clade A. Conversely, all clones from *A. sp. M2* ( $n = 27$ ) had the clade B restriction pattern (*XmnI*+). The majority of clones from *A. hibernicum* were also of the clade B type, but some clade A (*XmnI*-) clones were found in 15 of the 18 individuals sampled (mean number of clones screened per individual =  $18.3 \pm 1.1$  SD). The frequency of clade A clones per individual ranged from 0.00 to 0.22, with a mean of 0.09 ( $\pm 0.07$  SD), but was somewhat higher in individuals from the IOM population (mean =  $0.13 \pm 0.06$ ) than in those from CHA (mean =  $0.07 \pm 0.05$ ). Five of six individuals of *B. bocagei* had *AluI*+ in addition to *AluI*- clones (mean number of clones screened per individual =  $21.8 \pm 7.6$  SD). The frequency of *AluI*+ clones ranged from 0.00 to 0.65 per individual with a mean of 0.26 ( $\pm 0.24$  SD). All *AluI*+ clones were subsequently sequenced and confirmed to be recombinant variant *Bbo2*.

## Discussion

### Hybrid origin of *Alcyonium hibernicum*

The phylogenetic distribution of *ITS-1* variants suggests that *Alcyonium hibernicum* is a hybrid species that originated from a cross between *A. coralloides* and *A. sp. M2*. The *ITS-1* sequences found in *A. coralloides* and *A. sp. M2* are reciprocally monophyletic, with all *A. coralloides* sequences belonging to clade A and all *A. sp. M2* sequences belonging to clade B. The distribution of *ITS-1* variants in *A. hibernicum*, however, is additive. Over 80% of the individuals of *A. hibernicum* examined had *ITS-1* sequences belonging to both clades A and B, and the majority of these sequences were identical to the predominant variants in *A. coralloides* and *A. sp. M2*. No evidence was found, however, for introgression of clade A *ITS-1* sequences into *A. sp. M2* or clade B into *A. coralloides*, suggesting that the putative hybrid species has not backcrossed with either parent species.

The reproductive biology of *A. hibernicum* is consistent with a hybrid origin, as it appears to reproduce either by meiotic parthenogenesis or obligate selfing (Hartnoll 1977; McFadden 1999). Both of these types of reproduction are rare among marine invertebrates, but often occur in hybrid species (Bullini 1994). Inability to outcross would reproductively isolate *A. hibernicum* from its putative parental species, thereby precluding backcrossing and introgression of alleles into those species.

The diversity and phylogenetic distribution of *ITS-1* sequences found in *A. hibernicum* suggest that the hybrid origin of this species is not a recent event. Eleven *ITS-1* variants were found in *A. hibernicum* (four belonging to clade A and seven to clade B) that were not shared with either putative parent. Conversely, none of the sequence variants found in *A. sp. M2* or *A. coralloides* other than *Aco1* and *Ahi1* were shared by *A. hibernicum*. This mutually exclusive distribution of all but the two most common variants suggests that, following the hybrid origin of *A. hibernicum*, diversification of *ITS-1* repeats by mutation, recombination and concerted evolution has proceeded independently in hybrid and parental lineages.

The presence of unique alleles at three of 11 allozyme loci assayed previously (McFadden 1999) also suggests that *A. hibernicum* is not a recent hybrid, and that there has been time since its origin for new alleles to have arisen by mutation or recombination and for parental alleles to have been eliminated by drift or selection. The other eight allozyme loci provide equivocal support for a hybrid origin of *A. hibernicum*: one locus was fixed for an allele shared with *A. sp. M2*, one was fixed for an allele shared with *A. coralloides*, and six loci were fixed for alleles shared by both putative parental species. No allozyme loci exhibited polymorphism.

Although theory suggests that concerted evolution by gene conversion or unequal crossing-over should rapidly homogenize multicopy gene families such as *ITS-1* (Dover 1982; Arnheim 1983; Hillis & Dixon 1991), studies of a variety of hybrid taxa provide conflicting evidence. In some taxa, homogenization of *ITS* repeats following hybridization appears to have occurred rapidly, completely eliminating one parental *ITS* type from the genome (Hillis *et al.* 1991; Wendel *et al.* 1995a,b; Roelofs *et al.* 1997; Aguilar *et al.* 1999; Franzke & Mummendorf 1999). In other cases, however, homogenization of repeats has not followed hybridization, and hybrids maintain both parental DNA types, and, often, their recombinants (Sang *et al.* 1995; O'Kane *et al.* 1996; Waters & Schaal 1996; Campbell *et al.* 1997; Hugall *et al.* 1999). In particular, when divergent DNA repeat units are combined within a single genome, the rate of sequence homogenization by gene conversion may be greatly reduced (Modrich & Lahue 1996; Muir *et al.* 2001). This phenomenon may explain why both *ITS* types are still present in *A. hibernicum* despite parental alleles having already gone to fixation at single-copy allozyme loci. *Aco1* clones were, however, found at lower frequency than *Ahi1* clones in all *A. hibernicum* individuals sampled, suggesting that concerted evolution may be in the process of eliminating this parental *ITS* type from the hybrid genome.

Several recent studies have demonstrated that divergent *ITS* sequence types present in the genomes of hybrid species may not all be functional (Muir *et al.* 2001; Márquez *et al.* 2003). It is assumed that the *Aco1* and *Ahi1* sequences represent

functional genes since they are the predominant sequence types found in *A. coralloides* and *A. sp. M2*, respectively. The possibility that one of these variants has been silenced by nucleolar dominance and is evolving as a pseudogene in *A. hibernicum*, however, remains unexplored.

#### Recombination in *A. hibernicum*

The *Ahi9* and *Ahi10* variants found at low frequency in *A. hibernicum* appear to be recombinants between the parental *Aco1* and *Ahi1* ITS sequences (Fig. 4). Whether these recombinant sequences have arisen naturally in *A. hibernicum*, however, is unknown. Recent studies have demonstrated that recombinant sequences can be generated by PCR whenever a mixture of partially homologous template sequences is present in the reaction (Bradley & Hillis 1997; Cronn *et al.* 2002). Because copies of both the *Ahi1* and *Aco1* variants were present in the genomes of most of the *A. hibernicum* individuals sampled, the few recombinant sequences found could be PCR artefacts. If so, the generation of identical recombinant sequences (*Ahi9*) in PCR reactions from three different individuals implies a possible recombination 'hotspot' in *ITS-1* that promotes PCR-generated recombination at a particular location in the sequence.

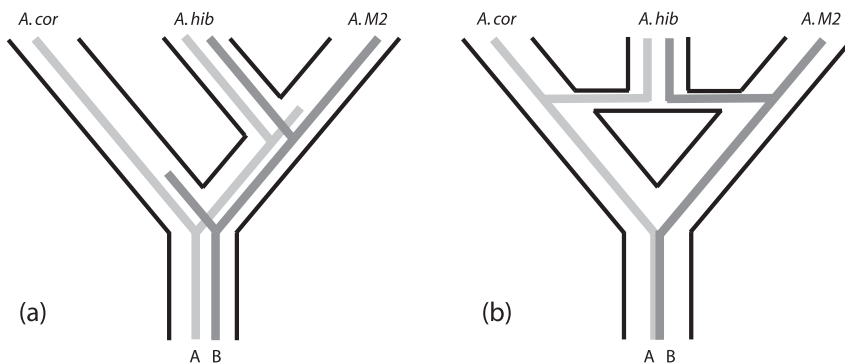
#### Geographic origin of *A. hibernicum*

The present-day geographic distributions of *A. hibernicum* and its presumed parents, *A. coralloides* and *A. sp. M2*, further support the molecular evidence that *A. hibernicum* is not a recent hybrid. At present, *A. sp. M2* is known to occur only in the western basin of the Mediterranean, where it is sympatric with *A. coralloides*. These two species often live in very close proximity to one another, and there are no extrinsic barriers to hybridization (McFadden 1999). *Alcyonium hibernicum* is, however, currently found only in the western British Isles and France; although it is sympatric with *A. coralloides* in northern France, its present geographic distribution is far from that of *A. sp. M2* (Fig. 1). All three species produce larvae with limited dispersal abilities

(McFadden *et al.* 2001), and it is difficult to imagine that the offspring of a hybrid cross in the Mediterranean could colonize the British Isles. Throughout the Quaternary, however, the western Mediterranean has been subject to periodic climatic fluctuations that have repeatedly altered its temperature, salinity and circulation patterns, and have promoted the immigration and survival of different faunas at different times (Pérès 1985). Both *A. coralloides* and *A. sp. M2* may have originated in the Atlantic and colonized the Mediterranean subsequently when conditions were favourable for immigration (McFadden 1999). If so, the hybridization event that gave rise to *A. hibernicum* could have taken place in the Atlantic, in closer proximity to the present-day geographic range of this species. With no fossil record, however, we can only speculate about the past geographic distributions of these species.

#### Incomplete lineage sorting of ancestral polymorphisms

In the absence of direct evidence for hybridization (e.g. breeding experiments), it can be difficult to distinguish cases in which polymorphisms are shared as a result of common ancestry from those resulting from hybridization. Retention of an ancestral polymorphism is therefore an alternative explanation for the presence of both clade A and B sequences in *A. hibernicum*. If *A. hibernicum* and *A. coralloides* diverged from a common ancestor that was polymorphic for both clade A and B type *ITS-1* sequences, *A. hibernicum* might still retain both *ITS* types while the clade B sequences have been lost from the *A. coralloides* genome by concerted evolution. Previous ribosomal DNA and allozyme phylogenies suggest, however, that *A. hibernicum* shared a common ancestor most recently with *A. sp. M2* or *Bellonella bocagei* (McFadden 1999; McFadden *et al.* 2001), both of which lack the *Aco1* variant. For *A. hibernicum* to share *Aco1* with *A. coralloides* by linear descent, the ancestral polymorphism would have to have persisted through one or more speciation events, and the *Aco1* variant would subsequently have to have been lost independently in both *A. sp. M2* and *B. bocagei* (Fig. 6). Whether *A. hibernicum* is a



**Fig. 6** Alternative explanations for the presence of clade A and B *ITS-1* variants in *Alcyonium hibernicum*. (a) Incomplete lineage sorting of an ancestral polymorphism. To explain the present-day distribution of *ITS* variants the polymorphism must have persisted through at least two speciation events. Because its phylogenetic position with respect to *A. hibernicum* and *A. sp. M2* is uncertain, *Bellonella bocagei* is not shown. (b) Hybridization between *A. coralloides* and *A. sp. M2* gives rise to *A. hibernicum*. The divergence of clade A and B sequence types could have occurred either before or after the initial speciation event.

hybrid species or shares *Aco1* with *A. coralloides* as a result of an ancestral polymorphism cannot be determined conclusively from the data presented here. Based on the available evidence, however, we favour the hypothesis that *A. hibernicum* is a hybrid species.

#### *Origin of recombinant ITS-1 in B. bocagei*

Of the two *ITS-1* variants that are predominant in *B. bocagei*, one (*Bbo1*) is a unique derivative of clade B, and the other (*Bbo2*) appears to be a recombinant between clade A and B sequence types (Fig. 4). The very high frequency of *Bbo2* among the clones sequenced in this study (Table 1) suggests that this variant is a naturally occurring recombinant rather than a PCR artefact. [The *Bbo3* recombinant found in a single clone may, however, be an artefactual recombinant formed by the interaction of *Bbo1* and *Bbo2* templates (Fig. 4), both of which were present in most PCR reactions involving *B. bocagei*.] Because *Bbo2* was not found in any other species, and neither half of the sequence resembles the predominant *B. bocagei* sequence (*Bbo1*), it is unlikely that it was introduced into the *B. bocagei* genome by recent hybridization. If at some time in the past, however, *B. bocagei* had both clade A (*Bbo6*) and clade B (*Ahi1*) sequences in its genome, either as the result of a past hybridization event or an ancestral polymorphism, recombination between them could have produced *Bbo2*. Concerted evolution to eliminate the parental sequence types would have had to occur subsequently to explain the observed distribution. Homogenization of *ITS-1* repeats to a recombinant sequence following hybridization has been documented previously in plants and fungi (Wendel *et al.* 1995b; Mummenhoff *et al.* 1997; Hughes & Petersen 2001). Whether *Bbo1* and recombinant *Bbo2* variants are both functional in *B. bocagei* remains unknown.

#### *Comparisons to other hybrid coral species*

The patterns of *ITS* polymorphism observed among these four soft coral species differ substantially from those documented to date among scleractinians. The most common pattern that has been attributed to reticulate evolution in scleractinians is one in which two or more species share several different *ITS* sequence types to form a paraphyletic clade (Odorico & Miller 1997; Hatta *et al.* 1999; Diekmann *et al.* 2001; van Oppen *et al.* 2001, 2002). These paraphyletic distributions have been postulated to result from occasional hybridization followed by backcrossing of hybrids with both parental species (van Oppen *et al.* 2002). A majority of the *ITS* variants in these hybrid complexes may have been subject to recombination (van Oppen *et al.* 2002). It is difficult to rule out incomplete lineage sorting, however, as an explanation for some of these patterns. In addition to having paraphyletic distributions of *ITS* variants, species

belonging to these hybridizing 'syngameons' typically share many allozyme alleles as well (Miller & Benzie 1997; Márquez *et al.* 2002).

At least one case has been documented in which hybridization among scleractinians has given rise to a hybrid species. In the Caribbean, *Acropora prolifera* appears to be a hybrid between *Acropora palmata* and *Acropora cervicornis*. Vollmer & Palumbi (2002) argue on the basis of the additivity of single-copy nuclear intron alleles that the majority of *Acropora prolifera* individuals are  $F_1$  hybrids that rarely reproduce. The *ITS* sequences of the two parental species and the hybrid, however, are very similar and form one large, unresolved clade with no detectable patterns of additivity (van Oppen *et al.* 2000). In contrast, the pattern seen here in *A. hibernicum* is one of additivity of divergent *ITS* alleles, whereby the majority of hybrid individuals have retained alleles from both parents.

#### *Conclusions*

Previous phylogenetic studies have been unable to resolve the relationships among the four species in this soft coral clade. Although allozymes support the monophyly of each species, they are uninformative with respect to the basal relationships among the four species (McFadden 1999). A phylogenetic analysis of the predominant ribosomal DNA sequence types supports the separation of *A. coralloides* from the other three species, but is unable to resolve the relationship among *A. hibernicum*, *A. sp. M2* and *B. bocagei* (McFadden *et al.* 2001). Mitochondrial genes have proven to be too invariant to resolve intrageneric relationships in most soft corals, including the species in this clade (McFadden *et al.* in press; C.S. McFadden, unpublished data).

The present study of *ITS-1* polymorphisms suggests that the lack of phylogenetic resolution within this clade may be a consequence of past reticulate evolution. The additivity of sequence variants in *A. hibernicum* supports the hypothesis that this species arose by hybridization between *A. coralloides* and *A. sp. M2*. Recombinant sequence variants in *B. bocagei* may also be signatures of a hybridization event in this species' past; alternatively, they may be the by-products of an ancestral sequence polymorphism. Whichever explanation is correct, the consequence of the recombination event is phylogenetic uncertainty.

Single-copy nuclear genes are an additional source of information that may in future help to resolve the relationships, reticulate or otherwise, within this clade. Unfortunately, no appropriately variable nuclear genes have yet been identified for these taxa (C.S. McFadden, unpublished data). Moreover, if sufficient time has passed since hybridization for parental alleles to have been eliminated by selection or drift, as suggested by the allozyme data, most single-copy loci in the hybrid genome may no longer retain the additive signature of hybridization. In such cases, where the alleles

of one parent species are fixed at some loci, and those of the other parent are fixed at other loci, distinguishing reticulate evolution from shared ancestry may be impossible.

### Acknowledgements

We thank Brandon Hadland for preliminary cloning studies; Michael McMillin, Linda Prince and the Rancho Santa Ana Botanic Garden for DNA sequencing; J. Mark Porter for discussion of recombination tests; Nancy Hamlett for assistance with graphics; and Michael Hart and six anonymous reviewers for comments on the manuscript. This study was supported by grants to the Harvey Mudd College Biology Department from the Frank M. Parsons and Arthur Vining Davis Foundations.

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