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# Genetic evidence for a protozoan (phylum Apicomplexa) associated with corals of the *Montastraea annularis* species complex

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## Introduction

Scleractinian corals associate with diverse microbial communities (e.g., Rowan 1998; Rohwer et al. 2001). Some eukaryotic microbes are common, well-known associates of corals; notably dinoflagellates of the genus *Symbiodinium* (e.g., Trench 1987), endolithic algae (e.g., Lukas 1974), and fungi (e.g., Bentis et al. 2000). Other eukaryotic associates have not received detailed study. Descriptions of these normal associations are a necessary first step towards identifying abnormal microbial associations (i.e., pathogens) which lead to coral disease (Richardson 1998).

Methods for molecular genetic analysis have revolutionized the study of marine microbes (e.g., Brinkmeyer et al. 2000) and have proven useful in the study of coral microbial communities (e.g., Rowan 1998; Rohwer et al. 2001). Here we give a brief account of a coral-associated eukaryote which was identified by molecular methods during a bleaching experiment with the Caribbean coral *Montastraea annularis* (Toller et al. 2001a). This symbiont, provisionally named genotype N, was identified during routine screening of *Symbiodinium* samples by restriction fragment length polymorphism (RFLP) analysis of small subunit ribosomal RNA genes (srDNA). Our data indicate that genotype N represents a coccidian protozoan (phylum Apicomplexa) which commonly associates with corals of the *Montastraea annularis* species complex.

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## Methods

Genotype N was identified by an RFLP that was distinct from those of *Symbiodinium* taxa known to associate with *M. annularis* (Fig. 1a). Here, we report only those methods specific to the identification and characterization of genotype N; methods for routine RFLP analysis of *Symbiodinium* srDNA, as well as methods for experimental manipulation of corals, are given elsewhere (Toller et al. 2001a, 2001b).

Initially, the srDNA of genotype N was detected following polymerase chain reaction (PCR) with a "host-excluding" primer combination (Rowan and Powers 1991): the primers were ss5 (a universal primer) and ss3Z (a primer designed to exclude cnidarian srDNA). We first observed genotype N in samples where *Symbiodinium* cell numbers were extremely low ($< 0.4 \times 10^5$ cells/cm$^2$) following experimental bleaching of host corals (~100-fold reduction in *Symbiodinium* numbers relative to controls). These samples yielded little srDNA when PCR-amplified in the usual (Toller et al. 2001b) manner. To obtain sufficient srDNA, we used two rounds of amplification as follows: srDNA was amplified from samples with host-excluding primers for 34 PCR cycles. Aliquots (10 µl) of those amplifications were electrophoresed on agarose gels (1.0% Nuseive GTG; FMC BioProducts, Rockland, ME), and faint bands of srDNA were excised and added to 100 µl water. The gel-purified srDNA was heated to 65 °C for 2 min, and then 1 µl of each was PCR-amplified with host-excluding primers in the same manner. The resulting re-amplified srDNA was then analyzed by RFLP as described elsewhere (Toller et al. 2001b).

To further characterize genotype N, srDNA was cloned and sequenced, as described previously (Toller et al. 2001b). Samples came from three different colonies of *M. annularis* in experiment I of Toller et al. (2001a). srDNA was amplified with universal PCR primers (clone $N^{0-1}$) and with host-excluding primers (clones $N^{0-2}$ and $N^{0-3}$). For each sample, a minimum of 12 recombinants was examined by RFLP with *Dpn* II and with *Taq* I. DNA sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/) under accession numbers AF238264 ($N^{0-1}$), AF238265 ($N^{0-2}$), and AF238266 ($N^{0-3}$).

srDNA sequences from genotype N, *Symbiodinium A*, *B*, *C*, and *E*, and the coral *M. annularis* were used to design "N-specific" PCR primers that amplify only srDNA of genotype N from samples that also contained coral and zooxanthellar srDNAs: The 5′ ends of primer 18N-F2 (5′-TAGGAATCTAAACCTCTTCCA-3′; forward primer) and of primer 18N-R1 (5′-CAGGAACAAGGGTTCCCGACC-3′;

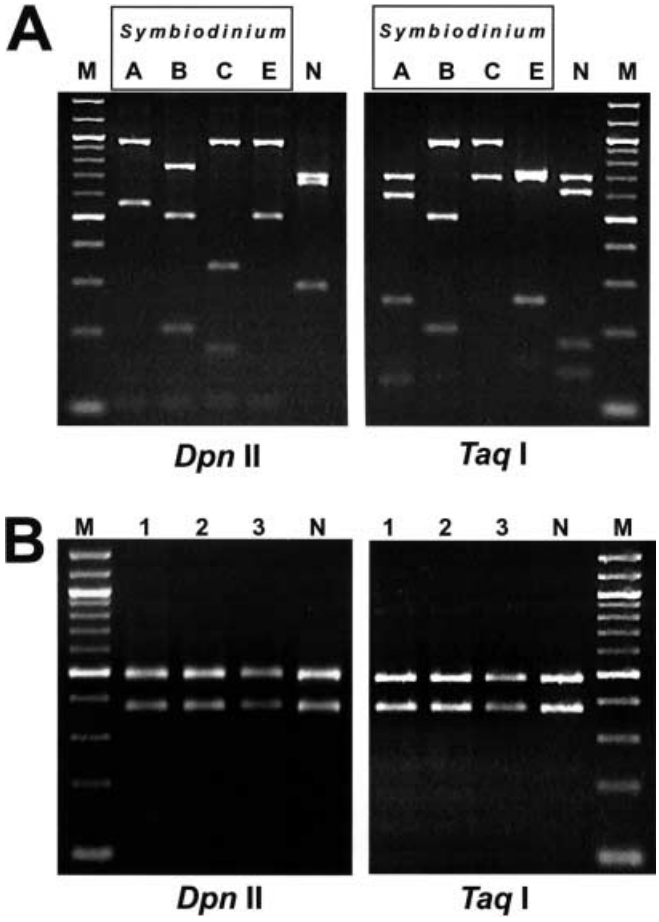


**Fig. 1. a** RFLP analysis of srDNA from genotype N and *Symbiodinium*. srDNA was amplified with host-excluding PCR primers from a coral sample (N) and from different srDNA clones representing *Symbiodinium A* (*A*), *Symbiodinium B* (*B*), *Symbiodinium C* (*C*), and *Symbiodinium E* (*E*). srDNA was digested with *Dpn* II (*left*) or *Taq* I (*right*). The sample containing RFLP genotype N was taken from *Montastraea annularis* following experimental bleaching (see text). *Lanes* marked *M* contain size standards of (from *top* to *bottom*) 1,500 bp, 1,200 bp, and then 1,000 bp to 100 bp in 100-bp increments. **b** RFLP analysis of srDNA obtained by PCR with N-specific primers. srDNA was amplified from coral samples (*lanes 1–3*) and an srDNA clone ($N^{0-1}$) and digested with *Dpn* II (*left*) or *Taq* I (*right*). Samples came from three different colonies of *M. annularis*. Size markers *(M)* are as above

reverse primer) correspond to nucleotides 515 and 1374 (respectively) in clone $N^{0-1}$. These primers were used in PCR amplifications as before, except that DNA synthesis steps were reduced to 1 min. PCR products were analyzed by RFLP with *Dpn* II and with *Taq* I.

For phylogenetic analysis, we aligned srDNA sequences with Clustal X software (Thompson et al. 1997) and used neighbor-joining reconstruction (Saitou and Nei 1987) with 1,000 bootstrap replications. We included srDNA sequences from representative alveolate taxa (apicomplexans, dinoflagellates, ciliates) which were used previously in similar phylogenetic analyses (McNally et al. 1994; Escalante and Ayala 1995).

## Results and discussion

Our initial observations of genotype N in experimentally bleached corals (see Methods) suggested that N might normally be present at undetectable levels, and "revealed" when *Symbiodinium* numbers fall. The design of that experiment enabled us to return to archived samples taken from the same corals, but prior to bleaching (i.e., prior to reduction of *Symbiodinium* numbers). Re-analysis of these samples with N-specific PCR primers confirmed that genotype N was present (in all cases) prior to experimental treatment, when *Symbiodinium* were abundant. Using the same method, we then screened samples from other corals. Approximately 90% of samples taken from apparently healthy, unmanipulated colonies of *M. annularis* (45 colonies) and *M. faveolata* (7 colonies) tested positive for genotype N, yielding a PCR product of ca. 860 bp which, by digestion with *Dpn* II and with *Taq* I, was indistinguishable from the PCR product obtained from clone $N^{0-1}$ (Fig. 1b). These data indicate that genotype N commonly associates with *M. annularis* and *M. faveolata*, and that its presence was previously masked in our analyses by the much greater abundance of *Symbiodinium*.

Sequence data confirm that the srDNA of genotype N is quite divergent (12–17%) from those of *Symbiodinium A*, *B*, *C*, and *E* – the zooxanthellae known to associate with *M. annularis* (see Toller et al. 2001a, 2001b). Sequence comparisons also explain why genotype N was amplified with host-excluding PCR primers – the primer ss3Z has an extensive match to the srDNA of genotype N (clone $N^{0-1}$; not shown; see also McNally et al. 1994). We performed experiments with mixtures of srDNA clones from genotype N (clone $N^{0-1}$) and *Symbiodinium C* (clone $C^0$; Toller et al. 2001b) and confirmed that our host-excluding protocol amplifies both srDNA with approximately equal efficiency (not shown). Thus, our method apparently detects both genotype N and *Symbiodinium* in proportion to the relative abundance of their srDNA.

Phylogenetic analysis (Fig. 2) indicates that genotype N is a member of the protozoan phylum Apicomplexa, a sister group to the dinoflagellates (Gajadhar et al. 1991; McNally et al. 1994). As far as is known, all apicomplexans are animal parasites (Levine 1982). Some, for example the apicomplexans *Plasmodium* and *Toxoplasma*, are notorious pathogens in humans and domestic animals. In our analysis, genotype N shared greatest similarity to *Toxoplasma*, *Neospora*, *Isospora*, and *Sarcocystis*, representative taxa from the apicomplexan class Coccidia (Levine 1982). From these data, we conclude that genotype N represents a coccidian protozoan that associates with corals.

Our three sequences from genotype N (from three different colonies of *M. annularis*) suggest that considerable srDNA nucleotide variation (1.0–1.7%) exists between samples obtained from different colonies. These srDNA sequences differ as much as comparisons between different species of coccidians (e.g., srDNA from *Toxoplasma gondii* and *Neospora caninum* differ by only 0.4%). However, srDNA heterogeneity is common in Apicomplexa in general (e.g., Li et al. 1997), and the accuracy and/or taxonomic significance of individual sequences may be suspect in such mixtures (see Wintz-

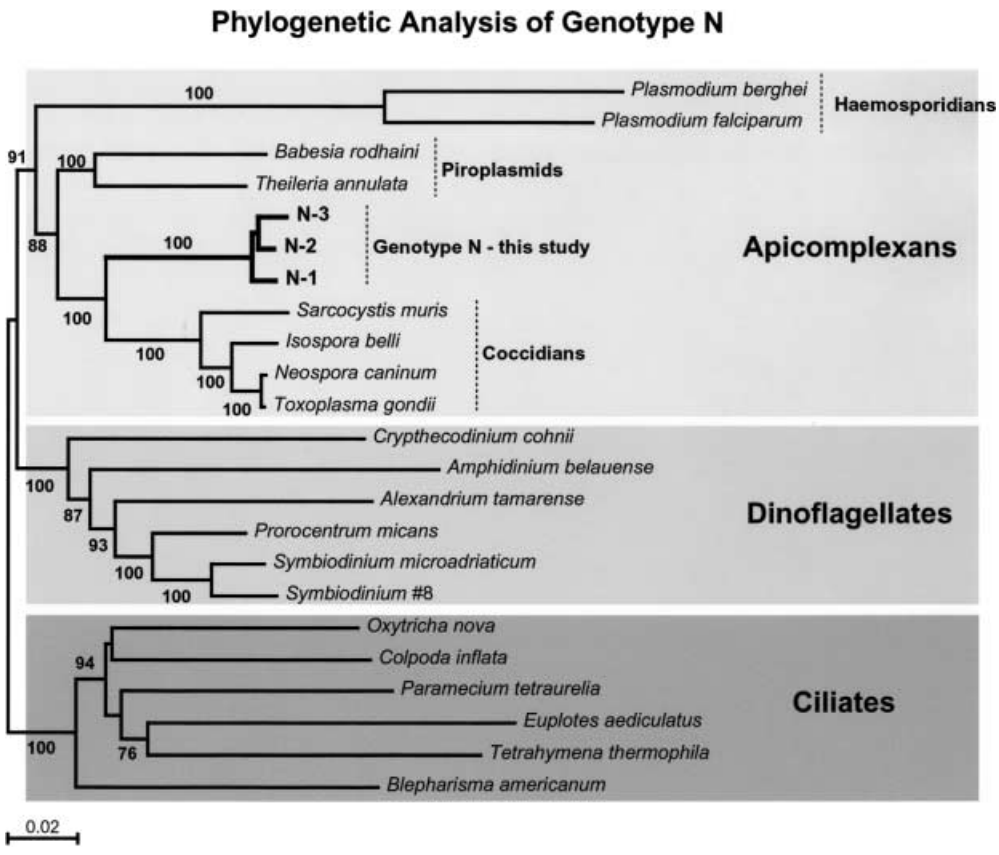


**Fig. 2.** Phylogenetic analysis of srDNA sequences from genotype N and representative protozoa. srDNA sequences were aligned and neighbor-joining reconstruction was used to explore the relationship of RFLP genotype N to members of the protozoan phyla Apicomplexa (apicomplexans, *light gray box*), Dinozoa (dinoflagellates, *medium-gray box*), and Ciliophora (ciliates, *dark gray box*). Bootstrap values (percent of 1,000 replicates) are shown where support for grouping exceeded 70%

ingerode et al. 1997; Toller et al. 2001b). In our limited examination of srDNA from genotype N, we found evidence of within-sample heterogeneity – approximately 25% of the srDNA clones obtained from each sample had restriction site changes not detected by RFLP of the original samples (not shown). srDNA heterogeneity within samples may contribute to the nucleotide variation that we observed between samples, and our data will serve as a starting point for clarifying this issue.

Does this coccidian associate with coral hosts outside of the *Montastraea annularis* species complex? The answer seems likely to be "yes." Upton and Peters (1986) used light microscopy to describe a coccidium, *Gemmocystis cylindrus*, from eight species of Caribbean corals. We made a preliminary analysis of two temperate, azooxanthellate coral species (*Balanophyllia elegans* and *Astrangia* sp. collected from San Diego, CA) and they also tested positive with N-specific PCR (not shown). We suggest that the association of coccidian apicomplexans, such as genotype N, with corals may be common.

We do not yet know if genotype N is a coral parasite. Clearly, it is related to a group of totally parasitic protozoa (Fig. 2), but its ecological relationship with corals remains obscure. We detected it in corals with no outward signs of pathology. Observations on *G. cylindrus* represent an analogous case, where this presumably parasitic coccidian was only infrequently associated with host coral pathology (Upton and Peters 1986). The use of in situ hybridization methods (e.g., Distel et al. 1995) will enable localization of N within host tissues and may further clarify the nature of their association.

Ultimately, it is likely that understanding the ecology of coral–apicomplexan associations will be more important than making a clear distinction between a benign association and a parasitic one. Coral pathogens, in some cases, may be otherwise benign microbes which become pathogenic only when their coral host becomes compromised, for example, under conditions of environmental stress (Mitchell and Chet 1975). Assays like ours can be used to rapidly assess the spatial and temporal patterns of coccidian distribution within and among host colonies. These methods can be used to test for the potential involvement of apicomplexans in coral diseases, which are of increasing concern worldwide (Richardson 1998).

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