South Pacific Studies Vol. 27, No. 2, 2007

Seasonal Changes in Morphological Condition of Symbiotic Dinoflagellates (*Symbiodinium* spp.) in *Zoanthus sansibaricus* (Anthozoa: Hexacorallia) in Southern Japan

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Abstract

Previous short-term (up to 3 months) work has shown that symbiotic dinoflagellates of the genus *Symbiodinium* Freudenthal (zooxanthellae) can be classified into several different conditions based on their external morphology, and that symbiont ratios of "healthy" to "stressed" conditions may be indicative of the holobiont's (host + symbiont) relative health. Here, for the first time, zooxanthellae morphological condition measurements over a wide range have been used in attempting to judge holobiont condition in situ. *Symbiodinium* spp. found in the encrusting anemone *Zoanthus sansibaricus* Carlgren at four sampling locations over a latitudinal range in southern Japan were examined monthly for 30 months for changes in their morphology. Percentage of "normal" zooxanthellae (NZ%) were calculated, and compared with a variety of collected environmental data (tide pool temperature, ocean temperature, time of day, salinity, pH, dissolved oxygen, rainfall, sunlight, visibility, and conductivity). NZ% decreases (<70%) were consistently correlated with 2-week average ocean temperatures <18.0℃ and >28.5℃. No other environmental data set had such consistent correlations with NZ%. The two northern sampling sites showed significant NZ% decreases in winter, while two southern locations had significant NZ% decreases in summer, especially during the ENSO event of summer 2001. The utility of the NZ% method and its relation to temperature are discussed.

Key words: bleaching, degradation, latitudinal variation, symbiosis, zoanthid, zooxanthellae

Received: 14th April 2006
Accepted: 8th November 2006
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Introduction

The symbiotic dinoflagellates of the genus *Symbiodinium* (Order Suessiales) (FREUDENTHAL 1962) are found in symbioses with a wide variety of marine hosts, including foraminifers (POCHON *et al*. 2001), cnidarians (ROWAN and POWERS 1991), and bivalves (CARLOS *et al*. 1999). Recent work into *Symbiodinium*-host relationships has been spurred to a large degree by the increasing frequency of coral bleaching worldwide, in which the holobiont (host + symbiont) symbiosis breaks down due to environmental stress(es). A wide variety of environmental stresses, ranging from high irradiance and UV levels to runoff to pollution, have been shown to induce bleaching in a variety of different host species (*e.g*. SALIH *et al*. 1998). However, the main cause of bleaching is generally agreed to be high ocean temperatures (GLYNN 1996). Whether the host, symbiont, or both partners are responsible for the occurrence of bleaching remains the subject of debate.

Until recently, bleaching was believed to have been the result of a decrease in internal *Symbiodinium* concentration in the host, but this has been shown to be only partially true (KUROKI and VAN WOESIK 1999). It has been shown that bleaching can also be caused by a decrease in the number of intraorganism symbionts with a "healthy" or "normal" condition or morphology despite overall *Symbiodinium* levels remaining the same (KUROKI and VAN WOESIK 1999). Previous work has demonstrated that there may be changing daily patterns of *Symbiodinium* morphological conditions (TITLYANOV *et al*. 1996, MARUYAMA and HESLINGA 1997), and that *Symbiodinium* morphology can change due to environmental stress (SALIH *et al*. 1998). It appears that *Symbiodinium* undergoes degradation inside the host (TITLYANOV *et al*. 1996).

Symbiodinium can be divided into several morphological "conditions", some "healthy" and some "stressed" or "degraded", and the relative ratios of these conditions may reflect the holobiont's (symbiont + host) health (KUROKI and VAN WOESIK 1999, MISE and HIDAKA 2003). Studies conducted in laboratories (KUROKI and VAN WOESIK 1999) and *in situ* (MISE and HIDAKA 2003) investigating temperature and light influences on *Symbiodinium* morphology have been conducted over short-term periods (up to 3 months in MISE and HIDAKA 2003). Seasonality of endosymbiotic *Symbiodinium* density has been reported by FAGOONEE *et al*. (1999); they observed apparent seasonality in *Symbiodinium* density in the staghorn coral *Acropora formosa* in a lagoon in Mauritius over a 6-year period, with *Symbiodinium* levels being lowest in summer months. Similar long-term results have also been reported by STIMSON (1997) in the shallow-water Hawaiian coral *Pocillopora damicornis*. However, there remains a lack of long-term and wide-ranging research from *in situ* hosts investigating *Symbiodinium* morphology, which could potentially further reveal seasonal trends in symbiotic *Symbiodinium* populations.

Here, we conducted a long-term (spanning a time frame of 30 months) investigation utilizing designated multiple, conspecific (according to sequences from three genetic markers - see REIMER *et al*. 2006a, 2007) host *Zoanthus sansibaricus* colonies over a 400 km latitudinal range to obtain monthly *Symbiodinium* morphological condition data. We then examined whether *Symbiodinium* morphological condition data could be correlated with any of our obtained environmental data (ocean temperature, tide pool temperature, dissolved oxygen concentration, pH, salinity, conductivity, sunlight, rainfall, visibility, colony size) or with geographical location, and whether or not there were significant seasonal differences in *Symbiodinium* morphological data at extreme summer (hot) and winter (cold) temperatures.

Zoanthus spp. encrusting anemones make ideal research subjects for investigations into host-symbiont symbioses, as they have been shown to obtain the large majority of their energy budget from their symbiotic *Symbiodinium* (REIMER 1971). Worldwide in distribution, *Zoanthus* spp. form clonal colonies or mats on rocks and other hard substrates in the tidal and sub-littoral zones in tropical and sub-tropical waters, allowing undemanding sampling and simple experimental processing, unlike related hard corals. Individual polyps (clonemates) can be simply sampled without undue damage to the entire colony.

Materials and Methods

Sampling, Sampling Locations

Samples of conspecific (according to obtained cytochrome oxidase I (REIMER *et al*. 2004), mitochondrial 16S ribosomal DNA (REIMER *et al*. 2006a) and internal transcribed spacer sequences (REIMER *et al*. 2007) *Zoanthus sansibaricus* containing *Symbiodinium* spp. were collected monthly between April 2001 to September 2003 from four sampling sites in southern Japan (Fig. 1, Table 1) representing a variety of habitats and ranging 400 km north to south. *Zoanthus* at the two northern sites of Kokubu and Sakurajima exists only in the sub-tidal zone (ONO *et al*. 2005) and samples at these sites were collected by snorkeling. *Zoanthus* at Yakushima and Amami sites exists in the inter-tidal zone, and samples were collected during extreme low tides.

For each sampling, one polyp was removed with scissors from the center of five designated *Zoanthus* colonies ranging from high/inland to low/ocean locations (at inter-tidal Yakushima and Amami sites) or ranging from shallow (<1m) to deep (approx. 4m) at the infra-littoral Kokubu and Sakurajima sites. The same five colonies were sampled at each site for the duration of the experiment. Due to dangerous conditions (*i.e.* large waves) at inter-tidal Yakushima and Amami sites, occasionally only three or four colonies were sampled during monthly visits. Polyps were placed intact into appropriately numbered glass vials.

Symbiodinium morphological condition data collection.

Immediately after sampling (within 15 minutes) each sampled *Zoanthus* polyp was placed in a Petri dish. *Symbiodinium* were squeezed out of the gastrointestinal cavity and surrounding tissue using forceps. The *Symbiodinium* “slurry” was diluted with 0.1ml of ambient temperature seawater, and extracted into a 1ml syringe (0.45 X

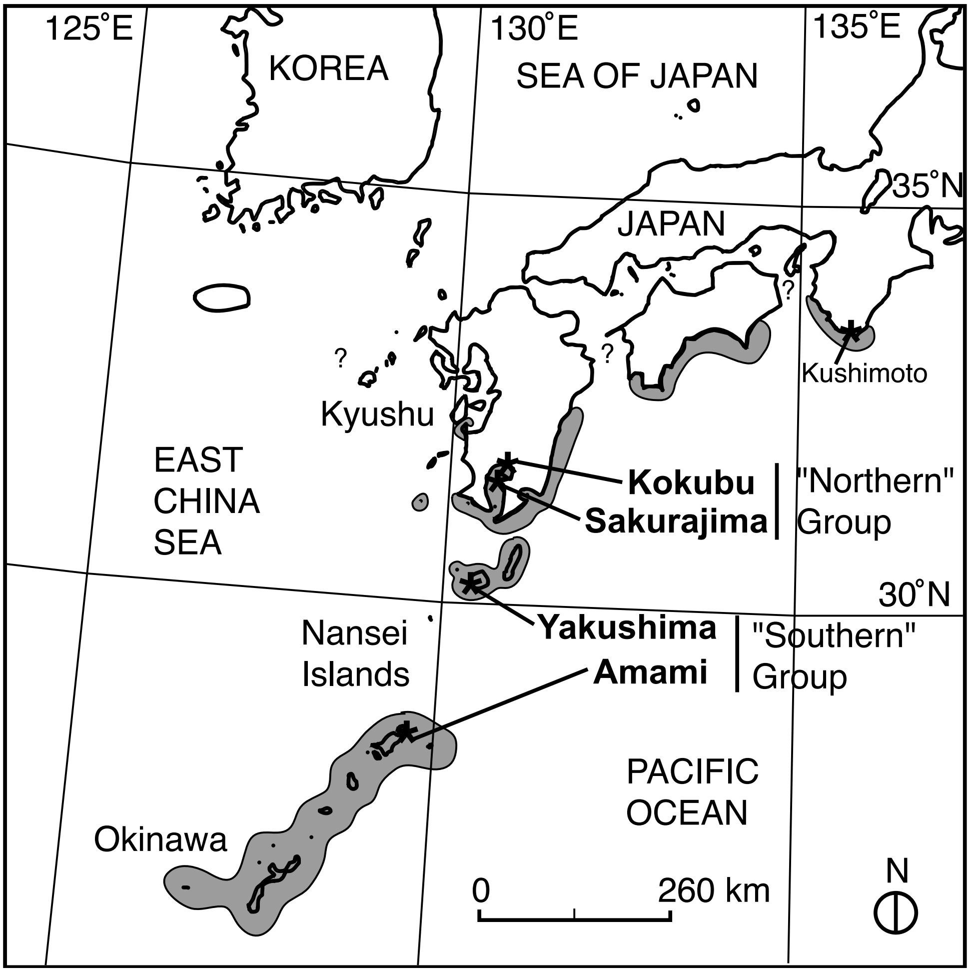

Fig. 1. Map of sampling locations. The distance from the northernmost location Kokubu to the southernmost location at Amami is approximately 400km, and ranges from inland temperate waters in Kagoshima Bay in the north to sub-tropical coral reefs on the Pacific Ocean in the south. Shaded areas indicate known *Zoanthus* spp. range. Question marks indicate areas where *Zoanthus* spp. presence is unknown.

13mm, Terumo, Tokyo, Japan). The syringe was thoroughly shaken, and the contents placed on a Thoma EKDS hemocytometer (1/10 mm depth, 1/400 mm^2), and observed under a microscope using a 40X objective lens. Individual *Symbiodinium* were divided into two groups, "Normal Zooxanthellae" (NZ) or "Stressed Zooxanthellae" (SZ) (Fig. 2, Table 2) based on our personal observations and previous papers (SALIH *et al.* 1998, KUROKI and VAN WOESIK 1999, MISE and HIDAKA 2003). Within these two groups further subdivisions were made (Fig. 2, Table 2). For each polyp, five predetermined fields of view (corresponding to 12, 3, 6, 9 o'clock positions of the hemocytometer as well as the center position) were selected (usually resulting in over 100 individual *Symbiodinium* counted per polyp) and the frequency of each *Symbiodinium* condition counted. In addition, syringes of seawater were also examined during each

Table 1. Sampling location characteristics and temperature data summary.

Sampling group	Sampling site	Location	Description	Observed max (℃)[1]	Observed min. (℃)[1]	Tide pool variation (℃)[2]	Expected annual max. (℃)[1,3]	Expected annual min, (℃)[1,3]
Northern	Kokubu	31° 41' N, 130° 48' E	Volcanic rock, moderate current, little wave action. Sub-tidal.	30.57 (2001.8.9-10)	15.53 (2003.2.13-14)	not applicable	27.98	15.83
	Sakurajima	31° 35' N, 130° 36' E	Recent volcanic rock (<100 y.o.), strong current, little wave action. Sub-tidal.	30.57 (2001.8.9-10)	15.53 (2003.2.13-14)	not applicable	27.98	15.83
Southern	Yakushima	30° 16' N, 130° 25' E	Granite rock, some hard coral. Strong current & wave action. Intertidal.	30.74 (2001.8.7-8)	18.28 (2003.2.5-6)	±16.59	28.57	19.88
	Amami	28° 27' N, 129° 44' E	Coral reef outer front, moderate current, strong wave action. Intertidal.	31.33 (2001.8.11-12)	19.91 (2003.3.7-8)	±24.83	28.55	20.74

[1]Ocean temperature data (April 2001-October 2003) from Kagoshima Prefectural Fisheries Experimental Center (KPFEC): Sakurajima and Kokubu data from Kagoshima Shinko site (31°30' N, 130°35' E), Yakushima data from Yakushima site (30°24' N, 130°19' E), and Amami data from Kasarisaki site (28°32' N, 129°32' E), taken by the ferry Naminoue, all at a depth of 5m.

[2]Data obtained from sampling location field measurements (July 2002-September 2003).

[3]Calculated from KPFEC data, 1982-2002.