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Molecular Identification of Thermally-Tolerant Symbiotic Dinoflagellates from Hard Coral (*Scleractinia*) in Biawak Island, Indonesia

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Symbiodinium is phototrophic dinoflagellates that occur as endosymbionts in various marine invertebrates and protists, forming mutualistic symbiosis with their hosts. The composition of *Symbiodinium* populations may also play an important role in the tolerance or sensivity of corals towards bleaching. Therefore, this research aims to identify thermally-tolerant *Symbiodinium* of scleractinian corals in Biawak Island using molecular techniques. Sampling was carried out from Acropora sp and Porites sp, from the depth of 3-5 meters. *Symbiodinium* was isolated through metagenomic approach using ZR Soil Microbe DNA Kit™ and 28S nrDNA gene was amplified using Polymerase Chain Reaction (PCR). The resulted sequences were processed using BioEdit™ software and MEGA™ 5.2 for phylogenetic tree construction. The results indicated that isolates of ZX-ACP-ST.1 and ZX-PORITES-ST.3 have similiarity to registered sequences of *Symbiodinium thermophilum* with identity value of 99% and 98%. BLAST™ analysis of ZX-PORITES-ST.2 isolate indicated high identity (99%) to sequences of *Symbiodinium* sp. clade C while ZX-PORITES-ST.3 isolate has 99% similiarity to *Symbiodinium* sp. CG8. Phylogenetic analysis using UGPMA method showed that all isolates had a very close relationship and predicted to have come from *Symbiodinium* clade C.

Key words: 28S nrDNA gene, Dinoflagellates, *Symbiodinium*, Thermally-tolerant

Symbiodinium merupakan dinoflagelata fototrofik yang muncul sebagai endosimbion pada berbagai invertebrata laut dan protista, membentuk simbiosis mutualisme dengan inangnya. Komposisi populasi dari *Symbiodinium* juga memiliki peran penting dalam mempengaruhi toleransi atau sensitivitas karang dalam menghadapi *bleaching* (pemutihan). Oleh karena itu, penelitian bertujuan untuk mengidentifikasi *Symbiodinium* yang memikili toleransi terhadap kenaikan suhu pada karang-karang keras di perairan Pulau Biawak menggunakan teknik molekuler. Pengambilan sampel Dinoflagelata dilakukan dari karang keras Acropora sp dan Porites sp, dari kedalaman 3-5 meter. Symbiodinium diisolasi melalui pendekatan metagenomik dengan menggunakan ZR Soil Microbe DNA Kit™ dan gen 28S nrDNA diamplifikasi dengan teknik *Polymerase Chain Reaction* (PCR). Hasil sequensing diolah dengan software BioEdit™ dan MEGA™ 5.2 digunakan untuk mengkonstruksi pohon filogenetik. Hasil penelitian menunjukkan bahwa isolat ZX-ACP-ST.1 dan ZX-PORITES-ST.3 memiliki kesamaan dengan *Symbiodinium thermophilum* dengan nilai identik masing-masing sebesar 99% dan 98%. Hasil analisis BLAST™ dari isolat ZX-PORITES-ST.2 menunjukkan kesamaan yang tinggi (99%) dengan *Symbiodinium* sp. clade C, sementara isolat ZX-PORITES-ST.3 memiliki 99% nilai kesamaan dengan *Symbiodinium* sp. CG8. Hasil analisis filogenetik menggunakan metode UPGMA menunjukkan bahwa semua isolat memiliki hubungan yang sangat dekat dan diprediksi berasal dari *Symbiodinium* clade C.

Kata kunci: Dinoflagellates, gen 28S nrDNA, *Symbiodinium*, toleran suhu

Zooxanthellae are members of the phylum dinoflagellata includes the genus *Symbiodinium*, which are found in the tissues of the phyla Mollusca (Tridacna clams, nudibranchs), Platyhelminthes (flatworms), Porifera (sponges), Protozoa (foraminifera), and Cnidaria (corals, sea anemones, hydroids, jellyfish) (Venn *et al.* 2008). Zooxanthellae play an important role in coral reefs ecosystem because it is nearly impossible to find a living coral without zooxanthellae symbiosis (Veron 1995). Zooxanthellae provide corals with nutrients and organic molecules as a result from photosyntesis. The products then used by coral host for its growth, reproduction and physiology process. Instead, the coral host provides zooxanthellae with protection in their polyps tissues. The composition of zooxanthellae populations may also play an important role in the tolerance or sensivity of corals towards bleaching because separation of zooxanthellae from its host may caused coral bleaching phenomenon, which if continued, results in coral death (Baker 2003; Baker *et al.* 2004).

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The *Symbiodinium* genus includes numerous lineages, also referred to as clades or phylotypes, as well as a wide diversity of genetic sub-clades and sub-phylotypes (Casado-Amezúa *et al.* 2014). There were 8 different genetic clades that have been identified named A, B, C (Rowan and Powers, 1991), D, E (Baker 2003), F, G and H (Pochon *et al.* 2004) all over the world. It has been determined that different clades of *Symbiodinium* have different physiological resistance against environmental conditions and stresses, therefore different survival rates of corals have been observed (Baker 2003).

Most of studies on Symbiodinium diversity stem from the Caribbean (Rowan *et al.* 1997; Diekmann *et al.* 2003; Baker and Rowan 1997) and Pacific Ocean (LaJeunesse *et al.* 2003; Glynn *et al.* 2001; Loh *et al.* 1998; van Oppen *et al.* 2001). Relatively few studies have been undertaken in the Indian Ocean. These studies were from corals of Eastern Africa (clade C and D; Baker *et al.* 2004; Visram *et al.* 2006), the Red Sea (clade A and C; Baker *et al.* 2004) and the Saudi Arabian coast of the southern Persian Gulf (clade A, C and D; Baker *et al.* 2004). The information of zooxanthellae diversity in Indonesia has yet to be known really well, therefore, it is important to identify *Symbiodinium* in Indonesian waters. Biawak Island is representing of tropical waters area located in Indramayu, Indonesia that have not been widely explored. Moreover, the composition of coral reef ecosystem on Biawak Island is very dense and dominated by scleractinian corals (KKJI 2004).

The development of species identification methods has begun from morphological identification to molecular identification, called a "DNA barcode" (Hebert *et al.* 2003). Molecular identification of *Symbiodinium* generally use gene analysis of ribosomal RNA in the sequence of specific molecular markers. These markers are as following: small subunit ribosomal RNA gene (SSU) (Brown *et al.* 2002; Rowan and Powers, 1992; Sadler *et al.* 1992), large subunit ribosomal RNA gene (LSU) (Pawlowski *et al.* 2001; Pochon *et al.* 2001; Savage *et al.* 2002), internal transcribed spacers (ITS1, ITS2), and 5.8S regions (Brown *et al.* 2002; LaJeunesse 2002; Pochon *et al.* 2004). Chloroplast rDNA sequence of ribosomal large subunit gene is also recently used to interpret from nrDNA as well (Santos *et al.* 2003). The partial 28S nuclear ribosomal (nr) DNA gene barcode was designed and has been used to identify the thermally-tolerant *Symbiodinium* (clade D and C) of Persian Gulf, Iran (Mostavafi *et al.* 2007). This is the first study on *Symbiodinium* diversity of scleractinian corals of Biawak Island. Therefore, the aim is to investigate the genetic diversity of *Symbiodinium* from coral species of Biawak Island for comparison with *Symbiodinium* from other coral communities worldwide, especially those with thermally-tolerant properties. The results will provide a valuable insight to the survival of Indonesian tropical coral reefs, especially in Biawak Island, and its management, in a future threatened by global warming.

MATERIALS AND METHODS

Sample Collection and Metagenomic DNA Isolation. Sampling was performed by scuba diving at 5-7 meters depths in Biawak Island waters, Indramayu, Indonesia, spotted in 3 sampling stations (ST1 : 108,372 Lgt -5,933 Lat.; ST2 : 108,375 Lgt -5,921 Lat; and ST3 : 108,391 Lgt -5,925 Lat) (Fig 1) on May 2016. Hardcoral fragments (Acropora and Porites) were preserved in ethanol 97% (Etnoyer *et al.* 2006) and transferred to laboratory and stored at -20°C. The 0.35 gr coral fragments from each samples was crushed using mortar, then genomic DNA was isolated through metagenomic approach (Agung and Moeis 2013) using ZR Soil Microbe DNA Kit™ (Zymo Research®).

PCR Amplification and Sequencing. The partial of large subunit (LSU) of nuclear ribosomal DNA (28S nrDNA) gene of *Symbiodinium* contained in metagemomic DNA was directly PCR amplified (Agung and Moeis 2013) using primers specifically designed for *Symbiodinium* species : Mos-Forward (ATATAAGTAAGCGGAGGAAAAG) and Mos-Reverse (CTTTCGGGTCCTAACACACATG) (Mostafavi *et al.* 2007). All PCR cocktails contained 10 ng DNA template and 7.5 pmol of each primer using 2G Robust Ready Mix™ (KAPA®). Amplification was performed using Eppendorf® Mastercycler DNA Engine Thermal Cycler and under following temperature profile: 5 minutes in 95 °C for 1 cycle and then 45 seconds in 95 °C, 1 min in 53 °C, 1 min in 72 °C for 30 cycles, and a final extension, 5 min in 72 °C was carried out for 1 cycle. The PCR products were visualized by electrophoresis in 1 % agarose gel (80 V, 45 min). Agarose gel was stained by GelRed™ (Biotium®) and observed under UV transilluminator (Fisher Scientific®). The amplicons then were sent to The 1st BASE® service in Singapore for direct sequencing analysis.

DNA sequences obtained from 4 coral samples were processed using BioEdit™. Resulted sequences

then identified by aligned the data available on Gene Bank at NCBI (www.ncbi.nlm.nih.gov) using BLAST™ and compared with reference sequences.

Phylogenetic Analysis. Phylogenetic analysis was carried out with MEGA™ 5.2 software. All sequences were aligned using ClustalW™ program. Trees were constructed using UPGMA (Unweighted Pair Group Method Using Arithmetic Average) methods. UPGMA tree were assessed with 1000 bootstrap replicates. *Symbiodinium* clades sequences were considered according to Baker (2003) and the reference *Symbiodinium* sequences belong to clade A to H. The phylogenetic trees generated in all analysis were visualized.

RESULTS

The 28S nrDNA PCR Amplifications. PCR amplification of *Symbiodinium* 28S nrDNA genes visualized on agarose gel resulted in products of approximately 780 bp from four coral samples (Fig 2).

Sequencing and BLAST™ Analysis. The results

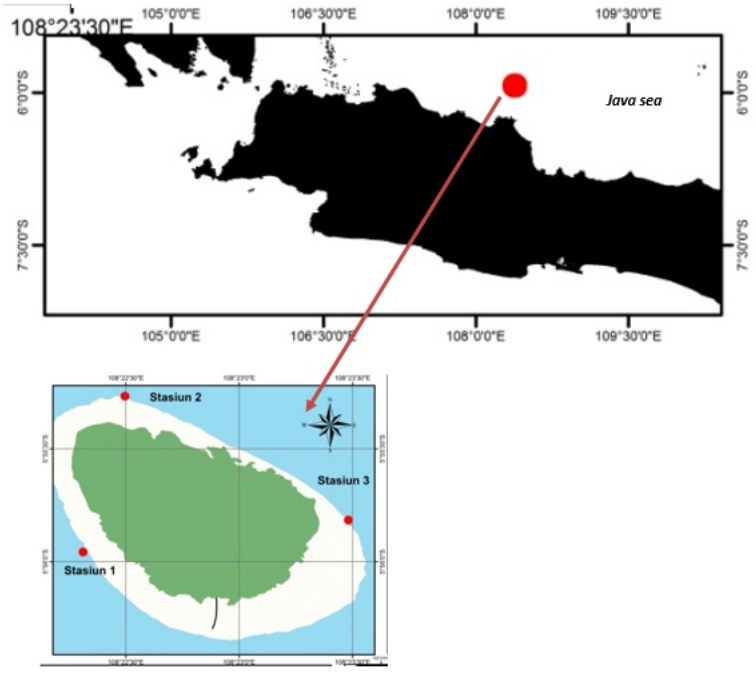

Fig 1 Map of Sample Collection Location at Biawak Island waters, Indramayu, Indonesia.

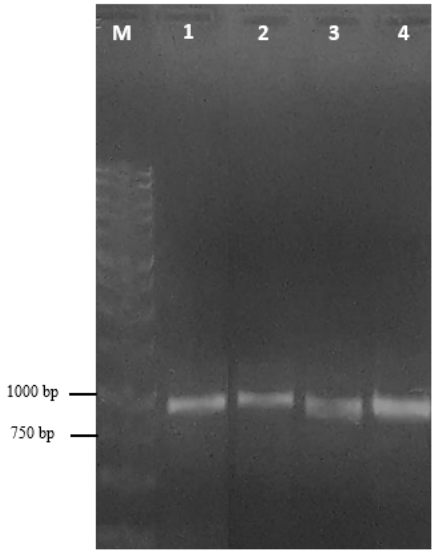

Fig 2 Gel electrophoresis (1%) was performed for PCR products of 28S nrDNA gene amplification.
M : DNA ladder 1 kb (FERMENTAS®); **1** : ZX-ACP-ST.1; **2** : ZX-PORITES-ST.1; **3** : ZX-PORITES-ST.2; and **4** : ZX-PORITES-ST.3.

of the 28S nrDNA gene analyzed with BLAST™ program showed that isolates ZX-ACP-ST.1, ZX-PORITES-ST.1, ZX-PORITES-ST.2 and ZX-PORITES-ST.3 had a similiarity (>98%) to *Symbiodinium* clade C (Table 1), and specifically BLAST™ result of ZX-ACP-ST.1 and ZX-PORITES-ST.3 showed that samples had similarity to database in Gene Bank (KR996308.1) and were identified as *Symbiodinium thermophilum* (>98% identity).

BLAST™ analysis of isolate ZX-PORITES-ST.1, showed that the sample had similarity to database Gene Bank (AJ830903.1), identified as *Symbiodinium* sp. clade C isolates partial 28S rRNA gene LII61 (99% identity), while in isolates ZX-PORITES-ST.2, BLAST™ results showed that the sample had similarity to database Gene Bank (AJ308892.1), identified as *Symbiodinium sp*. CG8 partial 28S rRNA gene, isolate CG8 with (99% identity).

Phylogenetic Analysis. Construction of a phylogenetic tree consists of *Symbiodinium* species worldwide (Gene Bank database) and four isolates from Biawak Island (ZX-ACP-ST.1, ZX-PORITES-

Table 1 BLAST™ result of query sequences aligned with Gene Bank databases

Isolate code	Closest Relative (28S nrDNA gene sequencing)	Identity	Query coverage	Gene Bank Accession number
ZX-ACP-ST.1	*Symbiodinium thermophilum* voucher B359 28S ribosomal RNA gene, partial sequence	99%	98%	KR996308.1
ZX-PORITES -ST.1	*Symbiodinium* sp. clade C partial 28S rRNA gene isolate LII61	99%	98%	AJ830903.1
ZX- PORITES -ST.2	Symbiodinium sp. CG8 partial 28S rRNA gene, isolate CG8	99%	95%	AJ308892.1
ZX- PORITES -ST.3	*Symbiodinium thermophilum* voucher B359 28S ribosomal RNA gene, partial seq uence	98%	99%	KR996308.1

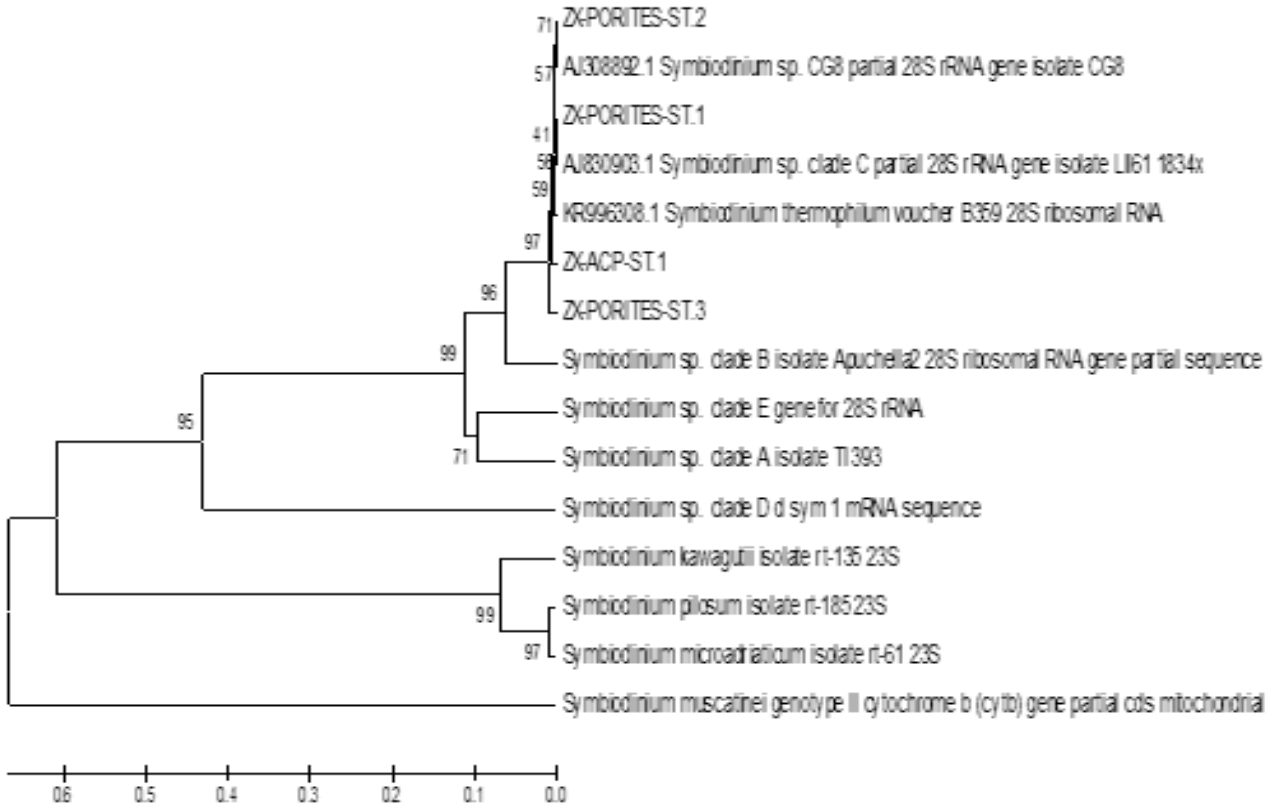

Fig 3 UPGMA tree of the *Symbiodinium* 28S nrDNA gene from scleractinian corals of Biawak Island; Clade controls (A, B, C, D, E, F, G and H) were included in the analysis.

ST.1, ZX-PORITES-ST.2, ZX-PORITES -ST.3) (Fig 3). Phylogenetic tree construction of *Symbiodinium* showed ZX-ACP-ST.1 and ZX-PORITES-ST.3 have a very close relationship with *Symbiodinium thermophilum* voucher B359 28S rRNA. Bootstrap value among these isolates showed a value as high as 97. Bootstrapping value above 70 indicates that the probability of> 95% of these organisms have in common a great and trustworthy (Hillis and Bull 1993).

DISCUSSION

The results showed that *Symbiodinium* populations were uniform among two coral species studied (Acropora and Porites) in Biawak Island and identified as *Symbiodinium* clade C. It is considered that there was flexibility in relationship between hosts and symbionts (Baker 2003). Different coral species in the similar area may have the same type of *Symbiodinium*. The dominance Clade C found in Biawak Island might be explained by its relatively high sea surface tempature (SST). Reported by Silalahi *et al.* (2015), the SST around Biawak Island waters reached 28 – 31 °C.

The presence of clade C identified as *Symbiodinium thermophilum* in Acropora (ST.1) and Porites (ST.3) (>98% identitiy). Specifically, *Symbiodinium thermophilum* identified as a type of symbiotic algae (zooxanthellae) which has a tolerance to high temperatures. It was first identified in 2015. The study was conducted in the Persian Gulf/ Arabian, the sea with the world's hottest temperature (~ 35 °C) and high salinity levels (Hume *et al.* 2015).

In the studies of Baker *et al.* (2004), most coral surveys in the Indo-Pacific showed a predominance of *Symbiodinium* clade C. It generally inhabit tropical latitudes and can be isolated from hard corals (scleractinia), among genus Acropora, Montipora, Pocillopora, Porites and others. There are more than 130 subclades on some types of *Symbiodinium* clade C is known to be thermally-tolerant (Riddle 2016).

As the spontaneous single hit on PCR amplification toward metagenomic DNA in this research, it might be explained that there was dominant species in the environment (Kim and Bae 2011, Agung and Moies 2013) and *Symbiodinium* Clade C might be dominant species in most common coral species of Biawak Island. The fact that a wide range of coral genera from Biawak Island possess this Clade C may provide optimism to the future maintenance of coral diversity in Indonesia in the face of global warming.

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