Molecular Ecology (2004) **13**, 2763–2772 doi: 10.1111/j.1365-294X.2004.02265.x

Testing the utility of internally transcribed spacer sequences in coral phylogenetics

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Abstract

Reef-building corals often possess high levels of intraindividual and intraspecific ribosomal DNA (rDNA) variation that is largely polyphyletic between closely related species. Polyphyletic rDNA phylogenies coupled with high intraindividual rDNA variation have been taken as evidence of introgressive hybridization in corals. Interpreting the data is problematic because the rDNA cluster evolves in a complex fashion and polyphyletic lineages can be generated by a variety of processes — such as incomplete lineage sorting and slow concerted evolution — in addition to hybridization. Using the genetically characterized Caribbean *Acropora* hybridization system, we evaluate how well rDNA data perform in revealing patterns of recent introgressive hybridization in contrast to genetic data from four single-copy loci. While the rDNA data are broadly consistent with the unidirectional introgression seen in other loci, we show that the phylogenetic signature of recent introgressive hybridization is obscured in the Caribbean *Acropora* by ancient shared rDNA lineages that predate the divergence of the species.

Keywords: *Acropora*, concerted evolution, hybridization, reticulate evolution, ribosome

Received 22 March 2004; revision received 18 May 2004; accepted 18 May 2004

Introduction

A major focus of coral genetics has been to determine if mass-spawning corals are connected by a high degree of hybridization and genetic introgression (Wallace & Willis 1994; Veron 1995; Willis *et al*. 1997). Nuclear ribosomal DNA (rDNA) markers, spanning the internal transcribed spacer (ITS) regions and 5.8s, have been used most extensively to reconstruct phylogenetic relationships among corals (Hunter *et al*. 1997; Lopez & Knowlton 1997; Odorico & Miller 1997; Medina *et al*. 1999; van Oppen *et al*. 2000, 2002; Diekmann *et al*. 2001; Lam & Morton 2003; Marquez *et al*. 2003; Fukami *et al*. 2004) and to assess whether genetic data in potentially hybridizing corals are consistent with introgressive hybridization (Odorico & Miller 1997; van Oppen *et al*. 2000, 2002; Diekmann *et al*. 2001). Coral rDNA phylogenies are typically complex and polyphyletic among closely related congeners (*Acropora*, Odorico & Miller 1997; van Oppen *et al*. 2000, 2002; Marquez *et al*. 2003; *Montastrea*, Medina *et al*. 1999; Fukami *et al*. 2004; *Madracis*, Diekmann *et al*. 2001; but see *Platygyra*, Lam & Morton 2003), and individual coral colonies often harbour a high degree of intraindividual rDNA variation, both in the ITS regions and in 5.8s (*Acropora*, Odorico & Miller 1997; Van Oppen *et al*. 2000, 2002; Marquez *et al*. 2003; *Madracis*, Diekmann *et al*. 2001; *Plesiastrea*, Rodriguez-Lanetty & Hoegh-Guldberg 2002; *Montastrea*, Fukami *et al*. 2004). This combination of polyphyletic species lineages and intraindividual rDNA variation in corals has been interpreted as evidence of recent hybridization and introgression (Odorico & Miller 1997; van Oppen *et al*. 2000, 2002; Diekmann *et al*. 2001; Marquez *et al*. 2003; Miller & Van Oppen 2003).

However, attributing patterns in coral rDNA data to introgressive hybridization is problematic because of the diverse set of processes acting on nuclear ribosomal DNAs. First, nuclear rDNAs are a multigene family of tandem repeats that evolve via concerted evolution (Arnheim *et al*. 1980; Dover 1982). In most eukaryotes, the rate of concerted evolution is sufficient to homogenize the variation among rDNA repeats within individuals and species, but allow for the accumulation of differences between species (Hillis & Dixon 1991). However, if the rate of concerted evolution is slower than the rate of speciation, then ancestral rDNA lineages will be shared between species (Sanderson

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& Doyle 1992; Baldwin *et al*. 1995; Harris & Crandall 2000). Second, concerted evolution may not operate fully across rDNA arrays located in different nucleus organizer regions (Arnheim *et al*. 1980), generating fully or partially independent (i.e. paralogous) rDNA lineages (Baldwin *et al*. 1995). Third, rDNA phylogenies can include rDNA pseudogenes that have been silenced and are evolving independently from the rDNA cluster (Buckler *et al*. 1997). Divergent rDNA pseudogenes are quite common in plants (Buckler & Holtsford 1996; Buckler *et al*. 1997; Muir *et al*. 2001; Alvarez & Wendel 2003), and have been identified in corals (Marquez *et al*. 2003) and flatworms (Carranza *et al*. 1996). Finally, recombination among rDNA lineages can generate chimera sequences (Sanderson & Doyle 1992; van Oppen *et al*. 2002), even among rDNAs and their pseudogenes (Buckler *et al*. 1997).

Given these problems, it is not clear whether polyphyletic ITS data for corals provide an interpretable conclusion. This problem is most severe when ITS data provide the bulk of the genetic data for coral relationships because, in this case, the ITS patterns cannot be verified against independent data. Here we evaluate how well nuclear rDNA data perform in revealing patterns of recent introgressive hybridization in contrast to genetic data from four other loci for the Caribbean staghorn corals genus *Acropora*. Ribosomal DNA data from the ITS1–5.8s–ITS2 gene region and a *Pax*C intron provided the first evidence that the three sympatric species of Caribbean *Acropora* exchanged genes through hybridization (van Oppen *et al*. 2000). Polyphyletic rDNAs coupled with high intraspecific and intraindividual variation (up to 13% in ITS1; van Oppen *et al*. 2000) were taken as clear evidence of introgressive hybridization because of the unhomogenized rDNA variation within species and the lack of fixed differences between species separated by ≥ 6 million years and 325 000 generations (van Oppen *et al*. 2000; Miller & van Oppen 2003). Subsequent genetic analyses using data from the mitochondrial DNA (mtDNA) control region and three single-copy nuclear loci (MiniCollagen, Calmodulin, and *Pax*C) showed that three Caribbean *Acropora* comprise a natural hybridization system composed of two distinct species, *A. palmata* and *A. cervicornis*, that hybridize to form a first-generation (F_1) hybrid called *A. prolifera* (Fig. 1) (Vollmer & Palumbi 2002). Rare backcrossing of the hybrids with *A. cervicornis* then allows for the unidirectional introgression of *A. palmata* genes into *A. cervicornis* at three of the four assayed loci (mtDNA control region, *Pax*C and Calmodulin), but not at the MiniCollagen locus. These patterns of genetic exchange provide a background against which we can compare data from ITS. We show that ribosomal DNAs in the Caribbean *Acropora* fail to show a clear effect of recent introgressive hybridization because ancient shared rDNA lineages that predate the divergence of the species obscure phylogenetic inference.

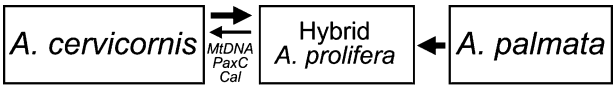

Fig. 1 Diagram of the Caribbean *Acropora* hybridization system revealed by multilocus genetic data (after Vollmer & Palumbi 2002). *Acropora palmata* hybridizes with *A. cervicornis* forming first-generation hybrids called *A. prolifera*. Hybrids backcross unidirectionally with *A. cervicornis* allowing *A. palmata* genes to introgress at three out of four genetic loci (mtDNA control region, a *Pax*C intron, and a Calmodulin intron).

Materials and methods

Nuclear rDNA sequences were obtained from 11 colonies of Caribbean *Acropora* including three *A. palmata*, six *A. cervicornis* and two F_1 hybrid *A. prolifera*. Each individual was genotyped at the mitochondrial control region and three nuclear intron loci (i.e. Mini-Collagen, Calmodulin, and *Pax*C). Based on these multilocus data, six colonies of *A. cervicornis* were chosen to reflect three different introgression histories (pure, nuclear introgressed and mitochondrial introgressed). Three *A. cervicornis* were from pure backgrounds (AcJa1, AcPRg4 and AcPRs6), two possessed at least one introgressed nuclear gene (AcPRm1 and AcPRg3), and one possessed an introgressed mitochondrion (AcJa4). Two distinct morphotypes of hybrid *A. prolifera* were also chosen — one palmate hybrid (AprPRs19) and one bushy hybrid (AprPRs8). Three Caribbean locations were represented: Puerto Rico (PR), Panama (Pa) and Jamaica (Ja).

DNA was extracted using a CTAB (hexadecyltrimethylammonium bromide) buffer, proteinase K (100 µg) and standard phenol–chloroform extraction methods. An approximately 700-base-pair (bp) region of ribosomal DNA containing a 130-bp fragment of 18s, ITS1 (*c*. 97 bp), 5.8s (163 bp), ITS2 (*c*. 122 bp) and a 194 bp fragment of 28s was amplified using published primers (Acf and Acr, van Oppen *et al*. 2000) and GeneAmp XL PCR kits (Perkin Elmer) under normal polymerase chain reaction (PCR) conditions, 35–38 cycles and annealing temperatures of 52 °C. Amplified PCR products were then cloned using pGEM-t cloning kits (Promega) and amplified with vector primers (T7 and M13R). Eight to 12 clones per individual were sequenced using the vector primers and ABI cycle sequencing chemistry. Direct sequencing of PCR products was also attempted for each individual.

Sequences were manually aligned to correspond with published alignments (Van Oppen *et al*. 2000). Phylogenetic analyses and genetic distance calculations were implemented in MEGA version 2.1 (Kumar *et al*. 2001). Independent phylogenetic analyses were performed for the combined rDNA gene fragments (18s, 5.8s and 28s) and each ITS region using uncorrected pairwise distances, and neighbour-joining searches. Gaps in the data because of insertions/

deletions (indels) and microsatellite regions primarily in ITS1/2 were treated as missing. However, coding gaps as separate evolutionary events did not have a significant effect on the resolution of the phylogenies (parsimony analyses not shown). Bootstrap support values were from 500 replicates. Genetic distance calculations were based on the pairwise treatment of sequences to account better for length variation as a result of indels and microsatellites.

Analyses of molecular variance (AMOVA) implemented in ARLEQUIN version 2.0 (Schneider *et al*. 2000) was used to estimate variance components within the hierarchical groups of *A. cervicornis*, *A. palmata* and *A. prolifera*. Variance components were estimated 'within individual', 'between individuals/within species' and 'between species' for the entire rDNA fragment and for each of the five gene regions (18s, ITS1, 5.8s, ITS2 and 28s). To assess the influence of introgression history on the signal of species divergence in rDNA, a second set of AMOVAs was run comparing each of the three categories of *A. cervicornis* (pure, nuclear introgressed, and mitochondrial introgressed) and the F_1 hybrid *A. prolifera* independently against *A. palmata*. In these four separate AMOVAs, the 'among-group' variance components served as a proxy of species divergence. In each AMOVA, gaps were coded conservatively as single evolutionary events (cost = 1).

Results

Within-individual rDNA variation

Eight to 12 clones of ribosomal DNA (rDNA) were sequenced per individual from 11 Caribbean *Acropora* (total $n = 107$ rDNA). The amplified rDNA fragment (up to 706 bp) contained partial fragments of 18s (130 bp) and 28s (194 bp) rDNA, all of 5.8s (162–163 bp), and the ITS regions ITS1 (80–97 bp) and ITS2 (97–122 bp). Length variation in ITS1 and -2 was the result of insertions/deletions (indels) and microsatellite repeat regions including $(ATCC)_n$ and $(GA)_n$ repeat regions in ITS1 and an $(A)_n$ repeat in ITS2, identified in Van Oppen *et al*. (2000). Every coral possessed multiple rDNA variants, and most possessed eight or more unique sequences out of the 8–12 sequenced clones. Sequence divergence among clones within individuals averaged 0.3–1.5% across the entire rDNA fragment (Table 1), which is far larger than expected for *Taq* cloning errors (*c*. 0.01%, Eckert & Kunkel 1990). Within-individual rDNA variation was commonly observed across all five of the ribosomal gene regions. The levels of within-individual variation were highest in the ITS regions, reaching up to 11.4% in ITS1 and 6.5% in ITS2. Among the three ribosomal genes, within-individual variation was highest in partial fragment of 18s rDNA (up to 3.8%).

Within-individual variation was highest in *A. cervicornis* (up to 2.7% overall, 11.4% in ITS1, and 3.8% in 18s) followed by hybrid *A. prolifera* (up to 1.7% overall, 8.3% in ITS1 and 3.1% in 18s) and then *A. palmata* (1.6%, 7.6%, and 0.8%, respectively). Average within-individual rDNA variation (Fig. 2) did not differ significantly between *A. cervicornis* and *A. palmata* (Mann–Whitney U, $P = 0.24$). Increased within-individual rDNA variation in *A. cervicornis* and hybrids is generally consistent with the expectation that the species receives added variation from introgressing *A. palmata* rDNAs. However, increased within-individual rDNA variation was not elevated in *A. cervicornis* individuals

Table 1 Ribosomal DNA variation in the Caribbean *Acropora*

Individual (History)		Clones (*n*)	Unique (*n*)	Overall average (range)	18s average (range)	ITS1 average (range)	5.8s average (range)	ITS2 average (range)	28s average (range)
Acropora palmata		22	16	0.8 (0–1.7)	0.1 (0–1.5)	3.5 (0–10.0)	0 (0–0.6)	1.5 (0–4.8)	0.2 (0–1.5)
palm_PRs2	pure	9	8	0.7 (0–1.0)	0.3 (0–0.8)	3.0 (0–5.4)	0.1 (0–0.6)	1.1 (0–4.8)	0
palm_PRa2	pure	11	10	0.8 (0–1.6)	0.1 (0–0.8)	3.2 (0–7.6)	0	2.3 (0–4.6)	0.1 (0–0.5)
palm_Pa1	pure	11	6	0.6 (0–1.3)	0	2.7 (0–7.5)	0	1.2 (0–3.7)	0.4 (0–1.5)
Acropora cervicornis		56	47	1.1 (0–3.0)	0.8 (0–5.4)	4.4 (0–13.2)	0.2 (0–1.2)	2.3 (0–6.8)	0.2 (0–1.5)
cerv_Ja1	pure	12	11	1.5 (0–2.7)	1.1 (0–3.1)	5.2 (0–10.2)	0	3.3 (0–6.5)	0.3 (0–1.0)
cerv_PRg4	pure	8	3	0.3 (0–1.2)	0	2.4 (0–9.7)	0	0	0.2 (0–0.5)
cerv_PRs6	pure	9	9	1.0 (0.1–2.1)	1.3 (0–3.8)	3.0 (0–8.3)	0.3 (0–1.2)	2.0 (0–3.9)	0.1 (0–0.5)
cerv_PRm1	nucDNA	8	8	0.7 (0.1–1.2)	0.7 (0–1.5)	0.9 (0–2.2)	0.3 (0–1.2)	2.3 (0–4.9)	0.3 (0–1.0)
cerv_PRg3	nucDNA	10	8	1.2 (0–2.3)	0.6 (0–1.5)	5.9 (0–10.5)	0.3 (0–1.2)	1.5 (0–4.7)	0.2 (0–1.0)
cerv_Ja4	mtDNA	9	8	0.9 (0–2.2)	0.3 (0–0.8)	3.4 (0–11.4)	0	2.3 (0–5.1)	0
Acropora prolifera		20	12	1.2 (0–2.2)	0.9 (0–3.1)	4.7 (0–9.7)	0.2 (0–1.2)	2.1 (0–5.6)	0.4 (0–1.5)
prol_PRs8	hybrid	10	7	0.9 (0–1.7)	1.1 (0–3.1)	1.3 (0–3.3)	0.2 (0–1.2)	2.0 (0–4.9)	0.4 (0–1.0)
prol_PRs19	hybrid	10	5	0.9 (0–1.7)	0.2 (0–0.8)	3.8 (0–8.3)	0.2 (0–0.6)	2.0 (0–4.7)	0.2 (0–0.5)
Overall		107	71	1.2 (0–3.0)	1.0 (0–5.4)	4.5 (0–13.2)	0.1 (0–1.2)	2.4 (0–7.4)	0.2 (0–2.1)

Average pairwise sequence divergence (%) among clones showing levels of rDNA variation observed within individual corals, within the species as a whole, and between the species.

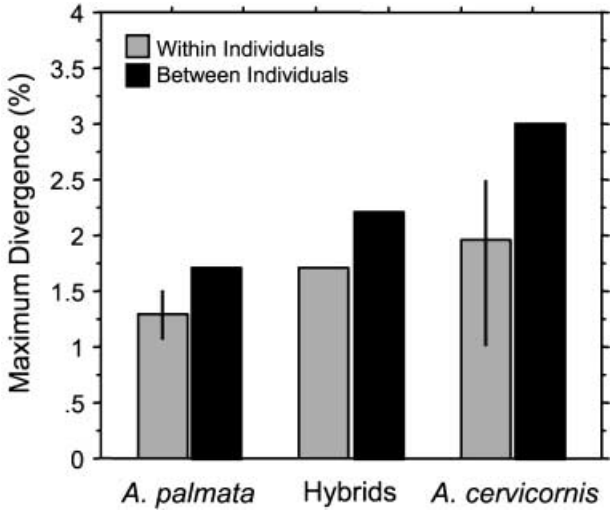

Fig. 2 Maximum rDNA sequence divergence (%) observed between individuals within each species compared to rDNA variation observed among clones within individual coral colonies. Within-individual sequence divergences presented as the average (range).

with introgressed backgrounds. Instead, rDNA variation was comparable between pure and introgressed individuals, and the highest within-individual variation overall was observed in an *A. cervicornis* individual with no apparent introgression history (cerv_Ja1 at 2.7%).

rDNA variation within and between species

Maximum sequence divergence within the Caribbean *Acropora* measured 3% overall, and 13.2% in ITS1, and 5.4% in 18s (Table 1). As observed for the within-individual rDNA variation, intraspecific rDNA variation was highest in *A. cervicornis* (3%, 13.2% and 5.4%, respectively), intermediate in hybrid *A. prolifera* (2.2%, 9.7%, and 3.1%, respectively), and lowest in *A. palmata* (1.7%, 10%, and 1.5%, respectively). Comparisons of intraspecific variation to the within-individual variation (Fig. 2) shows that the levels of rDNA variation observed within species are only slightly greater than the rDNA variation found within individual coral colonies. In ITS1, for example, rDNA variation within individual coral colonies encapsulated up to 86% of the variation in *A. cervicornis* and 76% of the variation in *A. palmata*.

rDNA phylogenies

Independent phylogenies for the combined rDNA genes (18s, 5.8s and 28s), ITS1, and ITS2 were polyphyletic with few well-supported nodes (Fig. 3). High within-individual variation observed in both species contributed to the phylogenies being complex and polyphyletic because rDNA variants from individuals are spread across all three trees. Despite the complexities of the trees, it was notable that species-specific clusters of *A. cervicornis* and *A. palmata* rDNA variants occurred on each phylogeny. Species-specific clusters in *A. palmata* rDNAs are most surprising given that recent hybridization and introgression should pass *A. palmata* rDNAs to *A. cervicornis*. As expected, rDNA variants from hybrid *A. prolifera* were well distributed across the phylogenies, including within the species-specific rDNA clusters. Shared rDNA variants between *A. cervicornis* and *A. palmata* were common. One shared variant occurred on the rDNA phylogeny, four were shared at ITS1, and two were shared at ITS2. These shared rDNA variants were relatively equally distributed among *A. cervicornis* individuals from the three introgression histories (pure, nuclear introgressed and mitochondrial introgressed). For example, out of the seven shared rDNA variants, three were found in *A. cervicornis* individuals from the three different backgrounds, three variants were found in individuals with nuclear introgressed backgrounds, and one was found in an individual with a pure background.

Partitioning of genetic variation

AMOVA were conducted to assess how much rDNA variation is attributed to 'within-individual', 'between-individual/within-species' and 'between-species' variation (Table 2). Ribosomal DNA variation found 'within-individual' coral colonies accounted for the majority of rDNA variation in the data (67.8% overall), especially in the highly variable ITS regions (81.9% for ITS1 and 80.1% for ITS2). However, the AMOVA results also show that a significant fraction of rDNA variation (15.4%) partitions 'between species'. Thus, despite polyphyletic rDNA data and high within-individual variation, there exists a strong species-specific signal in the data as a result of the species-specific rDNA clusters and frequency differences in shared related alleles. Importantly, the amount of 'between species' variation differed by gene region with the strongest species-specific signal occurring in the 18s rDNA (35.4%) and the poorest signal occurring in both ITS regions (1.2% and 9.8% for ITS1 and ITS2, respectively).

Independent AMOVAs comparing *A. palmata* to the three different introgression classes of *A. cervicornis* (pure, nuclear and mitochondrial introgressed) and the hybrid *A. prolifera* failed to show a strong signature of introgression in *A. cervicornis* individuals with introgressed backgrounds (Fig. 4). Instead, the 'among-group' variation was comparable among *A. cervicornis* individuals with pure (29.59%), nuclear introgressed (24.97%) and mitochondrial introgressed (32.26%) backgrounds. There was however, a substantial reduction in the 'among-group' variation in hybrid *A. prolifera* (9.94%), indicating that the hybrids were genetically intermediate between the species.

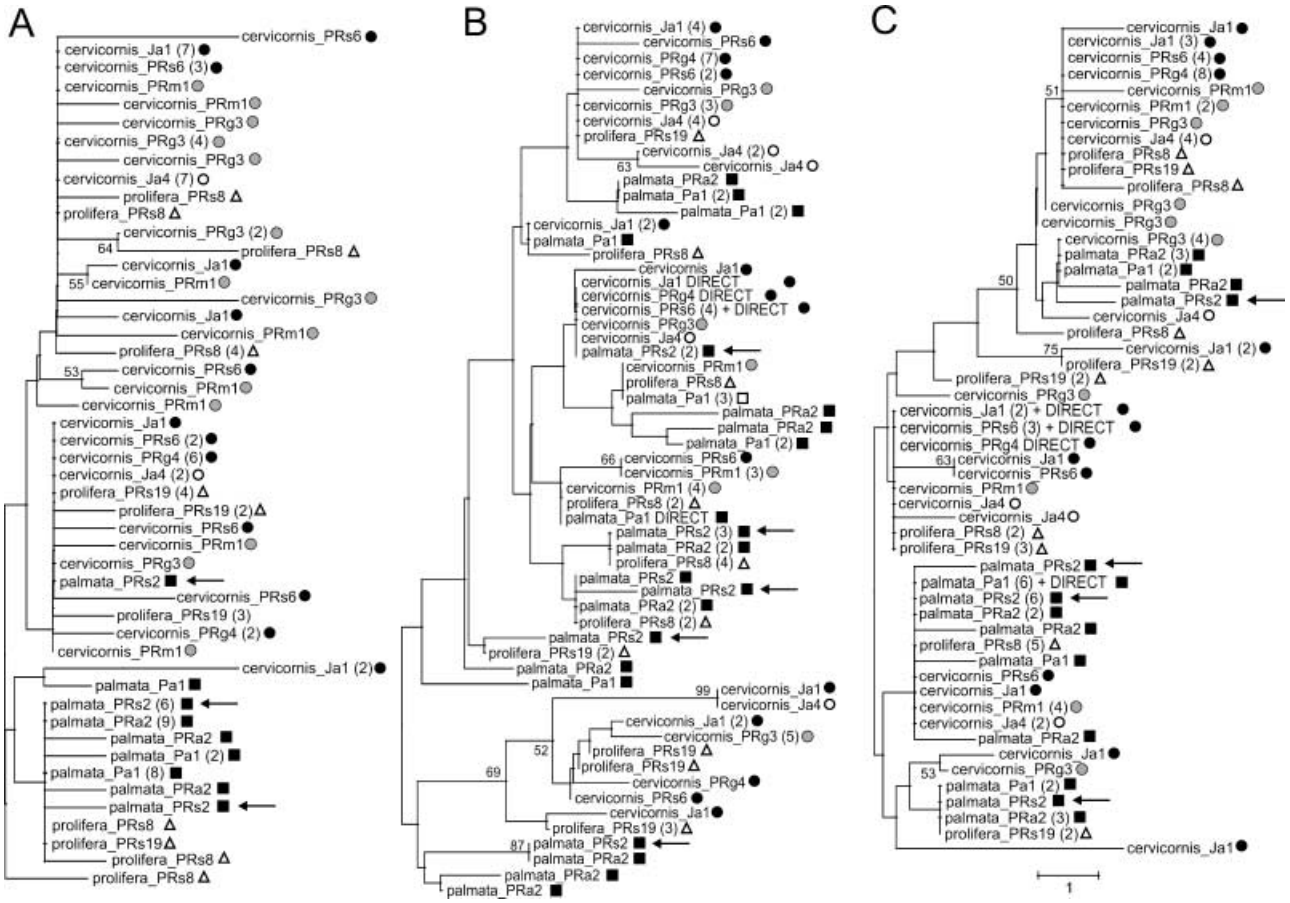

Fig. 3 Neighbour-joining trees for the rDNA gene region (a), ITS1 (b), ITS2 (c) based on the number of substitutions. *Acropora cervicornis* genotypes are labelled by introgression class: pure (●), nuclear introgressed (◍), and mitochondrial introgressed (○). *Acropora palmata* genotypes are labelled as ■ and full hybrid *A. prolifera* as △. Individuals are identified by a _ followed by a location designation (PR = Puerto Rico, Pa = Panama, and Ja = Jamaica) and individual ID. Samples sizes per individual are indicated in parentheses. Bootstrap support values (500 replicates) 50% or greater are labelled on relevant nodes. Arrows show the level of intraindividual variation in the *A. palmata* individual 'palmata_PRs2'. Sequences available on GenBank (accession numbers AY676202–AY676308).

Table 2 Analysis of molecular variance (AMOVA) comparison of variation 'within individual', 'between individuals/within species', and 'between species'

	Within individual				Between individual/ within species				Between species				
Locus	SSD	d.f.	Vc	%	SSD	d.f.	Vb	%	SSD	d.f.	Va	%	*P*
rDNA	312.057	96	3.251	67.81	87.946	8	0.804	16.77	70.642	2	0.739	15.41	**
18s	21.167	96	0.220	49.13	7.907	8	0.080	17.77	11.692	2	0.149	33.10	**
ITS1	38.319	96	0.399	81.87	9.336	8	0.080	16.35	2.962	2	0.009	1.78	**
5.8s	9.781	96	0.102	95.56	1.119	8	0.004	3.70	0.334	2	0.001	0.73	ns
ITS2	36.715	96	0.382	80.07	6.789	8	0.048	10.13	4.776	2	0.047	9.79	**
28s	19.802	96	0.206	88.66	2.860	8	0.016	6.75	1.422	2	0.011	4.60	*

Hierarchical groupings are by individual and by the species' groupings: *A. cervicornis*, *A. palmata*, and first-generation hybrid *A. prolifera*. AMOVAs across the entire rDNA fragment (rDNA) and for each of the five loci.
*$P < 0.05$; **$P < 0.001$; ns = not significant.

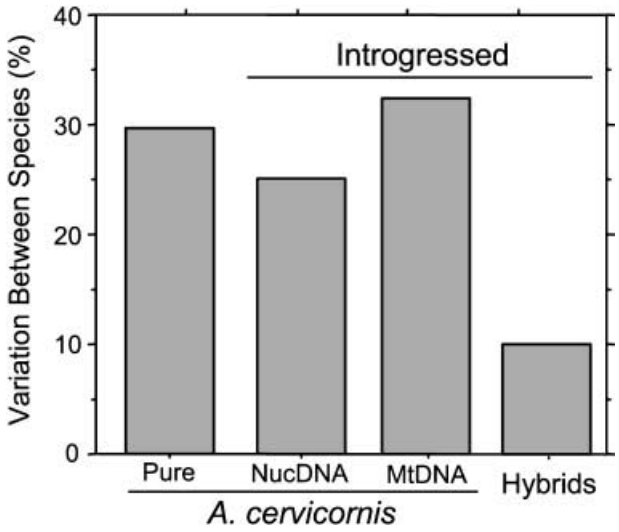

Fig. 4 Proportion of the variation explained 'among groups' between the three *Acropora cervicornis* introgression classes (pure, nuclear introgressed and mitochondrial introgressed) and first-generation (F_1) hybrid *A. prolifera* compared with *A. palmata* in independent analyses of molecular variance (AMOVA).

Direct sequencing of rDNA variation

Direct sequencing of PCR products has become a common method of screening rDNA variation in corals based on the assumption that successful sequencing indicates a lack of within-individual rDNA variation (Hunter *et al*. 1997; Takabayashi *et al*. 1998a,b, 2003; Medina *et al*. 1999). To test this assumption, we attempted to sequence PCR products directly from all 11 Caribbean *Acropora*. Direct sequences across ITS1–5.8s–ITS2 were obtained from four out of the 11 corals (cerv_Ja1, cerv_PRs6, cerv_PRg4 and palm_Pa1), even though cloning and sequencing revealed that each individual possessed multiple rDNA variants and length polymorphisms in their ITS regions, which should have caused the sequences to become unreadable. Direct sequencing for the remaining seven corals failed at the $(ATCC)_n$ length polymorphism in ITS1. The four successfully sequenced samples are shown on the ITS phylogenies (Fig. 3 in bold) and captured only a small subset of the actual rDNA diversity revealed by our cloning and sequencing efforts.

Discussion

Phylogenetic signal

Added insights gained from single-copy nuclear and mitochondrial genes allowed us to ask if the rDNA are consistent with the expectations of unidirectional introgression in the Caribbean *Acropora* hybridization system (Vollmer & Palumbi 2002). While far from clear, we did find that the rDNA data are broadly consistent with three expectations. First, we found elevated levels of intraindividual and intraspecific rDNA variation in *A. cervicornis* that could result from input of introgressed rDNA variation from its hybrid partner *A. palmata*. Second, both the phylogenies and AMOVAs showed that the hybrid *A. prolifera* are a mix of the parent species. Third, AMOVA results showing that the species are genetically distinct are consistent with rare introgression in the hybridization system (Vollmer & Palumbi 2002) because high rates of introgression would have swamped this signal. However, other expectations were not met. For example, there was no clear evidence of recent introgressive hybridization in the rDNA data in *A. cervicornis* individuals from introgressed nuclear or mitochondrial backgrounds. The rDNA phylogenies failed to show distinct clusters of native *A. cervicornis* alleles that were characteristic of unidirectional introgression on the single-copy gene phylogenies (Vollmer & Palumbi 2002). Unlike the Bayesian coalescent approaches that can be applied for single-copy genes (Nielsen & Wakeley 2001; Vollmer & Palumbi 2002), no framework exists to evaluate whether the shared variation among rDNA repeats evolving via concerted evolution is the result of shared ancestral variation (i.e. incomplete lineage sorting) or introgressive hybridization.

Further analysis of our data suggests that a large portion of the intraindividual and intraspecific rDNA variation represents ancestral variation that has not been homogenized by concerted evolution. Because introgression is unidirectional from *A. palmata* to *A. cervicornis*, high levels of intraindividual and intraspecific rDNA variation in *A. palmata* (up to 7.6% and 10% in ITS1, respectively) can be attributed to unhomogenized ancestral rDNA variation, and not to introgressed rDNA variation. This demonstrates that the rate of convergent evolution has not been sufficient to homogenize rDNA variation in *A. palmata*, and calls into question the assumption that the lack of homogenization in coral rDNA variation results from introgressive hybridization (Odorico & Miller 1997; van Oppen *et al*. 2000, 2002; Diekmann *et al*. 2001). It is difficult to make similar inferences about levels of intraindividual and intraspecific rDNA diversity in *A. cervicornis* (up to 11.4% and 13.2% in ITS1, respectively) because it receives introgressed genes from *A. palmata*. Some fraction of the rDNA variation in *A. cervicornis* probably represents unhomogenized, ancestral variation. Native sequence diversity in the mtDNA control region is identical for both species (0.5%), but is doubled by mtDNA introgression in *A. cervicornis* (Vollmer & Palumbi 2002). By comparison, intraindividual and intraspecific rDNA diversity in *A. cervicornis* was only one-third and one-quarter higher than in *A. palmata*. Shared ancestral rDNA variation is one explanation for why the levels of rDNA variation are not higher in *A. cervicornis*.

Ancient rDNA lineages

Comparisons of sequence divergence rates between the rDNAs and single-copy nuclear genes indicate that the Caribbean *Acropora* rDNA lineages are quite ancient and predate the split of the species. Sequence divergences between the species' lineages in intron regions of the three single-copy nuclear genes — MiniCollagen, Calmodulin and *Pax*C — ranged between 0.6% and 2.1% (calculated from Vollmer & Palumbi 2002). By comparison, sequence divergence at ITS1 (up to 13.2%) is at least six times higher. This suggests that the most divergent ITS lineages in the Caribbean *Acropora* are much older than the species, and predate their divergence over 6.6 million years ago (Budd *et al*. 1994). Because these shared ancient rDNA lineages predate the species divergence, it is not possible to make phylogenetic inferences about recent genetic exchange via hybridization in the Caribbean *Acropora*.

Shared rDNA lineages among species can be generated by a variety of processes – incomplete concerted evolution, pseudogenes, hybridization and polyploidization – which are not mutually exclusive (reviewed in Alvarez & Wendel 2003). Slow rates of concerted evolution can cause shared rDNA lineages to persist among species, within populations and within individuals (e.g. Vogler & Desalle 1994; Harris & Crandall 2000; Duran *et al*. 2004). This is probably the case for the shared rDNA variation in the Caribbean *Acropora*. Recent evidence has also shown that ancient pseudogenes contribute to the unprecedented rDNA diversity in the Pacific *Acropora* (Marquez *et al*. 2003). Divergent paralogous rDNA lineages are also common in many plant lineages (Buckler *et al*. 1997). In one case, the additivity of divergent parental rDNA lineages has been used to infer the hybrid origins of plant species (*Paeonia*, Sang *et al*. 1995). It is more common that divergent rDNA lineages predate speciation events and cannot be used to infer patterns of recent introgressive hybridization (Buckler *et al*. 1997; Muir *et al*. 2001; Alvarez & Wendel 2003). While corals are similar to plants in this respect, the levels of intraspecific and intraindividual rDNA variation corals appears to be without precedent (Odorico & Miller 1997; Marquez *et al*. 2003).

Utility of coral rDNA markers

Even though nuclear rDNA markers have provided some of the first genetic data for reef-building corals, these markers have often proved to be phylogenetically uninformative at the species- and population-levels because of high levels of intraindividual and intraspecific variation (summarized in Table 3). For example, out of six species-level comparisons, polyphyletic species lineages were common among closely related coral congeners in five comparisons (Table 3). Although these polyphyletic lineages have largely been attributed to introgressive hybridization, subsequent genetic data from single-copy mitochondrial and nuclear genes often show a clearer picture of species relationships. Here we have shown that rDNA data fail to show the clear pattern of unidirectional introgression in the Caribbean *Acropora* because ancient shared rDNA lineages predate the species divergence. In the *Montastrea annularis* species complex (*sensu* Knowlton *et al*. 1992), a mitochondrial intron and amplified fragment length polymorphism loci clearly distinguish *M. faveolata* from its close congeners (Fukami *et al*. 2004) whereas rDNA data were polyphyletic and equivocal (Lopez & Knowlton 1997; Medina *et al*. 1999; Fukami *et al*. 2004). In contrast, genetic data from mtDNA control region and a *Pax*C intron from the Pacific *Acropora* are often congruent with rDNA phylogenies (van Oppen *et al*. 2001, 2002; Marquez *et al*. 2003). There is one example in which rDNA data preformed better than single-copy nuclear data. Recent rDNA work shows that *Platygyra sinensis* and *P. pini* are genetically distinct (i.e. reciprocally monophyletic) at ITS1–5.8s–ITS2 (Lam & Morton 2003), even though previous allozyme data failed to show clear differences between the species (Miller & Benzie 1997).

Adequate genetic sampling has also been a problem in the coral rDNA literature (Table 3). In particular, intraindividual rDNA variation in corals requires that the variation within coral colonies be adequately sampled by cloning PCR products and sequencing multiple clones (e.g. 5–10 clones per colony), adding an expensive and time-consuming step. However, even though intraindividual rDNA variation has been detected in each of the five coral genera in which it has been assayed using cloning and sequencing (Table 3), most coral rDNA variation data have been assayed by directly sequencing amplified PCR products, under the assumption that concerted evolution has homogenized rDNA variation within a single coral individual to a single sequence type (Hunter *et al*. 1997; Lopez & Knowlton 1997; Takabayashi *et al*. 1998a, 1998b, 2003; Medina *et al*. 1999). Our direct sequencing results show that this assumption is incorrect. We were able to sequence ITS1–5.8s–ITS2 rDNA directly from both Caribbean *Acropora* species, even though the sequenced individuals had high intraindividual variation that included multiple-length polymorphisms in ITS1/2. We have also been able to directly sequence ITS1–5.8s–ITS2 from the Pacific staghorn *Acropora valida* (Vollmer & Palumbi, unpublished data), which has some of the highest reported intraindividual variation (up to 29% in ITS1 *et al*. 1997). It is unclear why we were able to obtain direct sequences from staghorn corals with high intraindividual variation. It could simply be the differential amplification of rDNA repeats because of PCR artefacts, or that we amplified a common rDNA repeat. In *A. valida*, it is possible that the species identifications and/or levels of rDNA variation differed between Guam (here) and the previous study from the Great Barrier Reef (Odorico & Miller 1997). More importantly, these results suggest that intraindividual

Table 3 Summary of reef-building coral nuclear rDNA datasets spanning ITS1–5.8s–ITS2

Taxon	Corals	Clones	Intra-specific	Intra-individual	Conclusions	Ref.
Acropora						
A. cervicornis	14	(3–6)	Yes	Yes	Polyphyletic lineages due to introgressive hybridization	1
A. palmata	13	(3–6)	Yes	Yes		
A. prolifera	9	(3–6)	Yes	Yes		
A. hyacinthus	2	2	6.0%	6.0%	Introgressive hybridization between *A. cytherea* and *A. hyacinthus*	2
A. cytherea	1	4	5.0%	5.0%		
A. valida	1	3	29.0%	29.0%		
A. formosa	1	3	16.0%	16.0%		
A. longicyathus	1	4	10.0%	10.0%		
A. aspera	10	Yes	23.6%*	Yes	Broad polyphyly due to introgressive hybridization, both recent and ancient. Evidence of putative F_1 hybrids	3
A. millepora	15	Yes	21.1%*	Yes		
A. papillare	6	Yes	16.2%*	Yes		
A. pulchra	12	Yes	22.9%*	Yes		
A. spathulata	16	Yes	21.7%*	Yes		
10 Pacific + 3 Caribbean species	73	Yes	Yes	Yes	5.8s pseudogenes in Pacific *Acropora*	4
Madracis						
M. mirabilis	4	(1–5)	Yes	0.0%	Introgressive hybridization between *M. decactis–formosa–pharensis*	5
M. decactis	8	(1–5)	Yes	2.4%		
M. formosa	7	(1–4)	Yes	2.2%		
M. senaria	6	(1–4)	Yes	3.7%		
M. pharensis	7	(1–5)	Yes	4.9%		
Montastrea						
M. annularis	(9–10)	(5–10)	1.7%	Yes[8]	Polyphyletic rDNAs do not resolve species relationships	6,7,8
M. franksi	(6–10)	(5–10)	0.9%	Yes[8]		
M. faveolata	(7–10)	(5–10)	1.1%	Yes[8]		
Platygyra						
P. sinensis	37	No	c.1%	No	Genetically distinct species; Reciprocally monophyletic lineages	9
P. pini	6	No	c.2.5%	No		
Plesiastrea versipora	49	4	Yes	*c*. 1%	Broad geographical structure	10
Stylophora pistillata	122	No	31.0%	?	No geographical structure	11
Seriatopora hystrix	3	No	?	?	Phylogenetically variable	12
Goniopora tenuidens	3	No	15.0%	?		
Porites lobata	3	No	?	?		
Heliofungia actiniformis	3	No	2.0%	?		
Porites						
P. compressa	1	No	?	?	Phylogenetically informative	13
P. lobata	2	No	Yes	?		
P. evermanni	2	No	Yes	?		
P. astreoides	1	No	?	?		
P. porites	1	No	?	?		

Sequence divergences (%) at ITS1; Ψ denotes divergence across ITS1–5.8s–ITS2

1. Van Oppen *et al*. (2000), 2. Odorico & Miller (1997), 3. Van Oppen *et al*. (2002), 4. Marquez *et al*. (2003), 5. Diekmann *et al*. (2001), 6. Lopez & Knowlton (1997), 7. Medina *et al*. (1999), 8. Fukami *et al*. (2004), 9. Lam & Morton (2003), 10. Rodriguez-Lanetty & Hoegh-Guldberg (2002), 11. Takabayashi *et al*. (2003), 12. Takabayashi *et al*. (1998b), 13. Hunter *et al*. (1997).

rDNA variation may have been overlooked in a large number of coral taxa. At the very least, our results demonstrate that future coral rDNA work must first check for underlying intraindividual rDNA variation before employing direct sequencing.

Conclusion

Multiple lines of evidence demonstrate that nuclear ribosomal DNAs perform poorly as a species- and population-level phylogenetic marker in reef-building corals. First, high intraindividual rDNA variation in corals can overlap with the within- and between-species rDNA variation, and obscure the phylogenetic signal in the data. Second, shared rDNA variation cannot be attributed to either ancestral variation or introgression because a framework does not exist to test these competing hypotheses. Third, even in a well-characterized coral hybridization system like the Caribbean *Acropora* (Vollmer & Palumbi 2002), a discernible signature of introgression could not be detected because the shared rDNA variation is ancient and predates the species divergence. Therefore, we suggest that multilocus genetic data from mitochondrial and nuclear genes (e.g. Wang *et al*. 1995; van Oppen *et al*. 1999, 2000; Vollmer & Palumbi 2002; Fukami *et al*. 2004; Mackenzie *et al*. 2004), which are better able to reconstruct the species-level phylogenetic relationships in corals (Fukami *et al*. 2004) and characterize coral hybridization systems (Vollmer & Palumbi 2002), should be favoured over nuclear ribosomal DNA markers in corals.

Acknowledgements

Many thanks to those who assisted with field collections including H. Ruiz, E. Weil, C. Zilberberg and S. Genovese, and to S. Belliveau and two anonymous reviews for valuable comments on the manuscript. Research was supported by National Science Foundation grants to S.R.P. and a National Institutes of Health Genetics Training Grant Fellowship to S.V.

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