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A cryptic species in the *Pteroclava krempfi* species complex (Hydrozoa, Cladocorynidae) revealed in the Caribbean

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Abstract Symbiotic relationships on coral reefs involving benthic hosts other than scleractinian corals have been poorly investigated. The hydroid *Pteroclava krempfi* is a widespread species known to be mainly associated with alcyonacean octocorals in the Indo-Pacific. In the present study, *P. krempfi* was discovered in association with octocorals of the genus *Antillogorgia* (Gorgoniidae) at two localities in the Caribbean Sea (St. Eustatius in the eastern Caribbean and Bocas del Toro in the western part), updating its host range with an additional genus and family. The Caribbean specimens showed no morphological differences and the shape of their polyps was consistent with the original *P. krempfi* description. A multi-locus phylogeny reconstruction of the *P. krempfi* species complex based on both mitochondrial and nuclear loci revealed three separate molecular clades. Two of them were composed of *P. krempfi* associated with the families Plexauridae and Alcyoniidae from the Maldives, whereas a new highly supported molecular lineage included all Caribbean specimens of *P. krempfi* associated with the family Gorgoniidae. These three divergent molecular clades represent distinct cryptic taxa within the *P. krempfi* species complex, in which the main interspecific difference consists of their host families.

Keywords Symbiosis · Hydroids · *Antillogorgia* · Systematics · 16S · COI · Diversity

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Introduction

Interspecific associations are very common in coral reef ecosystems and many of these involve small animals that live as endobionts in the skeletons of scleractinian corals, as epibionts on their surface or in between their tentacles (Hoeksema and Fransen 2011; Stella et al. 2011; Hoeksema et al. 2012; Bos and Hoeksema 2015). Hence, these corals construct a multitude of habitats for a diverse cryptobenthic fauna estimated to be over 172,000 species, belonging to a multitude of phyla (Reaka-Kudla 1997; Ruppert et al. 2004). However, invertebrates have received little attention, even though they account for the vast majority of animal species on coral reefs (Stella et al. 2011; Fisher et al. 2015). Moreover, many nominal species of invertebrates contain a cryptic diversity that can be unveiled only by using molecular techniques (Knowlton 2000), making the potential number of species living in tropical ecosystems such as coral reefs higher than previously estimated (Bickford et al. 2007; Fisher et al. 2015). Research on cryptic species has grown dramatically over the last three decades, and the increasing availability of DNA sequences has allowed taxonomists to unravel the hidden genetic diversity of several taxa (Bickford et al. 2007; Appeltans et al. 2012). This is also the case for hydrozoans, among which cryptic species seem to be common, especially for those showing few distinctive morphological features (e.g. Miglietta et al. 2009; Moura et al. 2012; Montano et al. 2015b; Maggioni et al. 2016).

Hydrozoans can live in symbiosis with several other organisms, including other cnidarians, such as scleractinian corals and octocorals (Puce et al. 2008a). To date, 13 nominal species of hydrozoans are known to live in association with at least 22 species of octocorals (Maggioni et al. 2016), which is less than the 34 scleractinian host species counted recently (Montano et al. 2015c, 2016). Among these hydrozoans, the worldwide-distributed species *Pteroclava krempfi* (Billard, 1919) is associated with seven alcyonacean genera belonging to two families, namely, *Cladiella*, *Lobophytum*, *Sarcophyton*, *Sinularia* belonging to the family Alcyoniidae, and *Astrogorgia*, *Plexaurella*, *Paraplexaura* of the family Plexauridae (Billard 1919; Boero et al. 1995; Puce et al. 2008b; Varela 2010; Seveso et al. 2015; Maggioni et al. 2016). Recently, Maggioni et al. (2016) provided a preliminary molecular study of *P. krempfi* (sensu lato) associated with alyonaceans from the Maldives, observing an evident species crypticism likely driven by host-specificity. However, little is known about *P. krempfi* in the Caribbean, and except for the first occurrence reported by Varela (2010) from Cuba, no ecological or molecular data are available from this region. The present study is aimed at clarifying the molecular diversity and relationships of *P. krempfi* based on specimens collected from two different Caribbean localities in order to shed more light on the cryptic diversity in the *P. krempfi* species complex.

Materials and methods

Octocoral-associated *Pteroclava* specimens were collected during two separate biodiversity expeditions, one conducted at St. Eustatius (Dutch Caribbean) in June 2015 and one at Bocas del Toro (Panama) in August 2015 (Fig. 1). After being observed in situ, host colonies with hydroids were collected by scuba diving and placed in outdoor tanks. For each host colony, small fragments bearing hydrozoans were fixed in 4 % formalin for morphological analyses and in 99 % ethanol for molecular analyses. Polyps were collected one by one using syringe needles and micropipettes. Morphological observations and measurements were performed using a Leica EZ4 D stereomicroscope and a Zeiss Axioskop 40 microscope, both equipped with a Canon G11 camera. All hydrozoans

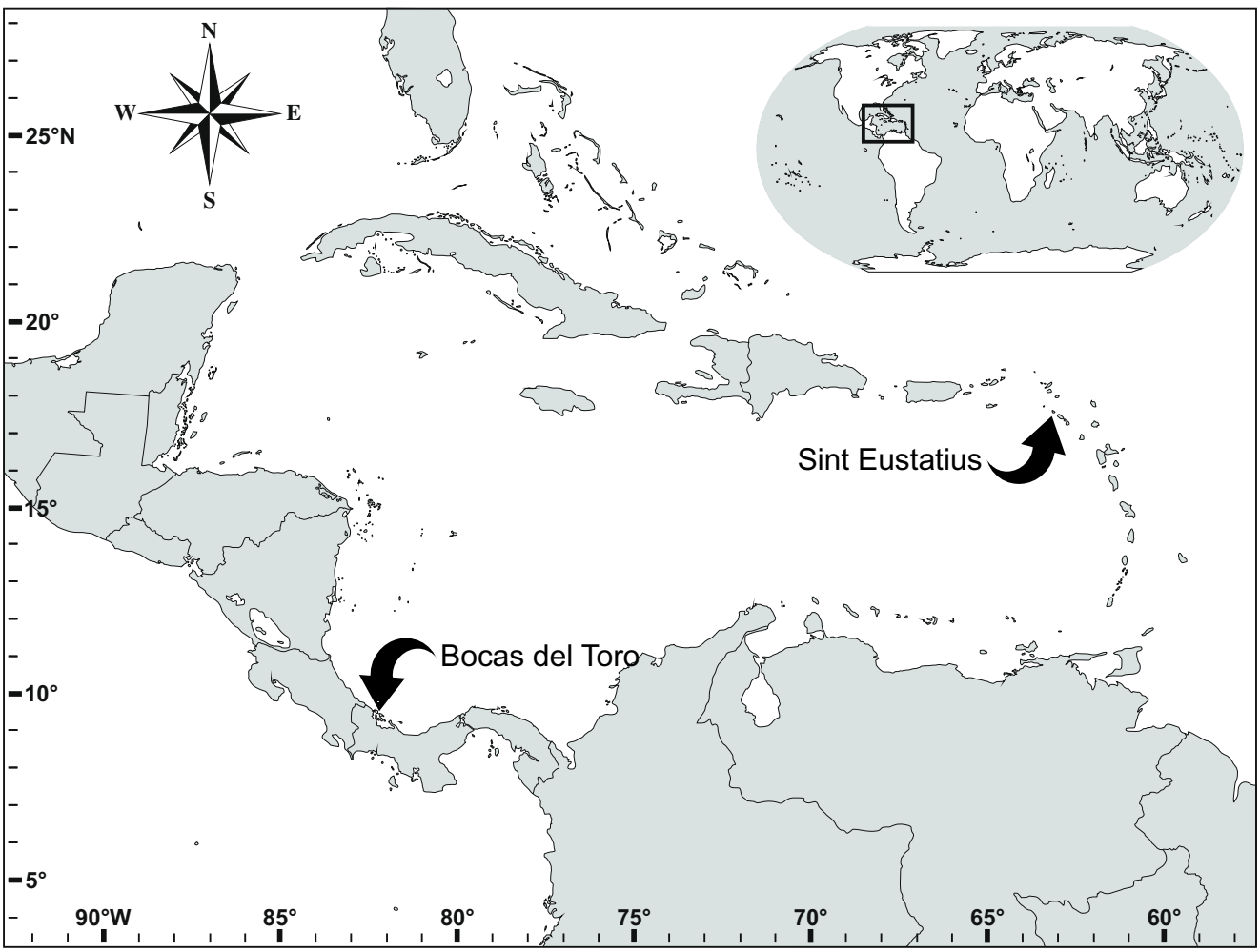


Fig. 1 Map of the Caribbean, with the *arrows* indicating the two sampling localities

were identified to the species level according to Boero et al. (1995), whereas the octocorals were identified morphologically at the genus level according to Fabricius and Alderslade (2001) and Williams and Chen (2012).

DNA of ethanol-fixed *Pteroclava* specimens from St. Eustatius ($n = 2$) and Bocas del Toro ($n = 1$) was extracted following a protocol modified from Zietara et al. (2000). Four molecular markers were amplified: a portion of the nuclear 28S rRNA (~1700 bp) and 18S rRNA (~1700 bp), and a portion of the mitochondrial 16S rRNA (~400 bp) and COI (~700 bp). The polymerase chain reaction (PCR) assays were set up using the same protocols and primers described in Maggioni et al. (2016), Medlin et al. (1988), Fontana et al. (2012) and Folmer et al. (1994), respectively. All PCR products were purified and directly sequenced in forward and reverse directions using an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The sequences obtained in this study were deposited with the European Molecular Biology Laboratory (EMBL) and the accession numbers are listed in Table 1. Sequences were aligned using MAFFT 7.110 (Katoh and Standley 2013), using the E-INS-i option, and the obtained alignments were run through Gblocks (Castresana 2000; Talavera and Castresana 2007) to remove ambiguously aligned regions using the default 'less stringent' settings. The 28S, 18S, 16S and COI sequences were combined in a concatenated dataset and PartitionFinder 1.1.1 (Lanfear et al. 2012) was used to determine the partition scheme and the molecular models (16S: HKY + G; COI_1st,2nd: HKI + G; COI_3rd: GTR + I; 18S, 28S: GTR + I). Phylogenetic analyses were performed using MrBayes 3.2 (Ronquist et al. 2012) for Bayesian inference and Garli 2.01 (Zwickl 2006) for maximum likelihood, as described in Maggioni et al. (2016). The genetic distances (Kimura two-parameter, 500 bootstraps) within and among the obtained clades were also estimated for each separated molecular locus using MEGA 6 (Tamura et al. 2013).

Results

During our survey, we found three *P. krempfi* colonies associated with the gorgonian *Antillogorgia* sp. (Fig. 2) at depths of between 4 and 17 m. For each *Pteroclava* colony, we observed only polyps. All specimens were morphologically identified as *P. krempfi*, showing the typical features of the species, such as a perisarc-covered pedicel, moniliform tentacles and euryteles patches. Moreover, no morphological variation among polyps collected from different hosts was found, as described by Seveso et al. (2015) and Maggioni et al. (2016). The total alignments of the concatenated datasets was 4355 bp long (4381 bp before the Gblocks treatment). The general topology of the tree (Fig. 3) was consistent with the available most comprehensive phylogeny of Cladocorynidae (Maggioni et al. 2016). *Pteroclava* sequences formed a monophyletic clade and they were the sister

Table 1 List of specimens included in the analysis, along with GenBank accession numbers

Species	Specimen code	Alcyonacean host	Locality	GenBank accession numbers			
				COI	16S	18S	28S
Pennaria sp.	XPS1	–	China	KF962169	KF962510	KF962290	KF962370
Zanclea gallii	MA058	–	Maldives	**LT593889**	LN714107	**LT593874**	**LT593869**
Cladocoryne haddoni	MA200	–	Maldives	**LT593890**	**LT593865**	**LT593875**	**LT593870**
Pteroclava krempfi	MA181	*Sinularia* sp.	Maldives	LT158199	LT158209	**LT593876**	LT222039
Pteroclava krempfi	MA182	*Sinularia* sp.	Maldives	LT158200	LT158210	**LT593877**	LT222040
Pteroclava krempfi	MA183	*Sinularia* sp.	Maldives	LT158201	LT158211	**LT593878**	LT222041
Pteroclava krempfi	MA184	*Sarcophyton* sp.	Maldives	LT158202	LT158212	**LT593879**	LT222042
Pteroclava krempfi	MA185	*Sarcophyton* sp.	Maldives	LT158203	LT158213	**LT593880**	LT158219
Pteroclava krempfi	MA186	*Lobophytum* sp.	Maldives	LT158204	LT158214	**LT593881**	LT222043
Pteroclava krempfi	MA187	*Lobophytum* sp.	Maldives	LT158205	LT158215	**LT593882**	LT222044
Pteroclava krempfi	MA188	*Lobophytum* sp.	Maldives	LT158206	LT158216	**LT593883**	LT158220
Pteroclava krempfi	MA189	*Paraplexaura* sp.	Maldives	LT158207	LT158217	**LT593884**	LT158221
Pteroclava krempfi	MA190	*Paraplexaura* sp.	Maldives	LT158208	LT158218	**LT593885**	LT222045
Pteroclava krempfi	BT006	*Antillogorgia* sp.	Bocas del Toro	–	**LT593866**	**LT593886**	**LT593871**
Pteroclava krempfi	SE012	*Antillogorgia* sp.	Sint Eustatius	**LT593891**	**LT593867**	**LT593887**	**LT593872**
Pteroclava krempfi	SE013	*Antillogorgia* sp.	Sint Eustatius	**LT593892**	**LT593868**	**LT593888**	**LT593873**

Boldface indicates newly obtained sequences

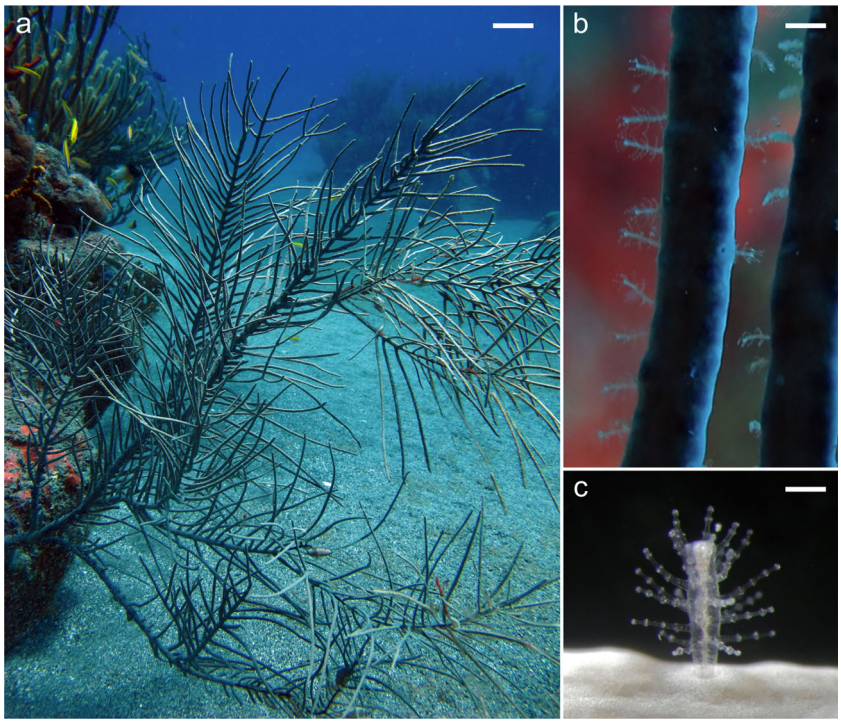


Fig. 2 Photographs of the symbiosis between *Pteroclava krempfi* and *Antillogorgia* sp. **a** Colony of *Antillogorgia* sp. from Sint Eustatius. **b** Branches of the same colony hosting *P. krempfi*. **c** Close-up of a polyp from Bocas del Toro. Scale bars: **a** 3 cm; **b** 1.5 mm; **c** 0.3 mm

group of *Cladocoryne haddoni*, a previously unsampled representative of the family Cladocorynidae. The *P. krempfi* species complex was subdivided into three monophyletic and highly supported molecular lineages (Fig. 3). The three lineages were recovered with high statistical supports also in each of the single-locus phylogenies (Fig. S1). In addition to the two clades already described by Maggioni et al. (2016), a third more basal new group was identified. This clade was composed by all the specimens collected in the Caribbean Sea and associated with octocorals of the genus *Antillogorgia*. The genetic divergence over mitochondrial sequences between the Caribbean *P. krempfi* and the two Maldivian clades were extremely high, with mean genetic distances ranging from 9.5 to 9.9 % for 16S rRNA and from 19.7 to 21.4 % for COI. The genetic distances among nuclear sequences were lower, being included in a range from 0.6 to 1.2 %. Also, the intra-clade divergence was low for all

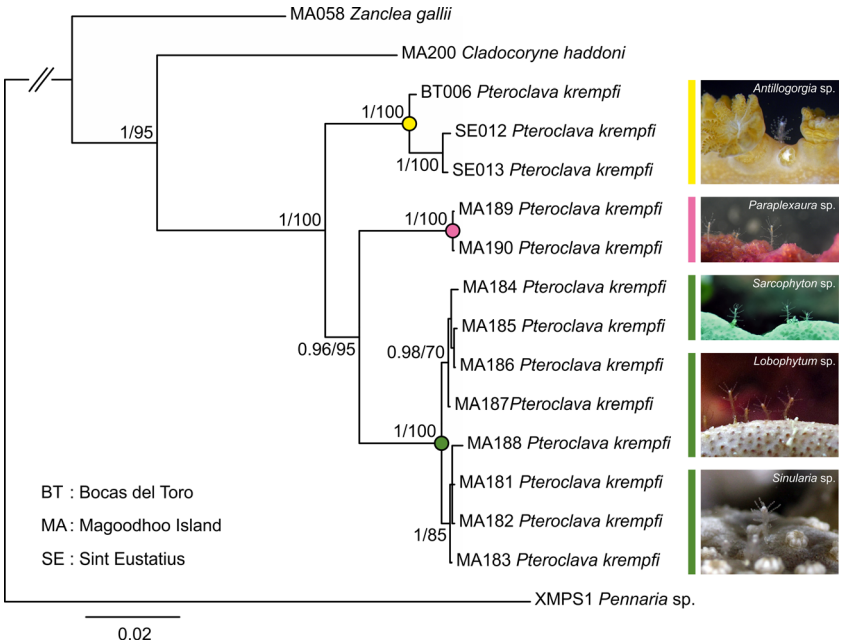


Fig. 3 Phylogenetic tree of the *Pteroclava krempfi* species complex based on the concatenated dataset of COI, 16S, 18S and 28S genes. Clades' support values are posterior probabilities from Bayesian inference and bootstrap values from maximum likelihood analyses, respectively. Different colours highlight the three cryptic species of *P. krempfi*. On the right, the photographs represent polyps associated with different hosts

four molecular markers, reaching a maximum of 1.9 % for the 16S rRNA.

Discussion

Clarifications of taxonomic problems in hydroids are traditionally carried out with the hydrozoan barcode gene 16S rRNA, which is useful for resolving phylogenies from species to subfamily levels (Moura et al. 2008), and with the two nuclear genes 18S rRNA and 28S rRNA, which are more effective for phylogeny reconstructions at higher taxonomic levels (e.g. Bridge et al. 1995; Collins 2000; Collins et al. 2006). Moreover, in some cases, also the universal barcode gene COI has been successfully used to infer intra-generic relationships and to detect cryptic lineages in several hydrozoan species (e.g. Schuchert 2014; Montano et al. 2015b). In the present study, a remarkable genetic differentiation was detected among *P. krempfi* specimens collected from different host families and from different localities. A multi-locus phylogeny reconstruction based on both mitochondrial and nuclear loci revealed a new genetic lineage comprising *P. krempfi* specimens associated with the family Gorgoniidae (genus *Antillogorgia*), plus two already described clades associated with the families Alcyoniidae (genera *Lobophytum*, *Sarcophyton*, *Sinularia*) and Plexauridae (genus *Paraplexaura*), respectively. Both the phylogenetic and genetic divergence analyses demonstrated a strong genetic diversification of these clades, indicating the presence of three different cryptic species. Indeed, the genetic distances among 16S rRNA sequences, as well as COI sequences, were comparable or even exceeded the intraspecific threshold values found for other hydrozoan taxa (e.g. Miglietta et al. 2009; Schuchert 2014; Zheng et al. 2014; Montano et al. 2015b). According to genetic evidence, *P. krempfi* from both St. Eustatius and Bocas del Toro, therefore, represent a new lineage of the *P. krempfi* species complex, composed now of three different cryptic species, with the main difference, other than the sequences, being their host.

Recently, several hydroids belonging to the genus *Zanclea* were discovered to grow on scleractinian corals (Boero et al. 2000; Pantos and Bythell 2010; Hirose and Hirose 2011; Montano et al. 2013, 2014, 2015a, c). Molecular analyses demonstrated that some of these hydrozoans that appeared morphologically similar or even identical were actually genetically highly divergent (Montano et al. 2015b). This genetic structure could be partially explained by the host specificity of these hydrozoans, similar to that presently observed among *Pteroclava* hydroids, where cryptic species grow on different octocorals with various levels of fidelity. In a similar way, parasitic snails (*Leptoconchus* spp.) living inside scleractinian corals also showed little morphological variation but a genetic differentiation at the species level that reflects the associations with their various host corals (Gittenberger and Gittenberger 2011).

The present results provide new insights into the *P. krempfi* genetic diversity, which is higher than previously known. As hypothesised by Maggioni et al. (2016), the variety of hosts and the widespread distribution of this hydrozoan species can be explained by a hidden genetic diversification, which seems to be only detectable by using molecular phylogenetics. The addition of a cryptic species associated with the alcyonacean family Gorgoniidae from new sampling localities thus confirms the idea that the nominal species *P. krempfi* could actually be a species complex. However, no molecular data are available for the originally described *P. krempfi*, which was reported to live in association with the alcyoniid *Cladiella krempfi* (Hickson, 1919). Consequently, the type species may actually belong to one of the three herein identified molecular clades or it could, conversely, represent another cryptic species where the geographic area rather than the host specificity may determine the difference. Additional morphological analyses of the medusa stages as well as molecular characterisations of specimens from other recorded localities are, therefore, strongly needed.

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