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NOTE

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Molecular evidence demonstrating the basidiomycetous fungus *Cryptococcus curvatus* is the dominant microbial eukaryote in sediment at the Kuroshima Knoll methane seep

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Abstract The Kuroshima Knoll, located in the southern Ryukyu Arc, is known to actively bubble with gas containing methane and hydrogen sulfide from numerous fissures in the large carbonate pavement. Although ecological studies regarding macrobenthos and bacteria from Kuroshima Knoll have been intensively conducted, the community structure and ecological importance of microbial eukaryotes (protists) have not yet been investigated. In the present study, we directly extracted DNA from sediment of the Kuroshima Knoll at a depth of 640 m and constructed genetic libraries of PCR-amplified eukaryotic small-subunit ribosomal DNA (SSU rDNA). Although the SSU rDNA sequences of several types of benthic foraminifers were retrieved from the surface of the sediment, all other sequences (just below the sediment surface to approximately 9 cm below sediment surface) were derived from the basidiomycetous yeast *Cryptococcus curvatus*. Furthermore, sequences of the internal transcribed spacer of rDNA (ITS-rDNA) retrieved from the same sediment were identical to that of *C. curvatus* originating from terrestrial habitats. The diversity of microbial eukaryotes in the Kuroshima Knoll sediment seems to be extremely low and significantly different from that of other marine environments previously reported.

Keywords Deep-sea · Eukaryotes · Foraminifers · Fungus · ITS-rDNA · Methane seep · SSU rDNA

Recently, culture-independent molecular analyses have demonstrated the diversity and ecological importance of microbial eukaryotes (protists) in various marine environments. In particular, there has been increasing interest in the diversity of microbial eukaryotes from extreme marine environments. For example, phylogenetic analyses of eukaryotic small subunit ribosomal DNA (SSU rDNA) retrieved from the sediment and ambient seawater of chemosynthetic ecosystems (i.e. deep-sea hydrothermal vents) have detected unexpectedly diverse phylotypes, including many novel ones at a high-taxonomic level (Edgcomb et al. 2002; López-García et al. 2003). However, some of these high-taxonomic level novel phylotypes have been suggested to be artifacts caused by the failure to detect chimeric sequences, phylogenetic misplacement of fast-evolving sequences, and/or incomplete sampling of described but as of yet unsequenced eukaryotes (Berney et al. 2004). Furthermore, Moreira and López-García (2003), based on these molecular analyses, have argued that parasitic protists inhabiting areas around deep-sea hydrothermal vents, such as Apicomplexa and Kinetoplastida, are possibly hosted by dense animal populations, and these parasites may cause sudden massive mortality of host animals. Our recent molecular survey of anoxic sediment around fumaroles on a submarine caldera floor at a depth of 200 m, with conditions similar to conventional deep-sea hydrothermal vents, also suggested the existence of taxonomically diverse protists, including a few novel eukaryotes at high-taxonomic levels (Takishita et al. 2005). Remarkably, some sequences from this environment were closely related to those of eukaryotic parasites, such as Phytomyxea and Ichthyosporea, although it was not clear whether these organisms occurred in free-living or parasitic forms (Takishita et al. 2005).

However, the genetic diversity of microbial eukaryotes occurring at cold seeps (chemosynthetic ecosystems where energy-rich fluids seep out of the ocean floor due to the geology of the underlying sediments and rock layers) has not yet been investigated. The Kuroshima Knoll, located in the southern Ryukyu Arc near Ishigaki Island, Japan, at a depth of about 650 m, is one of the most well-described cold methane seep sites, character-

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ized by active bubbling from numerous fissures in the large carbonate pavement. The site has large-scale colonies of the vesicomyid clam genus *Calyptogena* and the mytilid mussel genus *Bathymodiolus* (Machiyama et al. 2001). *Calyptogena* spp. and *Bathymodiolus* spp. harbor sulfur-oxidizing lithotrophic symbionts and methanotrophic symbionts, respectively, and the growth of both symbiont types depends on the bubbles released from the fissures (Inagaki et al. 2004). Furthermore, it has been shown that Kuroshima Knoll sediment contains abundant archaea, including both mesophilic methanogens related to the genus *Methanolobus* and ANME-2 members of *Methanosarcinales*, as well as epsilon-Proteobacteria, suggesting that both anaerobic methane oxidation and methanogenesis occur at this site (Inagaki et al. 2004). In the present study, to obtain information on the diversity of another ecological organism category (in addition to the previously surveyed macrobenthos, bacteria, and archaea), we have analyzed microbial eukaryotes (protists) from the sediment of this chemosynthetic ecosystem at the molecular level.

The dive survey was conducted at Kuroshima Knoll in Okinawa Trough using the ROV *Hyper-Dolphin* on April 24, 2004 (Cruise No. NT04-03, Hyper-Dolphin Dive #299). Sediment samples were collected with an MBARI-type push corer at two sites at a depth of 642 m: Site A (24°7.800′N, 124°11.455′E) and Site B (24°7.810′N, 124°11.385′E). The distance between the two sites was about 120 m. Both of the two core samples smelled strongly of hydrogen sulfide, and Site B's sample was colored black due to hydrogen sulfide. Both of the two core samples were composed mainly of planktonic foraminiferal ooze. Site A samples at 1, 9, and 16 cm from the sediment surface, and Site B samples at 1, 3, 9, and 17 cm from the sediment surface were fractionated in consideration of sediment facies. Total DNA from each sediment sample was extracted with UltraClean™ Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). According to the manufacturer's instructions PCR-amplification using the total DNA as a template was performed with HotStarTaq DNA polymerase (QIAGEN, Tokyo, Japan). Nuclear SSU rDNA were amplified by using different combinations of the eukaryotic SSU rDNA universal primers 18S-42F (5′-CTCAARGAYTAAGCCATGCA-3′), 18S-82F (5′-GAAACTGCGAATGGCTC-3′), 18S-1498R (5′-CACCTACGGAAACCTTGTTA-3′), and 18S-1520R (5′-CYGCAGGTTCACCTAC-3′), under the following thermal cycle conditions: 32 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, followed by a final elongation step of 10 min at 72°C. The amplified products were confirmed on 1.0% agarose gel electrophoresis. The PCR-amplified DNA fragments were cloned into the pCR2.1 vector of the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Positive transformants (more than 60 clones) for each sediment sample were screened by PCR amplification of inserts with InsertCheck-Ready-Blue (TOYOBO, Osaka, Japan). The clones from the libraries were sequenced with an ABI PRISM™ 3700 DNA Analyzer (PE Biosystems, Foster City, CA, USA) using a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems). Unexpectedly, all clones of all sediment fractions other than 16 cm of Site A and 17 cm of Site B had sequences identical to that of SSU rDNA from the saprophylic basidiomycetous fungus *Cryptococcus curvatus*. In addition, electrophoresis patterns of digestions by four base cutter restriction enzymes (*Hha*I, *Msp*I, and *Rsa*I) of the PCR-amplified products, as well as an insertion cloned into the plasmid vector and subsequently sequenced, showed no variation in results (data not shown), suggesting that the PCR-amplified products were derived from one sequence. No amplified-products were obtained from the samples at 16 cm of Site A and 17 cm of Site B. In the sediment samples from which the sequences of *C. curvatus* were obtained, yeast-like cells were frequently found during microscopic observation (but their density was considerably low when compared with that of prokaryotic cells). Figure 1 shows the maximum-likelihood (ML) tree based on the alignment including our obtained sequence 'KUROS1' (GenBank accession no. AB234889) along with previously reported SSU rDNA sequences from *C. curvatus* and its related fungal species (19 taxa, 1718 sites) constructed with a general time reversal model (Rodríguez et al. 1990) incorporating invariable sites and a discrete gamma distribution (eight categories) (GTR + I + Γ) using PhyML (Guindon and Gascuel 2003). PhyML bootstrap trees (500 replicates) were constructed using the same parameters as the individual ML trees. Furthermore, the internal transcribed spacer of rDNA (ITS-rDNA) was amplified with a set of primers pITS-F (5′-GTCGTAACAAGGTTAACCTGCGG-3′) and pITS-R (5′-TCCTCCGCTTATTGATATGC-3′) using each sample fraction DNA as templates. PCR conditions, cloning, and sequencing of ITS-rDNA were same to those of SSU rDNA mentioned above. Similarly, all clones (20 clones) of each sediment fraction, except for 16 cm of Site A and 17 cm of Site B, for which no amplified products were obtained, had sequences identical to that of ITS-rDNA from *C. curvatus* previously reported (accession no. AB035574 and AB035686-AB035696). The ITS-rDNA sequence obtained in this study 'KUROS2' was deposited in GenBank (accession no. AB234890).

In general, basidiomycetous yeasts often account for the majority of the total yeast population in oligotrophic oceanic waters, and among the basidiomycetous yeasts, some species of *Cryptococccus* and its teleomorphs have been found to be widespread across various oceanic regions (Nagahama 2005). In addition, yeast-like cells have been shown to be abundant in deep-sea sediments from quantitative surveys of microorganisms (Burnett 1981; Alongi 1987, 1992). A number of yeasts including the genus *Cryptococcus* were isolated during surveys of yeast distribution in deep-sea environments around the Pacific Ocean (Nagahama et al. 1999, 2001a, b, 2003a, b). However, based on molecular and other studies there has been no report of *C. curvatus* exclusively dominating not only yeast communities but also microbial eukary-

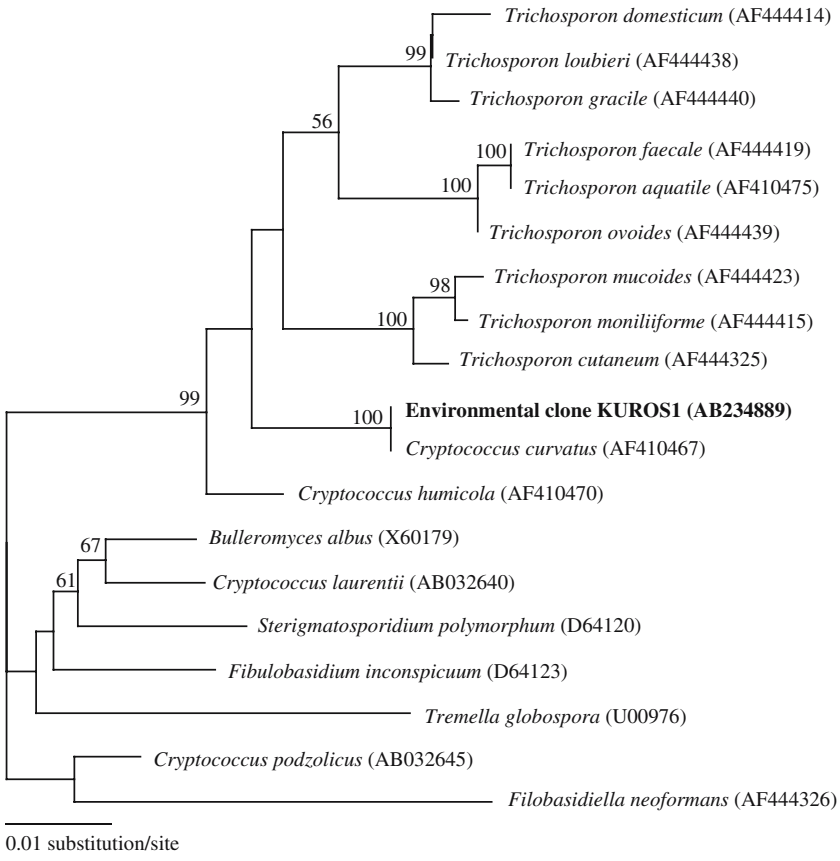

Fig. 1 The maximum-likelihood tree of SSU rDNA sequence from *Cryptococcus curvatus* and its related fungal species. Numbers at the nodes refer to the percentage (> 50%) of bootstrap support of maximum-likelihood analysis. GenBank accession numbers are in parentheses following species' names

otic communities in marine environments. Thus, the sediment of Kuroshima Knoll may be a unique marine environment with respect to the community structure of microbial eukaryotes. There has been a longstanding debate surrounding the questions: 'Are there indigenous marine yeasts?' and 'If some yeast species are indigenous, which species are indigenous?' (Kohlmeyer and Kohlmeyer 1979). According to a review by Nagahama (2005), the candidates for autochthonous marine yeasts are not thought to be ubiquitous, their occurrence being restricted to specific geographical latitudes or to associations with certain macroorganisms. ITS-rDNA sequences (known to be fast-evolving) retrieved from the Kuroshima Knoll sediment were exactly identical to sequences from *C. curvatus* isolated from terrestrial habitats, suggesting that the *C. curvatus* cells occurring in the sediment of Kuroshima Knoll are not autochthonous, but allochthonous. Why only allochthonous *C. curvatus* would be present at Kuroshima Knoll and not other microbial eukaryotes (including fungi) remains unclear. In addition, ecological interactions between *C. curvatus* and other organisms in the same environment (macrobenthos, bacteria, and archaea) are still enigmatic. *C. curvatus* is known to be a opportunistic pathogen of animals, including humans (Dromer et al. 1995). Because bivalves were found in the same sediment, *C. curvatus* may infect these animals. To properly investigate and clarify these questions, culturing, and subsequent physiological experiments of resident *C. curvatus* are necessary in the future.

In our microscopic observation not only yeast-like cells, but also foraminifer-like cells were found in the surface sediment of our samples. Although it is known that foraminifers are frequently found in various deep-sea sediments (Snider et al. 1984), the foraminifer-like cell density (frequency) at Kuroshima Knoll was extremely low. Because the sequences of the foraminiferal SSU rDNA are exceptionally divergent, eukaryotic SSU rDNA universal primers used in the present study failed to amplify foraminiferal SSU rDNA. Thus, with a set of primers specific to the foraminiferal SSU rDNA; s14f1 (5′-AAGGGCACCACAAGAACGC-3′) and sB (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) (Pawlowski et al. 1996), PCR amplifications were performed using all fraction samples of DNA as templates. PCR conditions were the same as mentioned above except 40 cycle steps were conducted. Products of the expected size (about 1,000 b.p.) were amplified only from samples 1 cm from the sediment surface at both sites. Consequently, three different sequences 'KUROS3-5' were obtained by cloning and sequencing (17 clones) (GenBank accession no. AB234891–AB234893). These sequences were aligned with those of various foraminiferal SSU rDNA (24 taxa, 685 sites), and were applied to ML analysis under the GTR + I + Γ model with PhyML. PhyML bootstrap analysis (500 replicates)

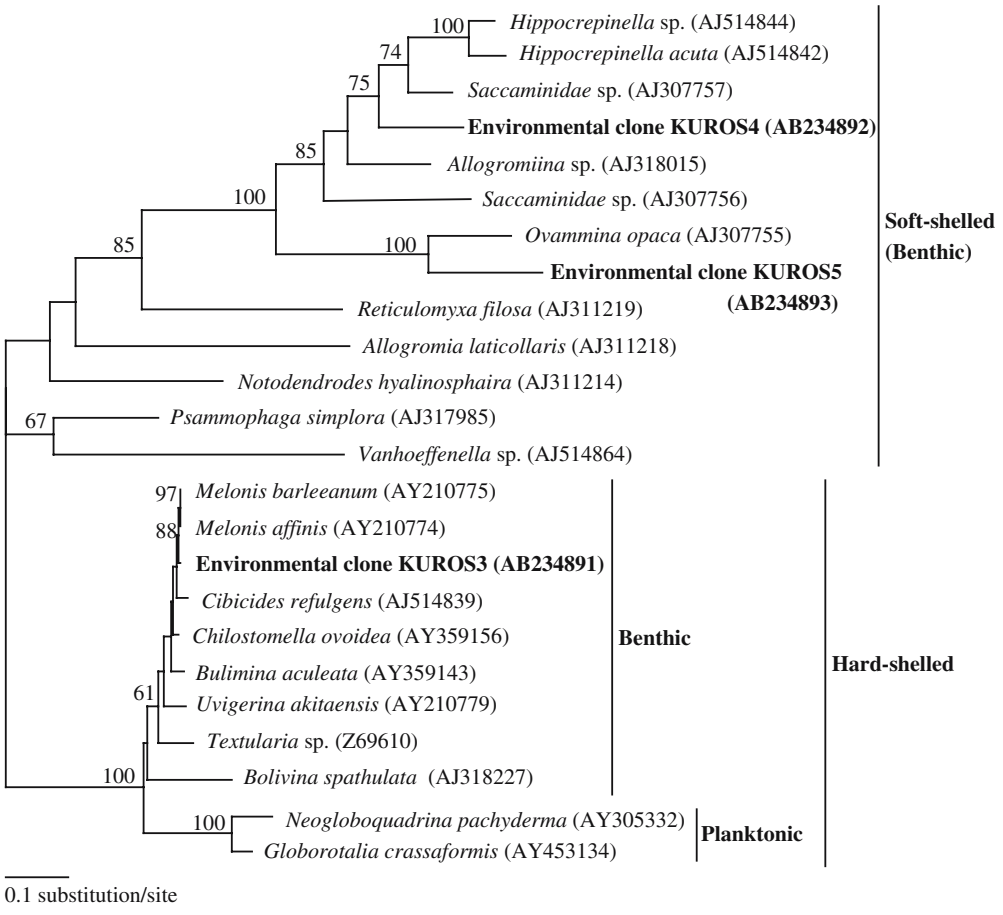

Fig. 2 The maximum-likelihood tree of SSU rDNA sequence from taxonomically diverse foraminiferal species. Numbers at the nodes refer to the percentage (> 50%) of bootstrap support of maximum-likelihood analysis. GenBank accession numbers are in parentheses following species' names

was also performed. In the phylogenetic tree (Fig. 2) KUROS3 was positioned within the radiation of the calcareous hard-shelled benthic foraminiferal lineage, while KUROS4 and KUROS5 were within the soft-shelled benthic foraminiferal lineage. Thus, it is highly likely that all the three sequences were derived from benthic foraminifers intrinsic in the sediment, and not from sinking planktonic foraminifers. Previously, many foraminiferal species have been found in a methane-seep in Monterey Bay, and hydrocarbon-seeps both at Blake Ridge, Atlantic Ocean and in the Gulf of Mexico (Bernhard et al. 2001; Rathburn et al. 2003; Robinson et al. 2004). However, whether the foraminiferal species having these sequences obtained in this study occur specifically at methane seeps remains unclear, because no other existing molecular information on foraminifers inhabiting other seep sites.

In summary, Kuroshima Knoll sediment is very unique with respect to the community composition of microbial eukaryotes, the fungus *C. curvatus* being dominant to a depth of 9 cm below the sediment surface, and with a minority of several benthic foraminiferal species just below the sediment surface.

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