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ARTICLE *in* JOURNAL OF HELMINTHOLOGY · OCTOBER 2012

Impact Factor: 1.42 · DOI: 10.1017/S0022149X12000715 · Source: PubMed

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Journal of Helminthology (2014) **88**, 50–63
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doi:10.1017/S0022149X12000715

An environmental assessment of the parasite fauna of the reef-associated grouper *Epinephelus areolatus* from Indonesian waters

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(Received 26 March 2012; Accepted 29 August 2012; First Published Online 25 October 2012)

Abstract

Sixty *Epinephelus areolatus* were examined for metazoan fish parasites in Indonesia, off Segara Anakan lagoon, Java and in Balinese waters. The study revealed 21 different parasite species, and 14 new host and locality records. The anisakid nematodes *Anisakis typica* and, for the first time in Indonesia, *Anisakis* sp. HC-2005 were identified by using molecular methods. Ecological parameters were calculated for both sites off the anthropogenically influenced Segara Anakan lagoon and the relatively undisturbed reference site at the southern Balinese coast. The fish from Segara Anakan demonstrated a significantly higher enzymatic activity (Hepatosomatic index) and a significantly reduced number of heteroxenous gut helminths (e.g. the digenean *Didymodiclinus* sp., the nematode *Raphidascaris* sp. and the acanthocephalan *Serrasentis sagittifer*). Other regional differences for *E. areolatus* included ecto-/endoparasite ratio, endoparasite diversity, the parasite species composition and prevalence of infection of the respective parasite species. We applied the stargraph method to visualize observed regional differences using grouper parasites as biological indicators for the sampled coastal ecosystems at both sampling sites.

Introduction

The maritime nation Indonesia can be considered as one of the centres of marine biodiversity (Roberts *et al.*, 2002). With over 80,000 km, it has the second largest coastline of any country worldwide. More than 130 million people live within 50 km of the coast. Marine resources, from fisheries, aqua- and mariculture, play a major role as protein resources, and generate substantial income and commercial profit for the nation (Rückert, 2006; MoMAF, 2009). After the collapse of shrimp cultures (tiger prawns, *Panaeus monodon*) between 1992 and 1998 (Harris, 2001) the culture of coral reef fishes of the genera *Epinephelus*, *Lates* and *Cromileptes* were promoted in Indonesia. As potential mariculture species, groupers

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(e.g. *Epinephelus coioides*, *E. fuscoguttatus*, *Lates calcarifer* and *Cromileptes altivelis*) are the main target species in Indonesia (Rimmer *et al.*, 2004).

Finfish mariculture often underlies economic losses caused by diseases and parasites (Rückert *et al.*, 2009b). Justine *et al.* (2010) recorded 146 parasite species in 28 investigated grouper species (Serranidae, Epinephelinae) from New Caledonia. For example, 23% of the then known 254 fish parasitic trypanorhynch cestodes have been recorded from Indonesian waters (Palm, 2004). Jakob & Palm (2006) recorded 38 fish parasite species from mainly deepwater fishes from Pelabuhan Ratu, southern Java coast. Fish parasitological studies in Indonesia have been intensified in recent years (e.g. Jakob & Palm, 2006; Rückert *et al.*, 2008, 2009a, b, 2010; Palm *et al.*, 2008, 2011; Kleinertz, 2010), resulting in faunistical treatments, revisions and several new species descriptions (e.g. Palm, 2000, 2008; Rückert *et al.*, 2008, 2009a, b, 2010; Bray & Palm, 2009; Kuchta *et al.*, 2009).

Palm *et al.* (2008) summarized the knowledge of the zoonotic nematode genus *Anisakis* from Indonesia, reporting 21 different and also commercially important fish species to be infested with this potentially harmful parasite. The authors studied the scombrid *Auxis rochei rochei* and the carangid *Decapterus russelli* for larval *Anisakis* spp., and detected three different genotypes of *Anisakis typica*. Recently, this common tropical nematode species has also been identified genetically from the South-west Atlantic (Brazil; Nadler *et al.*, 2005), North-west Atlantic (Florida; Mattiucci *et al.*, 2005) and the Mediterranean (North Africa; Farjallah *et al.*, 2008). So far, all authors recorded a high number of anisakids in the studied fish and discussed their zoonotic potential. However, due to high morphological similarities in 'sibling species', exact species identification was not possible at that time. According to a review on anisakid parasites in fish, Klimpel & Palm (2011) recognized eight different *Anisakis* sibling species that could be differentiated by using molecular methods. Theisen (2009) also detected *Anisakis* sp. from Javanese fishes (coastal zone off Segara Anakan lagoon), isolating the worms from three different commercially important fish hosts from the coastal zone off Segara Anakan lagoon.

The mangrove-fringed Segara Anakan lagoon is located in South Central Java, separated from the Indian Ocean by the rocky mountainous island of Nusakambangan (Jennerjahn *et al.*, 2009). For many decades the lagoon has been facing a number of environmental problems due to resource exploitation. Most important are overfishing, logging and high sediment input by the Citanduy River because of poor upland agricultural practices, agricultural runoff, potential pesticide and oil pollution (White *et al.*, 1989; Jennerjahn *et al.*, 2009). Recent investigations stated a high level of water and sediment contamination in the vicinity of the oil refinery of Cilacap, and a wide spectrum of contaminants in the tissues of aquatic organisms (Dsikowitzky *et al.*, 2011). Over 50 organic contaminants were found in water, sediment and macrobenthic invertebrates from the lagoon, mostly polycyclic aromatic compounds (PACs) (Dsikowitzky *et al.*, 2011). The hydrology of the mangrove-fringed Segara Anakan lagoon has a high water mass influx. The high freshwater input, mostly from Citanduy River (Holtermann *et al.*, 2009), is additionally governed by the tides (Jennerjahn *et al.*, 2009), directly linking the lagoon with the coastal ecosystem. The lagoon plays an important ecological role as nursery ground for several fish species, as it supports a large and productive mangrove ecosystem (Romimohtarto *et al.*, 1991).

Palm & Rückert (2009), Palm (2011) and Palm *et al.* (2011) developed a method to use fish parasites as biological indicators serving as biomarkers for the environmental conditions inside Segara Anakan lagoon and inside a grouper mariculture farm in the Thousand Islands, and they also discussed the potential use of grouper parasites to assess environmental change in the wild. *Epinephelus areolatus* is one of the most abundant grouper species in Indonesian fish markets (personal observation) and is also cultivated in commercial fish aquaculture (Heemstra & Randall, 1993; Froese & Pauly, 2010). Data on the parasite infection of this fish species, however, are scarce. The purpose of the present study is a first detailed analysis of the metazoan fish parasite fauna of *E. areolatus* from Indonesia, including new host and locality records. The acquired data are used to assess the environmental status at two different sampling sites, based on the observed regional differences in the parasite fauna of this commercially important fish.

Materials and methods

Collection and examination of fish

Samples were taken within the framework of the SPICE project (Science for the Protection of Indonesian Coastal Marine Ecosystems) during the dry season of 2009. A total of 60 *E. areolatus* were obtained fresh from local fishermen and studied from Javanese (off Segara Anakan lagoon) (108°46′–109°03′W; 08°35′–08°48′S) and Balinese waters (114°25′53″–115°42′40″W; 08°30′40″–08°50″48″S).

Fish were examined immediately after collection, or kept on ice and then frozen (*c.* −20°C) until subsequently dissected in the laboratory. Total fish length (L_T) and weight (W_T), weight at slaughter (W_S) and liver weight (W_L) were measured to the nearest 0.1 cm and 0.1 g (table 1) prior to the parasitological examination (see Rückert *et al.*, 2009a). Skin, fins, eyes, gills, mouth- and gill-cavity were studied for ectoparasites. Inner organs, such as digestive tract, liver, gall bladder, spleen, kidneys, gonads, heart and swim bladder, were separated and transferred into saline solution for microscopic examination; belly flaps and musculature were examined on a candling table. Isolated parasites were fixed in 4% borax-buffered formalin and preserved in 70% ethanol. Musculature was sliced into fillets 0.5–1 cm thick, and pressed between two Petri dishes to identify and isolate the parasites from the musculature. For identification purposes Nematoda were dehydrated in a graded ethanol series and transferred to 100% glycerine (Riemann, 1988). *Anisakis* spp. specimens were fixed and stored in 98% ethanol for subsequent molecular investigation. Digenea, Monogenea and Cestoda were stained with acetic carmine, dehydrated, cleared with eugenol and mounted in Canada balsam. Crustacea were dehydrated and

Table 1. Sampling periods, number (n) of dissected specimens, mean length and mean weight (range in parantheses) of free-living *Epinephelus areolatus*, sampled in dry season (ds) 2009 from Balinese and Javanese waters.

Season	Locality	n	L_T (cm)	W_T (g)	W_S (g)
2009; free-living; ds	SA 4	30	32.4 (27.3–38.1)	460.5 (316.0–740.0)	429.0 (305.9–702.0)
2009; free-living; ds	Bali	30	32.9 (31.0–36.0)	428.3 (350.0–573.0)	397.0 (310.0–551.0)

SA 4, coastline Segara Anakan lagoon.
L_T, total length; W_T, total weight; W_S, weight at slaughter.

transferred into Canada balsam. Parasite identification literature included original descriptions (for details see Palm *et al.*, 2011).

Parasitological parameters

Different ecological parameters were evaluated at both sampling sites, such as diversity indices like the Shannon–Wiener, Evenness and Simpson indices, fish ecological indices such as the Hepatosomatic index, and the parasitological parameters ecto-/endoparasite ratio and different prevalences of infection of metazoan parasites (see Palm & Rückert, 2009; Palm, 2011; Palm *et al.*, 2011). Parasitological calculations were made according to Bush *et al.* (1997).

The present study applies the method of Palm & Rückert (2009) and Palm *et al.* (2011) to monitor the parasite community of groupers in Indonesia. The Berger–Parker index (BP) characterizes the dominance of a respective parasite species within the sample: $BP = N_{max}/N$, with N_{max} being the number of specimens of the most dominant species in relation to the total number of collected parasites within the sample (N). The diversity of the metazoan endoparasite fauna of each fish species was determined by using the Shannon–Wiener diversity index (H') and, in addition to Palm *et al.* (2011), the Evenness (E) of Pielou (Magurran, 1988). Microsporean parasites were recorded qualitatively but not considered in the ecological analysis, because it was not possible to calculate their intensity or density. Based on calculations from Palm & Rückert (2009), and for comparison purposes, the ratio of ecto- to endoparasites was calculated [Ec/En ratio (R) = number of ectoparasite species/number of endoparasite species]. Species groups, which could not be further identified and might represent other recorded taxa (higher taxa such as Nematoda indet.), were omitted from the calculations (see Palm *et al.*, 2011). The Hepatosomatic index was calculated as a descriptor of a possible pollution impact on the fish host, which may affect increasing liver weights (W_L) in relation to the total weight (W_T) of the host [$HIS = (W_L/W_T) \times 100$] (Munkittrik *et al.*, 1994). The Simpson diversity index (D) was considered to be the 'better' diversity index to visualize regional differences $\left[D = 1/\sum_{i=1}^{s}(n_i/N)^2\right]$, where S = the total number of collected parasite species within the sample (ecto- and endoparasites included), N = the total number of collected parasite individuals within the sample and n_i = number of specimens of a single parasite species, i.

Visual integration

The visual integration of the fish parasitological data follows Palm & Rückert (2009) for the prevalence of trichodinids, ecto-/endoparasite ratio and endoparasite diversity after Shannon–Wiener, and fish parasite prevalence data according to Palm *et al.* (2011). Parameters that are herewith suggested to be useful as biological indicators (Simpson diversity index, Evenness index and Hepatosomatic index) were also applied (see Kleinertz, 2010). Values that indicate unnatural environmental conditions are orientated towards the centre of the stargraph. Values representing natural and unaffected environmental conditions are arranged towards the frame of the stargraph.

Molecular identification

Genomic DNA was isolated and purified from 20 individual *Anisakis* larvae using a genomic DNA extraction kit (Peqlab Biotechnology GmbH, Erlangen, Germany) according to the instructions of the manufacturer. The rDNA region comprising the internal transcribed spacer (ITS), ITS-1, 5.8S, ITS-2 and flanking sequences were amplified using the primers TK1 (5′-GGC AAA AGT CGT AAC AAG CT-3′) and NC2 (5′-TTA GTT TCT TTT CCT CCG CT-3′) (Zhu *et al.*, 1998, 2000a, b; Shih, 2004; Klimpel & Palm, 2011) (primer by Eurofins MWG Synthesis, Ebersberg, Germany). Polymerase chain reactions (PCR) (50 μl) included 25 μl 2 × master-mix (Peqlab Biotechnology GmbH) containing deoxynucleoside triphosphates (dNTPs), $MgCl_2$, buffer and *Taq* polymerase, 3 μl of each primer (5 pmol/μl), 14 μl water and 5 μl genomic DNA. Each PCR reaction was performed in a thermocycler (Biometra, Göttingen, Germany) under the following conditions: initial denaturation at 95°C for 15 min, 30 cycles of 94°C for 1 min (denaturation), 55°C for 1 min (annealing), 72°C for 1 min (extension) followed by a final extension at 72°C for 5 min. Samples without DNA were included in each PCR run. PCR products were checked on 1% agarose gels (Cambrex Bio Science, USA; www.cambrex.com/bioproducts). A 100 bp ladder marker (Peqlab Biotechnology GmbH) was used to estimate the size of the PCR products. To identify the anisakid nematodes, the PCR products were purified (EZNA Cycle-Pure Kit (Peqlab Biotechnology GmbH) and double strand sequenced (Seqlab, Göttingen GmbH, Germany). The obtained sequences were identified by BLASTN database search in GenBank and aligned with homologous sequences of *Anisakis* using CLUSTAL W (1.83)

(Thompson *et al.*, 1994) (see accession numbers AB432909.1 for *A. typica* and EU718474.1 for *Anisakis* sp. HC-2005 of the present study, and for the comparison data from Palm *et al.* (2008) see EU346093).

Data analysis

Univariate and multivariate statistical analyses were conducted with the programs Statistica (release 6, StatSoft Inc., Tulsa, Oklahoma, USA) and Primer (release 6, Primer-E Ltd., Ivybridge, Devon, UK), respectively. Homogeneously distributed (Levene's test) and normally distributed data (Shapiro test) were tested for significant differences with the *t*-test or with one- or two-factorial analysis of variances (ANOVA), using Tukey's HSD test for *post hoc* comparisons (see also Nordhaus *et al.*, 2009). The chi-square test was used to compare each year and site with another for all parameters showing parasite prevalence and ecto-/endoparasite ratio. All tests were considered statistically significant at $P < 0.05$. In order to compare the parasite community abundance, data were square-root transformed. A similarity matrix was constructed by using the Bray–Curtis similarity measure (Primer, release 6, Primer-E Ltd.). The relation between samples based on the comparison of similarity matrices was displayed by using multi-dimensional scaling (MDS). One-way analyses of similarity were applied to identify the differences in parasite species composition between the sampling sites (routine ANOSIM, values close to 1 indicate high differences and close to 0 indicate high similarity between species compositions). Routine Simper analysis was applied to test which parasite species contributed most to the shown differences between stations (Nordhaus *et al.*, 2009).

Results

Fish parasitological studies of *E. areolatus* from the coastal zone off Segara Anakan lagoon and in Balinese waters revealed 21 different metazoan parasite species belonging to the taxa Microsporea (1), Digenea (5), Monogenea (1), Cestoda (2), Nematoda (4), Acanthocephala (2) and Crustacea (6) (fig. 1). Fourteen new host and locality records could be established for *E. areolatus* (see tables 2 and 3), mainly in Balinese waters. Furthermore, the morphologically identified *Anisakis* spp. specimens were confirmed as mainly *A. typica* and a single case of *Anisakis* sp. HC-2005, using molecular methods (see fig. 4). The information on prevalence, intensity, mean intensity, mean abundance and relative occurrence of the collected

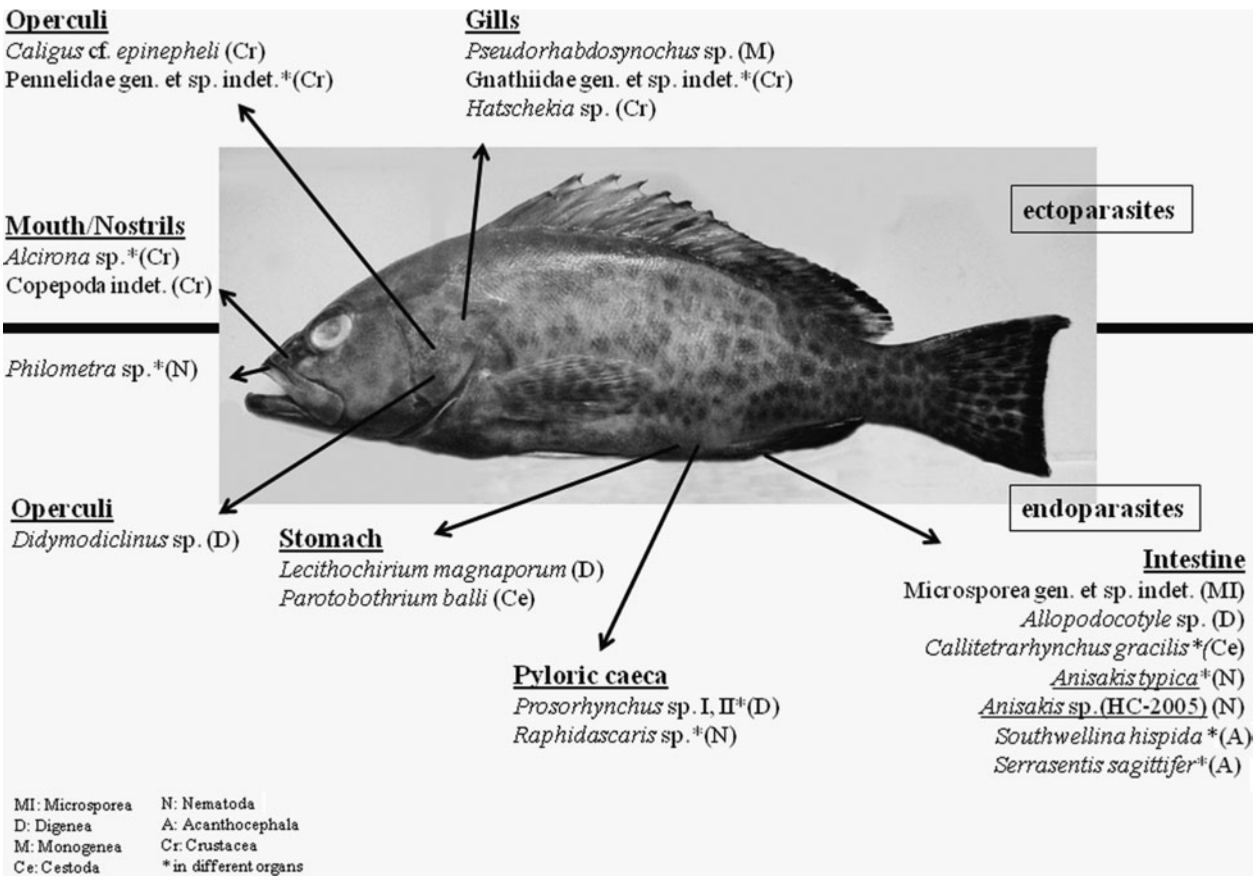

Fig. 1. 'The grouper habitat'. Given are all isolated parasite species/taxa from the investigated *Epinephelus areolatus* from Indonesian waters. Underlined parasites: anisakid nematodes with zoonotic potential. Above the bold, horizontal line, ectoparasites; below the line, endoparasites.

Table 2. A list of parasite species previously identified from *Epinephelus areolatus*.

Parasite species	Locality	References
Digenea		
Cainocraedium epinepheli	Arabian Gulf	Saoud *et al.*, 1986
Hirudinella ventricosa	Southern Ocean	Parukhin, 1976
Lepidapedoides levenseni	Southern Ocean	Parukhin, 1976
Monascus filiformis	Southern Ocean	Parukhin, 1976
Prosogonotrema bilabiatum	Southern Ocean; Kuwait, Arabian Gulf	Parukhin, 1976; Sey *et al.*, 2003
Prosorhynchus chorinemi	Southern Ocean	Parukhin, 1976
Prosorhynchus epinepheli	India	Hafeezullah & Siddiqi, 1970
Prosorhynchus ozakii	Red Sea; Gulf of Aden	Parukhin, 1970
Stephanostonum dentalum	Southern Ocean	Parukhin, 1976
Monogenea		
Diplectanum grouperi	Sumatra, Medan	Bu *et al.*, 1999
Pseudorhabdosynochus coioidesis	Sumatra, Medan	Bu *et al.*, 1999
Pseudorhabdosynochus lantauensis	Sumatra, Medan	Bu *et al.*, 1999
Cestoda		
Callitetrarhynchus speciosus	Arabian Gulf	Palm, 2004
Floriceps sp.	Arabian Gulf, Dubai, Sharjah	Palm, 2004
Pterobothriidae heteracanthum	Arabian Gulf	Palm, 2004
Pterobothrium sp.	Arabian Gulf, Dubai, Sharjah	Palm, 2004
Nematoda		
Philometra cf. *ocularis*	New Caledonia	Moravec & Justine, 2008
Anisakis sp. 2	Bali	Palm *et al.*, 2008
Crustacea		
Ergasilus sp.	Thailand	Purivirojkul & Areechon, 2008

parasite species is summarized in table 3. To analyse the parasite composition and environmental conditions at the respective sampling sites, the ecological parameters as suggested by Palm & Rückert (2009) and Palm *et al.* (2011) were considered as given in table 4. Regional differences for *E. areolatus* were found in terms of species composition (see fig. 2), endoparasite diversity, ecto-/endoparasite ratio, Hepatosomatic index and prevalences of infection of the metazoan parasites (see fig. 3).

Parasite diversity and levels of infection

Epinephelus areolatus from Bali exhibited higher parasite diversity (16 taxa) compared to Segara Anakan lagoon (7 taxa) (table 3). Lower ectoparasite richness was found off Segara Anakan in contrast to Bali (2 versus 6 taxa, table 3). Endoparasite richness was twice as high in Balinese fish, with *A. typica* (Segara Anakan, 73% prevalence) and *Didymodiclinus* sp. (Bali, 100% prevalence) (Berger–Parker index of 0.33–0.50, see table 4) being the most predominant taxa. Ecto-/endoparasite ratios ranged from 0.4 to 0.6, with a lower number of ectoparasites compared to the number of endoparasites. Regional difference of the ecto-/endoparasite ratio was not significant between the two sampling sites. Endoparasite diversity of *E. areolatus* ranged from 0.68 (Segara Anakan) to 0.96 (Bali) for the Shannon–Wiener index. Simpson diversity index for the whole parasite community was higher for grouper parasites off Segara Anakan (3.86) compared to Bali (2.83) (table 4). Highest Evenness for the endoparasites was recorded in Bali (0.44) in contrast to Segara Anakan (0.38), whereas the values for the Hepatosomatic index were regionally contrary (highest in Segara Anakan) (0.50–0.71) (table 4), with a significant difference (ANOVA: $F = 5.92$, $P < 0.05$, *post hoc* test: $P = 0.036$).

Prevalence of infection of the digenean *Didymodiclinus* sp. was significantly higher from Balinese waters (100 versus 0% prevalence, table 3) ($P = 0.000$) compared with Javanese waters. Trypanorhynch cestodes *Callitetrarhynchus gracilis* and *Parotobothrium balli* occurred in Balinese waters (6.7 and 3.3% prevalence, table 3). Because of the low prevalence of infection, regional differences for both cestodes were not significant ($P > 0.05$). Infection with the fish parasitic nematode *Raphidascaris* sp. was 36.7% in Bali (table 3). This displays a significant regional difference ($P = 0.002$). Infection with the acanthocephalan *Serrasentis sagittifer* was significantly higher in *E. areolatus* from Balinese waters (16.7 versus 0% prevalence, table 3) ($P = 0.020$).

Regional parasite composition and visual integration

Significant differences in species composition were found between the sampled *E. areolatus* from Balinese and Javanese coastal waters (ANOSIM: *R*, 0.642; P, 0.1%). Parasite species contributing most to the regional differences off Segara Anakan according to Simper analysis were *A. typica* (46.7%), *Hatschekia* sp. (34.3%) and Gnathiidae gen. et sp. indet. (18.8%), and those of Balinese waters were *Didymodiclinus* sp. (67.6%), *Hatschekia* sp. (12.5%) and *A. typica* (10.9%). There was a clear separation of the parasite communities at both sampling sites (fig. 2). Ten chosen bioindicators are visualized within a stargraph according to Bell & Morse (2003) and Palm & Rückert (2009) (fig. 3).

Molecular nematode identification

Molecular identification of 20 individual *Anisakis* larvae revealed 19 *A. typica*. Multiple sequence alignment of the *A. typica* indicated no genetic diversity within the

Table 3. Prevalence (P), intensity (*I*), mean intensity (*I*m), mean abundance (*A*m) and relative occurrence (*pi* (%)) of the respective parasite species of *Epinephelus areolatus*, sampled from Javanese and Balinese waters.

	SA 4 2009			B 2009		
Parasite species/-taxa	P (%)	*I*m (*I*) *A*m	p_i (%)	P (%)	*I*m (*I*) *A*m	p_i (%)
Ectoparasites						
Pseudorhabdosynochus sp. (M)	–	–	–	3.3	1.0 (1) 0.03	0.1
Alcirona sp. (Cr)*	–	–	–	3.3	1.0 (1) 0.03	0.1
Gnathiidae gen. et sp. indet. (Cr)*	50.0	2.5 (1–7) 1.23	24.3	63.3	4.7 (1–39) 3.00	9.1
Isopoda indet. (Cr)	3.3	1.0 (1) 0.03	0.7	–	–	–
Caligus cf. *epinepheli* (Cr)*	–	–	–	3.3	1.0 (1) 0.03	0.1
Caligidae gen. et sp. indet. (Cr)*	–	–	–	3.3	1.0 (1) 0.03	0.1
Hatschekia sp. (Cr)*	63.3	2.4 (1–6) 1.50	29.6	76.7	5.3 (1–19) 4.07	12.3
Pennelidae gen. et sp. indet. (Cr)*	–	–	–	33.3	6.7 (1–22) 2.23	6.8
Copepoda indet. (Cr)*	6.7	2.5 (1–4) 0.17	3.3	–	–	–
Endoparasites						
Microsporea gen. et sp. indet. (MI)	–	–	–	6.7	2.0 (2) 0.13	0.4
Didymodiclinus sp. (D)	–	–	–	100.0	16.4 (4–68) 16.40	49.6
Allopodocotyle sp. (D)*	–	–	–	3.3	1.0 (1) 0.03	0.1
Prosorhynchus sp. I (D)	6.7	3.5 (1–6) 0.23	4.6	–	–	–
Prosorhynchus sp. II (D)	–	–	–	23.3	5.9 (1–21) 1.37	4.1
Lecithochirium magnaporum (D)	3.3	1.0 (1) 0.03	0.7	–	–	–
Callitetrarhynchus gracilis (Ce)*	–	–	–	6.7	1.5 (1–2) 0.10	0.3
Parotobothrium balli (Ce)*	–	–	–	3.3	2.0 (2) 0.07	0.2
Cestoda indet. (Ce)	–	–	–	6.7	2.0 (2) 0.13	0.4
Anisakis typica (N)	73.3	2.3 (1–12) 1.67	32.9	73.3	5.4 (1–28) 3.97	12.0
Anisakis sp. (HC-2005) (N)*	3.3	1.0 (1) 0.03	0.7	–	–	–
Raphidascaris sp. (N)*	–	–	–	36.7	2.9 (1–6) 1.07	3.2
Philometra sp. (N)	–	–	–	10.0	1.0 (1) 0.10	0.3
Nematoda indet. (N)	3.3	3.0 (3) 0.10	2.0	10.0	1.3 (1–2) 0.13	0.4
Serrasentis sagittifer (A)*	–	–	–	16.7	1.0 (1) 0.17	0.5
Southwellina hispida (A)*	3.3	1.0 (1) 0.03	0.7	–	–	–
Acanthocephala indet. (A)	3.3	1.0 (1) 0.03	0.7	–	–	–
Ectoparasite species		2			6	
Endoparasite species		5			10	
Ecto-/endoparasite ratio		0.40			0.60	

SA 4, coastline Segara Anakan lagoon; B, Bali; *, new host record; A, Acanthocephala; Ce, Cestoda; Cr, Crustacea; D, Digenea; M, Monogenea; MI, Microsporea; N, Nematoda.

sequenced rDNA region. The ITS-1, 5.8S, ITS-2 rDNA was identical for all investigated specimens at both sampling sites, compared with the reference sequence AB432909. No difference was observed between our material and *Anisakis* (*typica*) sp. 2 genotype as given in Palm *et al.* (2008) (EU346093). A single specimen differed from the 19 identified *A. typica* by amplicon size and sequence. This individual was identified as *Anisakis* sp. HC-2005 (EU718474). A sequence alignment of the *A. typica* sequences from Java and Bali (AB432909) with the genotype *Anisakis* sp. HC-2005 is given in fig. 4. The total lengths of the PCR product from *Anisakis* sp. HC-2005 was 774 bp (ITS-1 358 bp, ITS-2 259 bp) compared with 859–860 bp for *A. typica. Anisakis* sp. HC-2005 was isolated first from Mauritian waters, and the present finding is a new locality record for this yet unspecified genotype from Indonesian waters.

Discussion

The Indonesian Archipelago is known to harbour an extremely high marine biodiversity on a global scale (Gray, 1997; Myers *et al.*, 2000; Allen & Werner, 2002; Roberts *et al.*, 2002; Palm, 2004), based on its geographical position and geological history (Rückert *et al.*, 2010). Earlier studies proved high parasite diversity for Indonesian waters, although the number of investigations is relatively low. According to Jakob & Palm (2006), up to 400 different parasite species have been recorded from Indonesian marine waters in 242 investigated fish species. Palm (2004) recorded 23% of the then known trypanorhynch cestode fauna from Indonesian waters.

The parasite fauna of some commercially important epinephelids from Indonesian waters, such as *E. coioides* and *E. fuscoguttatus*, has been studied in recent years (see Yuniar *et al.*, 2007; Palm & Rückert, 2009; Rückert *et al.*, 2009a, b, 2010). This contrasts with *E. areolatus*, a species with only few parasite records worldwide (19 records, Hafeezullah & Siddiqi, 1970; Parukhin, 1970, 1976; Sey *et al.*, 2003; Moravec & Justine, 2008; Purivirojkul & Areechon, 2008; 5 of them from Indonesian waters) (Bu *et al.*, 1999, Palm, 2004) (see table 2). So far, ten studies on the parasite fauna of *E. areolatus* revealed 19 parasite species/taxa, belonging to the Digenea (9 species), Monogenea (3 species), Cestoda (4 species), Nematoda (2 species) and a single crustacean (see table 2). Within the present study, 14 new host records could be established.

Table 4. Parasitological and ecological metrics from the studied *Epinephelus areolatus* used as biological indicators to visualize regional environmental change.

Parameter	SA 4 2009 ×	SA 4 2009 ± SE	B 2009 ×	B 2009 ± SE
Hepatosomatic index	0.71	0.09	0.50	0.04
Condition factor	1.33	0.03	1.20	0.03
Shannon–Wiener (endoparasites)	0.68		0.96	
Shannon–Wiener (total)	1.50		1.61	
Evenness (endoparasites)	0.38		0.44	
Evenness total	0.65		0.60	
Ec/En ratio	0.40		0.60	
Simpson index	3.86		2.83	
Berger–Parker index	0.33		0.50	
Dominant species	*Anisakis typica*		*Didymodiclinus* sp.	
Trichodinids (P %)	nd		nd	
Didymodiclinus sp. (P %)	0		100	
Callitetrarhynchus gracilis (P %)	0		6.7	
Parotobothrium balli (P %)	0		3.3	
Raphidascaris sp. (P %)	0		36.7	
Serrasentis sagittifer (P %)	0		16.7	

SE, standard error; SA 4, coastline Segara Anakan lagoon; B, Bali; Ec/En, ecto-/endoparasite; nd, no data.

Different parasite species were applied within the stargraph system (see Palm & Rückert, 2009). The digenean trematode *Didymodiclinus* sp. has already been recorded from Segara Anakan lagoon for *E. coioides* (Rückert *et al.*, 2009a), indicating lower host specificity and probably a wide zoogeographical distribution in Indonesia. Groupers represent final hosts in the life cycle of this parasite, becoming infected via smaller fishes, possibly *Johnius coitor* and/or *Nemipterus japonicus* (Rückert *et al.*, 2009b), both abundant hosts for that digenean at least in the Segara Anakan region (Yuniar, 2005; Rückert, 2006; Rückert *et al.*, 2009b; Theisen, 2009). According to Palm (2004) *Parotobothrium balli* is widely distributed in Indonesian waters. The genus *Callitetrarhynchus* has been isolated from *N. japonicus* off Segara Anakan by Theisen (2009). *Raphidascaris* sp. has been already recorded for *E. coioides* at both sampling sites with a similar prevalence of infection (2.9–8.6% in Segara Anakan region versus 20.0–28.6% in Bali) (Kleinertz, 2010). The recorded *Serrasentis sagittifer* (Acanthocephalan) had already been found in the Segara Anakan region by Theisen (2009) in three different fish species (*J. coitor*, *N. japonicus* and *Platycephalus arenarius*; see also Verweyen *et al.*, 2011). Rückert (2006) and Rückert *et al.* (2009b, 2010) suggested the epinephelids *E. coioides* and *E. fuscoguttatus* as paratenic and/or transport hosts for these species.

Because of its zoonotic importance, the first Indonesian research on anisakids in finfish started in Jakarta Bay around the 1980s (Burhanuddin & Djamali, 1978, 1983; Hadidjaja *et al.*, 1978; Hutomo *et al.*, 1978; Ilahude, 1980; Petersen *et al.*, 1993; Jakob & Palm, 2006; Palm *et al.*, 2008). Hadidjaja *et al.* (1978), Hutomo *et al.* (1978) and Ilahude *et al.* (1978) studied anisakid nematodes from East Sumatra and the North Java coast, and Burhanuddin & Djamali (1978) used anisakid nematodes for stock separation in the roundscad *Decapterus russelli* in the Java Sea. Ilahude *et al.* (1978) and Burhanuddin & Djamali (1983) recorded 23 different fish species from the northern Java coast that were infected with anisakid nematodes, including four different *Epinephelus* species. However, no infection was recorded from the fish musculature, most probably due to the preservation of the fish in 10% formalin. Other *Anisakis* sp. records originate from Indonesian mariculture (e.g. Asmanelli *et al.*, 1993). Palm *et al.* (2008) identified *A. typica* and *Anisakis* sp. 1 and 2 from *Auxis rochei rochei*, *Caesio cuning*, *Coryphaena hippurus* and *E. areolatus* by using molecular methods. They stated that larval *A. typica* or closely related siblings infest a wide range of clupeiform, perciform and also gadiform fish in warmer waters, and seem to be the most common anisakid nematodes in the Indonesian region. *Anisakis typica* is a common parasite of various dolphins of warmer temperate and tropical waters belonging to the families of Delphinidae, Phocoenidae and Pontoporidae (see Mattiucci *et al.*, 2002). A total of 21 fish species were known to harbour *Anisakis* spp. or *A. typica* in Indonesia, with 34 fish species known to be infected with anisakid nematodes (Palm *et al.*, 2008). The present study adds a new locality record from off Segara Anakan lagoon. *Anisakis* sp. HC-2005 is herewith identified from Indonesian waters for the first time. This genotype requires further attention, especially in order to estimate the potential risk for the fish consumer caused by fishery products from this geographical region that might include this nematode.

Fish parasites have been demonstrated to be useful as biological indicators to monitor environmental change (Vidal-Martínez *et al.*, 2010). They have been used to indicate, for example, bacterial biomass (Palm & Dobberstein, 1999; Palm & Rückert, 2009), heavy metals (Sures & Siddall, 2003) or environmental stress (Landsberg *et al.*, 1998) (for reviews on parasitological bioindicators, see Lafferty, 1997; Marcogliese & Cone, 1997; Overstreet, 1997; Williams & MacKenzie, 2003; Marcogliese, 2005; Palm, 2011). Sasal *et al.* (2007) utilized fish parasites to detect anthropogenic influences (urban and industrial pollution) in coral reefs, and Lafferty *et al.*

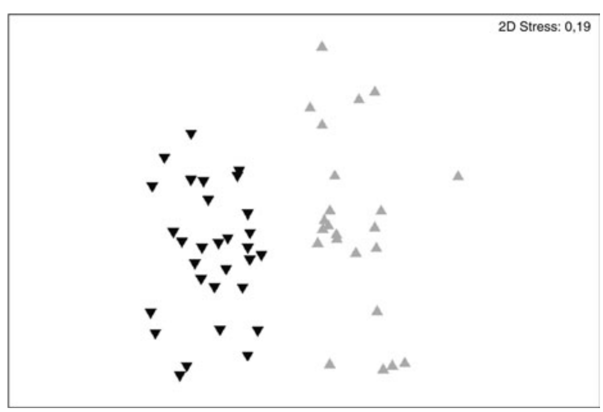

Fig. 2. Multi-dimensional scaling (MDS) plot for the parasite community of *Epinephelus areolatus* from Javanese and Balinese waters during the dry season, 2009. ▼, Bali; ▲, coastline of Segara Anakan lagoon.

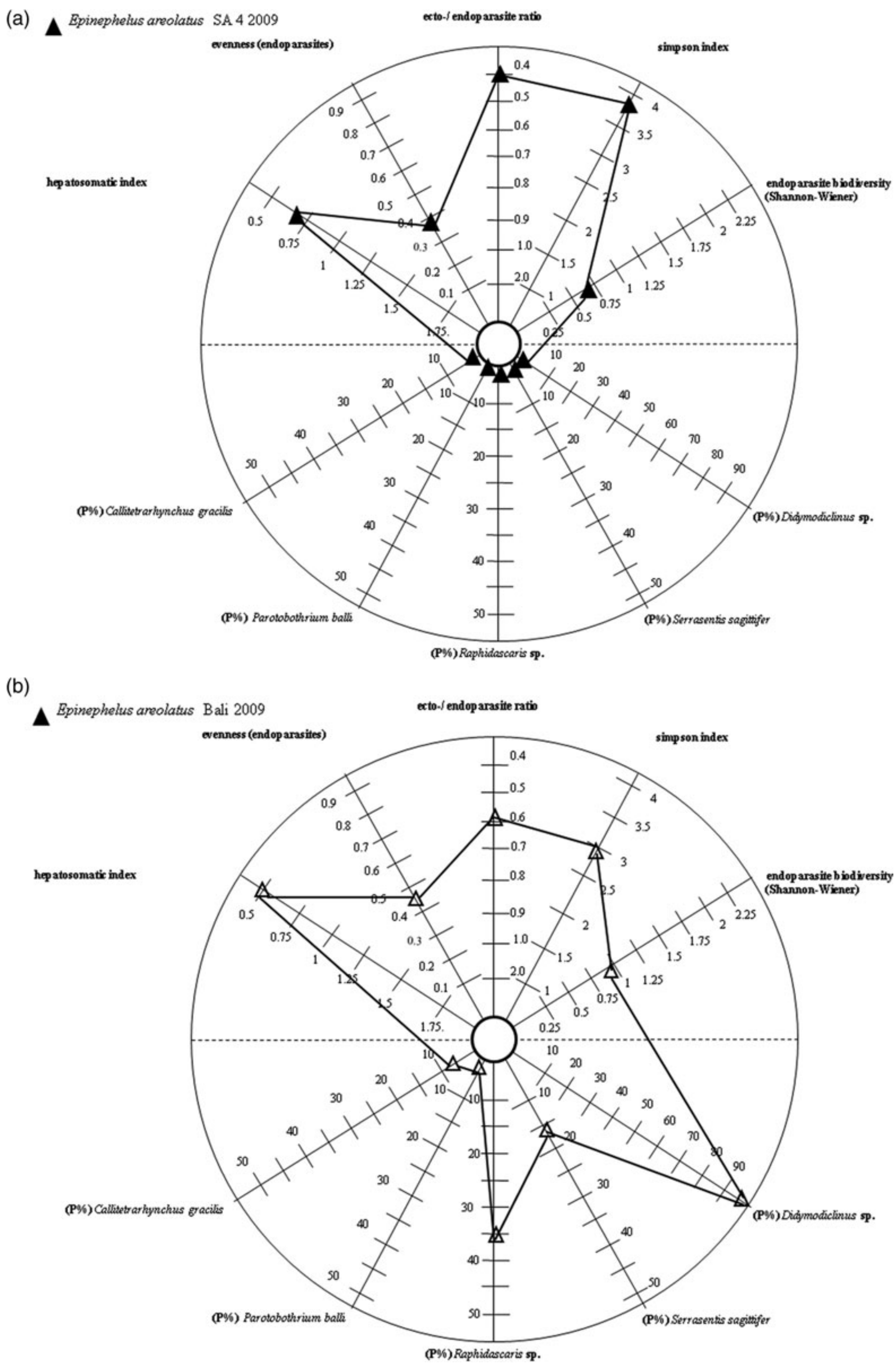

Fig. 3. Visual indicator integration for *Epinephelus areolatus* from Javanese (a) and Balinese (b) waters during the dry season, 2009. SA 4, coastline Segara Anakan lagoon.

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                         [ITS-1
Seq1_A.typ_             ATCGAGCGAATCCAAAACGAAAAAGTCTCCCAACGTGCATACCGCCCATTTACATGTTGT 60
Seq6_A.sp.HC_2005_      ATCGAGCTA-TCCAAAACGAAAAAGTCTCCCAACGTGCATACCATCCATTTGCATGTTGT 59
                        ******* * *********************************  ****** ********
Seq1_A.typ_             TGTGAGCCGCACGGAAACTCGTACACGTTTGTGGTGGTGATAGCCGTCTGCTGTGCGTTC 120
Seq6_A.sp.HC_2005_      CGTGAGCCGCATGGAAACTCATACACGC-----GTGGTGGTAGCCGTCTGCTGTGC-TTT 113
                         ********** ******** ******      ****** **************** **

Seq1_A.typ_             GTTGGGCAGACAATGGCTTACGAGTGGCTGTGCGCTTGTTGAACAACGGTGACCAATTTG 180
Seq6_A.sp.HC_2005_      ATCGTGCAGACAATGGCTTATGAGTGGCTGTGCGCTTGTTGAACAACGGTGACCAATTTG 173
                         * * *************** ***************************************

Seq1_A.typ_             GCGTCTACGCCGTATCTAGCCTCCGCCTGGACCGTCGGTAGCGATGAAAGATGCGGAGGA 240
Seq6_A.sp.HC_2005_      GCGTCTACGCCTCATCTAGCTTCCGCCTGGACCGTCGGTAGCGATGAAAGATGGGGAGAA 233
                        ***********  ******* ******************************** **** *

Seq1_A.typ_             AGTTCCTC-GT-----------------CAGAGTTGAGCAGACTTAATGAGCCACGCTCT 282
Seq6_A.sp.HC_2005_      AGTTCCTTTGTTTTGGTTCATTCCAGCGCAAAGTTGAGCAGACTTAATGAGCCACGCTC- 292
                        *******  **                 ** ****************************

Seq1_A.typ_             AGGTGGCCGCCAGAACCCAAAACACACCAATTGTTGTCATTTGACATTGTTGA-----TG 337
Seq6_A.sp.HC_2005_      -GGTGGCCGCCAAAACCCAAAACACAACCAGTCT----ATTTGACATTGTTCAGTATGTG 347
                         *********** ************* * * *    ************* *     **
                                     [5.8S
Seq1_A.typ_             ATGATTATGTACAAATCTTGGCGGTGGATCACTCGGTTCGTGGATCGATGAAGAACGCAG 397
Seq6_A.sp.HC_2005_      CTATTAATGTACAAATCTTGGCGGTGGATCACTCGGTTCGTGGATCGATGAAGAACGCAG 407
                         *  * ******************************************************

Seq1_A.typ_             CCAGCTGCGATAAATAGTGCGAATTGCAGACACATTGAGCACTAAGAATTCGAACGCACA 457
Seq6_A.sp.HC_2005_      CCAGCTGCGATAAATAGTGCGAATTGCAGACACATTGAGCACTAAGAATTCGAACGCACA 467
                        ************************************************************
                                                                            [ITS-2
Seq1_A.typ_             TTGCGCTATCGGGTTCATTCCCGATGGCACGTCTGGCTGAGGGTCGAATTGTGCTAGAGC 517
Seq6_A.sp.HC_2005_      TTGCGCTATCGGGTTCATTCCCGATGGCACGTCTGGCTGAGGGTCGAATTATGGCAAACT 527
                        ************************************************** **  * *

Seq1_A.typ_             ATCTTTGCAATCACTTCTCTCAGATTGTGATTGTGAAGCATTCGGCGAGCGATTGTTGTC 577
Seq6_A.sp.HC_2005_      ATCTTCGAAGTTTC--------------------GAAGCTTCCGGCAAGCAGTCGTT--- 564
                        ***** * * *  *                    ***** * **** ***  * ***

Seq1_A.typ_             GTGTTGTTGCTTAAGGTGACGATTGAATCGGCACCGCGCGACACGACACGGTTCCTTGCT 637
Seq6_A.sp.HC_2005_      GTGTTGTTG------------GTCCAATCGACAATACG---------GCGTCTCCTTGCT 603
                        *********             *  ***** **   **          **  ********

Seq1_A.typ_             TAGTTTGATGAACAAAAAGACGTCCCGCACACCCAACGTCTGCTAAACACTAGACTAGAG 697
Seq6_A.sp.HC_2005_      TAGTT-GTTGTGAAGAGTGTTGTGTGAAACTCTC----------------TAGAC----G 642
                        ***** * **   * *  *  **     ** * *                *****    *

Seq1_A.typ_             CTGGTGTCTAGAGGTGTTGGGTGTGATTTTGATGGTCACAAAAGTGCCGCCATTTCATAG 757
Seq6_A.sp.HC_2005_      TTAACACCGTACGGCG------GTGATATTGGTGGTCGCGTA-----TGCCGCTTCATAG 691
                         *     *    ** *      ***** *** ***** *  *      ***  *******

Seq1_A.typ_             TGGCAACAACCAGCATACGTCTATGATACTAGTAGGTTGGCTGGTTGATGAAACGGCAAC 817
Seq6_A.sp.HC_2005_      GGGCAACAACCAGCATACG--------ACAAGT---TCGGTTGGTTGATGAACTGGCAAC 740
                         ******************        ** ***   * ** ***********  ******

Seq1_A.typ_             GGAATGTGCGCATGCATGTGATCGAGAAGCGATAATGTTCGTA 860
Seq6_A.sp.HC_2005_      GGAGTA-GTG-ATCGATGTGATCAAGAA-------TGTTCGTA 774

                        *** *  * * **  ******** ****       ********
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Fig. 4. Alignment of the ITS-1, 5.8S and ITS-2 region from larval *Anisakis typica* (Seq1_A.typ) and larval *Anisakis* sp. HC-2005 (Seq6_A.sp.HC_2005) of *Epinephelus areolatus* from the coastline of Segara Anakan.

(2008b) proposed them as a convenient method for assessing spatial variation in their final host distribution. Because their occurrence is dependent on the composition of the food web, a low biodiversity could impair parasite transmission by reducing the availability of hosts required by parasites with complex life cycles (Marcogliese, 2005; Lafferty *et al.*, 2008b). Consequently, heteroxenous fish parasites (multiple hosts) with complex life cycles are generally useful tools to indicate food web relationships in unaffected marine habitats (e.g. Palm, 1999; Klimpel *et al.*, 2006; Palm *et al.*, 2007; Lafferty *et al.*, 2008a). Thus, they reflect the status of the marine food web that might be affected through environmental change. The occurrence of endoparasites decreases in polluted waters (Nematoda; Kiceniuk & Khan, 1983), whereas ectoparasitic parasites such as monogeneans are increasing (Monogenea, Khan & Kiceniuk, 1988; *Trichodina*, Khan, 1990; Palm & Dobberstein, 1999; Ogut & Palm, 2005). These effects can be used in order to analyse and monitor environmental conditions, especially where other information, such as water quality parameters, pollution or anthropogenic influences, are scarce.

Ten different bioindicators were chosen and visualized in a single stargraph, according to Bell & Morse (2003) and Palm & Rückert (2009), demonstrating regional differences in the parasite infection of *E. areolatus* at both sampling sites (fig. 3). We applied: (1) the ratio of ecto- versus endoparasites, which has been suggested to indicate the common parasite infection of free-living groupers in two Indonesian coastal ecosystems (Vidal-Martínez *et al.*, 1998; Dzikowski *et al.*, 2003; Jakob & Palm, 2006; Rückert *et al.*, 2009a); (2) the endohelminth parasite diversity, to indicate successful parasite transmission and host biodiversity (Rückert *et al.*, 2009a; Palm *et al.*, 2011); (3) the Evenness of endohelminths, to assess the availability of potential host organisms in the surroundings and/or other external factors resulting in low diversity and high dominance levels (Kleinertz, 2010); (4) the Simpson diversity index for both ecto- and endoparasite species (see 2 and 3 above); (5) the Hepatosomatic index to indicate high enzymatic activity in relation to stress (e.g. caused by pollution) (Munkittrik *et al.*, 1994). According to Lafferty *et al.* (2008b), using tetraphyllidean cestodes of elasmobranchs as biological indicators, and Dzikowski *et al.* (2003) on the transmission of parasites with complex life cycles through a chain of different host species, we applied endoparasite prevalences to indicate regional differences at the chosen sampling sites. Moreover, we applied: (6) the prevalence of the digenean *Didymodiclinus* sp. (Hechinger *et al.*, 2007); (7 and 8) the prevalence of trypanorhynch cestodes, *C. gracilis* and *P. balli*, that utilize elasmobranchs as final hosts (Palm, 2004; Lafferty *et al.*, 2008b); (9) the prevalence of the nematode *Raphidascaris* sp. (Palm & Rückert, 2009, Palm *et al.*, 2011); and (10) the prevalence of the acanthocephalan *S. sagittifer* (Dzikowski *et al.*, 2003; Marcogliese, 2003, 2005; Hechinger *et al.*, 2007; Lafferty *et al.*, 2008b; Kleinertz, 2010; Palm, 2011). Four of the chosen bioindicators demonstrated significant regional differences (Hepatosomatic index, prevalences of *Didymodiclinus* sp., *Raphidascaris* sp. and *S. sagittifer*). According to the chosen parasite bioindicators, the environmental conditions at both studied sampling sites differed significantly, reflecting either different abiotic conditions, such as regular salinity changes or anthropogenic pollutants (in Segara Anakan, see below), and/or biotic conditions resulting in different food webs and availability of prey organisms (off the Balinese coast).

Palm *et al.* (2011) applied the same methodology to monitor the parasite community of groupers from a mariculture facility in the Thousand Islands, Indonesia. By using six different parasite metrics from *E. fuscoguttatus* presented in a single figure, a significant change in parasite composition and abundance was recorded over six consecutive years. Their study was the first to use fish parasites to assess long-term changes in holding conditions within a commercially run tropical finfish mariculture farm. Their results suggested that groupers can also be used as biomarkers to monitor environmental change in the wild, requiring more detailed information on the parasite systematics and especially on taxonomy. Being directly linked to the surrounding invertebrate and vertebrate communities, fish parasites with multiple-host life cycles (Hechinger *et al.*, 2007) are sensitive bioindicators of aquatic ecosystem health (Overstreet, 1997; Dzikowski *et al.*, 2003). They require unaffected environmental conditions to get access to the full range of potential parasite intermediate hosts, whereas monoxenous parasite species (single-host) may persist in highly perturbed, extreme environments (Dzikowski *et al.*, 2003; Hechinger *et al.*, 2007). Consequently, we can conclude that the observed parasite fauna in *E. areolatus* from Balinese waters reflects merely unaffected environmental conditions for this species in Indonesian coastal waters, while the parasite fauna off Segara Anakan lagoon is widely influenced by the specific environmental conditions at that sampling site (see below). Other available methods such as multi-dimensional scaling (MDS) for community analyses (see fig. 2) are likewise useful tools to demonstrate significant differences in parasite composition at different sampling sites. However, they fall short of providing any possible reasons for the observed differences.

According to the stargraphs, groupers from both sampling sites originate from two different habitats, and the differences in the metazoan parasite fauna are either a result of altered feeding behaviour or of food composition. For decades, Segara Anakan lagoon has been facing a number of environmental problems due to extensive resource exploitation (Jennerjahn *et al.*, 2009). Most important are overfishing, logging of mangrove wood, high sediment input through the Citanduy River because of poor upland agricultural practices, agricultural runoff, potential pesticide, oil pollution and organic contaminants in water, sediment and macrobenthic invertebrates (White *et al.*, 1989; Jennerjahn *et al.*, 2009; Dsikowitzky *et al.*, 2011). Because of the hydrological impact of the nearby mangrove-fringed Segara Anakan lagoon with high water mass influx, freshwater input mainly from Citanduy River (Holtermann *et al.*, 2009), tidal variability (Jennerjahn *et al.*, 2009) and two water exchange channels with direct connection to the ocean (Holtermann *et al.*, 2009), the coastal zone of Segara Anakan is more heavily influenced compared to the sampled Balinese waters. We are aware that it is difficult to correlate directly the observed parasite communities, without replication, to specific environmental or anthropogenic factors at both sampling sites. However, both localities that likewise offer suitable living conditions for *E. areolatus*, are distinctly different, a situation that is reflected by the recorded parasite fauna.

Acknowledgements

We are thankful to Professor Dr E. Yuwono from Universitas Jenderal Soedirman (UNSOED), Purwokerto, Java, for providing laboratory space and institutional support. Special thanks to Mr A. Rinto Suncoko and Mr E. Hermawaran from UNSOED for their personal initiative and organizational support during fieldwork. We are thankful to Professor Dr S. Klimpel (Senckenberg Biodiversity and Climate Research Centre (BIK-F), Frankfurt, Germany) for supporting the molecular nematode identification. Financial support was provided by the German Academic Exchange Service (DAAD), the German Ministry for Education and Research (BMBF Grant No. 03F0471) within the framework of the joint Indonesian–German research programme SPICE (Science for the Protection of Indonesian Coastal Marine Ecosystems, RISTEK research permit No. Surat Izin: 0037/FRP/SM/II/09) (S.K., H.W.P.), and the German Research Council (DFG PA 664/4-1 and 2, 664/6-1) for the molecular study (H.W.P.). This is publication No. 2 under the Memorandum of Understanding between the Faculty of Veterinary Medicine, Udayana University, Bali and Aquaculture and Sea-Ranching, University of Rostock, Germany, in order to promote research on fish parasites and biodiversity data collection in Indonesia.

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