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REPORT

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# An ITS region phylogeny of *Siderastrea* (Cnidaria: Anthozoa): is *S. glynni* endangered or introduced?

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**Abstract** The genus *Siderastrea* contains only five extant species, including *Siderastrea glynni*, which is one of the few recognized species of endangered stony coral. Cloned sequences of the internal transcribed spacer (ITS) region had low levels of intragenomic nucleotide diversity, and few alignment ambiguities, which allowed for the first species-level phylogenetic analysis of the genus. Results indicated an unexpected deep divergence between the Western-Pacific and Atlantic species. ITS region sequences indicated that *S. glynni* is not derived from *S. savignyana*, as previously thought. Instead, *S. glynni* shared identical sequence types with *S. siderea* in the Caribbean. Given a range of previously published evolutionary rates for the ITS region, it is unlikely that *S. glynni* represents the remnants of a population that was divided by the closure of the Central American Seaway (approximately 3–3.8 MYA). It is more likely that *S. glynni* originated by a breach of the Isthmus (approximately 2 MYA), or a contemporary introduction by ship.

**Keywords** *Siderastrea glynni* · ITS region · Endangered coral · Coral species phylogeny · Panamá Canal · Introduced species

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## Introduction

*Siderastrea* (starlet coral) is a circum-tropical genus that contains only five species. *Siderastrea siderea*, *S. radians*, and *S. stellata* are common in the Caribbean and Atlantic, and *S. savignyana* is the only member of the genus that occurs in the Indo-pacific. *S. glynni* is the only extant species that occurs in the eastern Pacific (Veron 2000). *S. glynni* is endemic to Panamá, and extremely rare, only five colonies have ever been discovered, and currently only four survive (Budd and Guzman 1994). *S. glynni* is one of very few (between two and five depending on the source) documented cases of endangered stony coral (Glynn and Ault 2000; Fenner 2001; Dulvy et al. 2003). *S. glynni* is morphometrically most similar to *S. savignyana*, therefore, Budd and Guzman (1994) hypothesized that *S. glynni* may have originated from a rare dispersal event from the central Pacific. One alternative hypothesis is that *S. glynni* is geminate to a Caribbean species, and represents the last relicts of a population that was fragmented either by the closure of the Tropical American Seaway, approximately 3.0–3.8 MYA (Kegwin 1982), or by a breach of the Isthmus during periods of high sea-level, approximately 2.0–2.3 MYA (Cronin et al. 1996)

It is also possible that *S. glynni* arose from contemporary introduction from the Caribbean. Although it seems unlikely that a soft-bodied organism could survive prolonged exposure to the fresh water of the Panamá Canal, several cases of marine introductions (including Cnidarians) have been documented from ballast, hull fouling, or catch byproduct from the Atlantic and Caribbean into Hawaii (DeFelice et al. 2001). It has also been observed that a member of the genus (*S. radians*) can survive prolonged burial (7–10 days) and severely low (15 ppt) salinity levels (Lirman et al. 2002). Additionally, all five known *S. glynni* colonies were discovered within a few square meters of each other, downstream and proximal to the Pacific opening of the Panamá Canal (Budd and Guzman 1994). All five colonies were small

(7–10 cm in diameter), and similar in size, suggesting that they may have originated from a single event (Budd and Guzman 1994). The ITS region was cloned and sequenced from all currently recognized extant members of the genus *Siderastrea*, in order to determine the species-level relationships in the genus and to evaluate alternative hypotheses about the origin of *S. glynni*.

## Methods

*S. glynni* was originally discovered near Isla Uraba in the Bay of Panamá, near the Pacific coast; however, after a mass-bleaching event during the 1998 El Niño, the four remaining colonies were moved to the Smithsonian Tropical Research Institute (STRI). Very small tissue scrapings (approximately 10–20 mg) were collected from all four surviving *S. glynni* specimens. *S. radians* and *S. siderea* were from Bocas del Toro on the Caribbean side of Panamá. *S. stellata* was collected from Pernambuco State Brazil. *S. savignyana* was collected from Taiwan. All specimens were preserved in ethanol. A few milligrams of tissue and skeleton were dried in a vacuum centrifuge for 20 *min*; then homogenized in a solution of 250 µl of 50 mM tris–HCL (pH 8.0) and 10 mM EDTA and incubated for 5 min at room temperature in 250 µl of 20 mM NaOH and 1% SDS. A volume of 350 µl of 3.0 M potassium acetate (pH 5.5) was added on ice then centrifuged at maximum speed. The top 500 µl of the cleared lysate was then transferred to a new tube and the DNA was precipitated by centrifugation in 1 ml of 20°C isopropanol.

The nuclear ribosomal ITS region (spanning a partial sequence of the 5′ end of the 18S rRNA gene, the complete sequence of ITS-1, 5.8S rRNA gene and ITS-2, and a partial sequence of the 3′ end of the 28S rRNA gene) was amplified using the Eukaryotic 'universal' primers; ITS-1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS-4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White et al. 1990). The PCR temperature profile was 96°C for 2 min, 30 cycles of 96°C for 10 s, 50°C for 30 s, and 70°C for 4 min. The PCR reaction produced a single clear band approximately 650 bp in *Siderastrea* species. PCR products were either directly sequenced or ligated into the PgemT-EZ cloning vector (Promega Inc.) and transformed into JM109 competent cells, followed by blue white colony screening. White colonies were screened for inserts, by PCR of white bacterial colonies using the M13 vector primers. Multiple molecular clones from each individual were sequenced using the M13 vector primers, in both the forward and reverse directions using ABI cycle sequencing chemistry. A BLAST query of the National Center for Biological Information's (NCBI) sequence database confirmed that the sequences are of the ITS region and most similar to coral sequences, some the closest of which (AF180110–AF180111, AY320289) were used as outgroups for this study. The sequences have been deposited in GenBank accession numbers: AY322575–AY322612,

Sequence alignment was performed using default parameters in CLUSTALX v.1.8 (Thompson et al. 1994) and adjusted by hand in Bioedit (Hall 1999). PAUP*v.4.0b10 (Swofford 2002) was used for pair-wise distance calculation, Neighbor-joining (Saitou and Nei 1987) and Maximum Parsimony methods. Neighbor-Joining trees and pair-wise distance calculations used the HKY85 distance measure with nodal support determined by 1,000 bootstrap replicates. The fast-heuristic method was used for Maximum Parsimony, with 1,000 bootstrap replicates. The best-fit maximum likelihood model (TrNef + G) was chosen using MODELTEST v3.06 (Posada and Crandall 1998), and used to set the initial parameters for a Bayesian analysis using MRBAYES v3.0b4 (Huelsenbeck 2000). The resulting model was run for $10^6$ generations, with a burn-in of 2,000. MEGA v2.1 (Kumar et al. 2001) was used to calculate pair-wise distances within and between intragenomic and intraspecies groups, and standard errors were estimated from 500 bootstrap replicates. DAMBE v4.1.19 (Xia and Xie 2001) was used to plot transitions and transversions against pair-wise genetic distance to determine if mutational saturation was severe enough to bias phylogenetic estimates (as can be indicated by an asymptote as genetic distance increases), the relationship was linear with no indication of mutational saturation (data not shown). This was important to examine, because the true outgroup of *Siderastrea* is unclear. If mutational saturation was evident, then the choice of outgroup taxa could significantly alter the resulting phylogeny.

## Results

Forty-three contiguous sequences for the complete ITS-1, ITS-2, and 5.8S regions were assembled, with at least three molecular clones for each *Siderastrea* species. Alignment gaps and ambiguities were rare among the *Siderastrea* sequences. Sample sizes, G + C, content and length variation are listed in Table 1, and sequence variation is indicated in Table 2. Intragenomic and intraspecies sequence diversity was low in all species sampled (averaging = $0.39 \pm 0.35\%$ and $0.34 \pm 0.33\%$, respectively). Average interspecies divergence was generally high ($12.7 \pm 11.1\%$), however, *Siderastrea radians* and *S. stellata* differed by only a few fixed nucleotide differences ($0.3 \pm 0.2\%$).

In addition to cloning, direct sequencing of the PCR product was also possible for *S. savignyana*, *S. radians*, and *S. stellata*, however *S. glynni* and *S. siderea* sequence chromatograms had several regions with double peaks. Subsequent cloning of the PCR product indicated that several PCR products were present in the latter two species. Surprisingly, exactly the same sequence types were found in all *S. glynni* and *S. siderea* individuals. All four *S. siderea* individuals shared at least one identical sequence with *S. glynni*. All *S. siderea* individuals contained a second sequence type, which was also detected

**Table 1** Length variation, percent G + C content, number of individuals, number of sequences, geographic region, collector and date for the ITS-1 and ITS-2 sequences collected for this study

| Species | Region | ITS-1 | | ITS-2 | | No. of Individuals | No. of Sequences |
|---|---|---|---|---|---|---|---|
| | | Length (bp) | Percentage of (G + C) | Length (bp) | Percentage of (G + C) | | |
| *S. glynni* | Panamá (EP)[a] | 211 | 44.6 | 233–234 | 51.9 | 4 | 16 |
| *S. siderea* | Panamá (ATL)[a] | 211 | 44.1 | 233–234 | 52.1 | 3 | 13 |
| *S. radians* | Panamá (ATL)[a] | 213–214 | 43.3 | 233 | 54.2 | 2 | 6 |
| *S. stellata* | Brazil (ATL)[b] | 213–214 | 44.3 | 233 | 53.8 | 1 | 3 |
| *S. savignyana* | Taiwan (WP)[c] | 234 | 56.3 | 231–233 | 66.8 | 5 | 5 |
| Total | | | | | | 15 | 43 |

The 5.8S gene had a constant length of 159nt, and a 48.4% G + C content
*WP* Western Pacific; *EP* Eastern Pacific; *ATL* Atlantic.
[a]Mate and Guzman (2001)
[b]Neves (2000)
[c]Chen (2004).

**Table 2** Pair-wise average difference (5.8S gene included) between species are listed on the lower diagonal matrix, standard deviation of the mean is listed in the upper diagonal matrix. Average within species means are listed on the diagonal in bold

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| *S. glynni* [1] | **0.001** | 0.002 | 0.008 | 0.008 | 0.016 |
| *S. siderea* [2] | 0.008 | **0.009** | 0.008 | 0.007 | 0.016 |
| *S. radians* [3] | 0.04 | 0.038 | **0.001** | 0.002 | 0.016 |
| *S. stellata* [4] | 0.038 | 0.037 | 0.003 | **0.001** | 0.016 |
| *S. savignyana* [5] | 0.195 | 0.198 | 0.194 | 0.195 | **0.004** |

in one of the *S. glynni* individuals; however, the molecular clone was only successfully sequenced in one direction and therefore was not included in the analysis (data not shown).

*Siderastrea* species were monophyletic relative to the outgroup taxa from GenBank. *Siderastrea savignyana* was deeply divergent from all other *Siderastrea* species (Fig. 1). *S. stellata* and *S. radians* had fixed differences, however, *S. glynni* and *S. siderea* shared identical sequence types, and together formed a monophyletic clade. Neighbor-Joining, Maximum Parsimony, and Bayesian methods revealed nearly identical topologies and nodal support values for all groupings; although Neighbor-Joining and Bayesian methods provided slightly greater resolution within the *S. glynni*/*S. siderea* clade.

## Discussion

Intragenomic and intraspecies ITS sequence diversity in *Siderastrea* averages less than half of one percent. In contrast, most other studies in Scleractinia have found values ranging from 2% to as high as 29%, (Lopez and Knowlton 1997; Odorico and Miller 1997; Medina et al. 1999; van Oppen et al. 2000, 2002; Diekmann et al. 2001; Takabayashi et al. 2003; Vollmer and Palumbi 2004, but see Forsman 2003, Forsman et al. submitted; Chen et al. 2004). The ITS region is a multicopy gene region; therefore, hybridization between species will result in additive sequence diversity. The majority of previous studies have selected species that are highly likely to hybridize (particularly *Acropora*). *Siderastrea* on the other hand has very low regional species diversity and therefore few opportunities for hybridization. Potential problems with the ITS region as a phylogenetic marker such as alignment ambiguity, or high intragenomic variation, do not appear to be problematic in *Siderastrea* (present study), or in Atlantic and east Pacific *Porites* species (Forsman 2003; Forsman et al. submitted).

The deep divergence between Pacific and Atlantic *Siderastrea* species was unexpected, however similar surprising results have recently been observed in several other coral genera (Fukami et al. 2004). The hypothesis that *Siderastrea glynni* originated from a founding dispersal event from *S. savignyana* in the central Pacific can clearly be rejected. The alternative hypothesis that *S. glynni* is a relict population that has survived since the formation of the Isthmus of Panamá is unlikely, given previous ITS region rate estimates (standardized by excluding the 5.8S gene, rates are per nucleotide cite per million years), which vary several fold depending on the taxanomic group, e.g.; 0.011–0.012 in *Drosophilla* (Schlotterer et al. 1994), 0.004 in Birch trees (Savard et al. 1993), 0.003625–0.00725 in Cucurbitaceae (Jobst et al. 1998). Complete closure of the Central American Seaway was estimated at 3.8 MYA by Kegwin (1982), which would correspond to between 1.5% and 5% sequence divergence (assuming the range of previously published rates), which is considerably larger than the observed differences.

The Isthmus may have been breached by high sea-level periods between 2.0 MYA and 2.3 MYA (Cronin et al. 1996). A two-million year separation corresponds to between 0.8% and 2.4%, which overlaps with the average observed distance between *S. glynni* and *S. siderea* ($0.8 \pm 0.2\%$). The intragenomic variation in *S. siderea* is 0.9%, and smaller differences may be undetectable. There are significant differences in the frequency of ITS region sequence types detected in

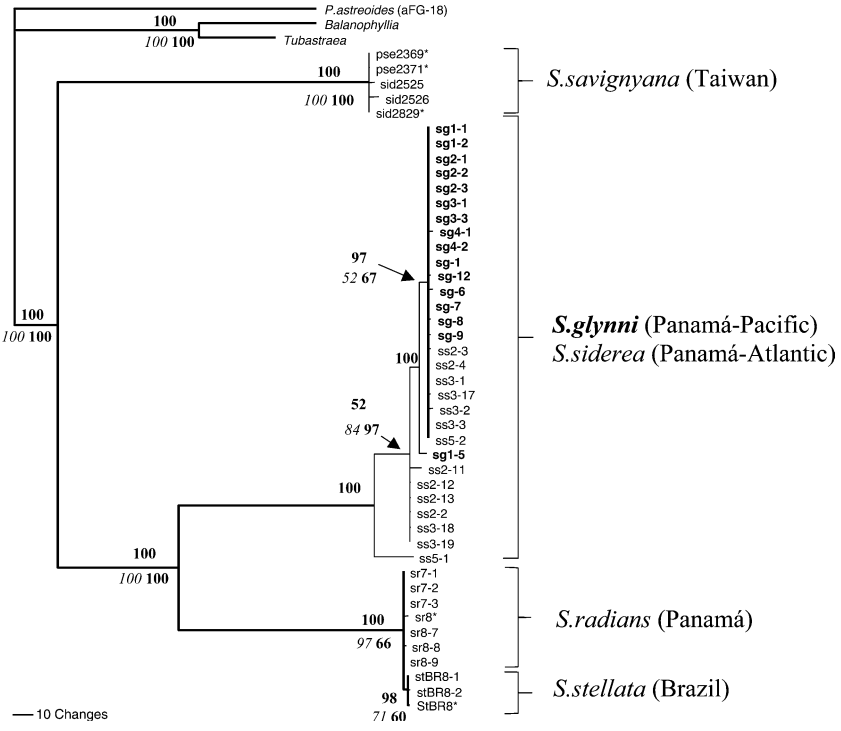


**Fig. 1** Bayesian tree of phylogenetic relationships among ITS region sequences from *Siderastrea* species. *Siderastrea glynni* sequences are indicated as *sg*, and in bold, while *S. siderea* is indicated as ss in regular script. *Bold lines* indicate agreement between Bayesian, Parsimony, and Neighbor-Joining methods. *Bold numbers* above the nodes indicate Bayesian posterior probabilities (generations = $10^6$, burnin = 2,000), *italic and bold numbers below the nodes*indicate Parsimony and Neighbor-Joining support, respectively, for 1,000 bootstrap replicates. *Sequences that were direct sequenced from PCR products as opposed to those sequenced from molecular clones

*S. glynni* and *S. siderea*, however, we are unable to interpret this result without additional information about the population dynamics of ITS region variation, or additional sampling of *S. siderea* populations. At present, we are unable to conclude if *S. glynni* originated from a relatively recent ( $\leq$ 2 MYA) speciation event, or from contemporary introduction by transport through the Panamá canal. Either hypothesis requires the occurrence of a rare event. The introduction of *S. glynni* appears to be a more parsimonious explanation, however further work is necessary to confirm the results from the ITS region, and to investigate the potential for phenotypic plasticity as an explanation for morphological differences between *S. siderea* and *S. glynni*.

This study underscores some of the challenges of discerning coral species (endangered or otherwise). Scleractinian molecular or morphologically based taxonomy is notoriously difficult at the species-level, which is an enormous challenge that will require an integrated approach. The ITS region may be a valuable component of such an approach. However, further studies are necessary to improve understanding of its evolutionary and population dynamics, the results of which will have important implications for understanding coral taxonomy, biodiversity, and conservation.

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