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The first report of the parasitic red alga *Gracilaria babae* (Rhodophyta: Gracilariaceae) from Singapore

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Abstract. The presence of a red alga *Gracilaria babae* growing on its congener *G. salicornia* in Singapore is reported for the first time. The parasitic *G. babae* is distinguished by having pigmented pustules without rhizoids penetrating into host tissue. The tissues between the parasite and the host appeared continuous. The Singaporean *G. babae* has tetrasporangia and deep spermatangial conceptacles formed at the periphery of the pustule. Based on the sequences of molecular markers belonging to different genomic compartments (nuclear ITS and LSU, mitochondrial *cox*1 and plastid *rbc*L), there was no genetic variation in the Singaporean *G. babae* collected from several different habitats.

Key words. *Gracilaria babae*, red algal parasite, ITS, LSU, *cox*1, *rbc*L.

INTRODUCTION

Yamamoto established the genus *Congracilaria* in 1986 to accommodate *C. babae* parasitic on *Gracilaria salicornia* (Dawson, 1954). This red algal parasite is characterised by the absence of rhizoids penetrating into the host tissues, by having a colouration similar to that of its host, the presence of bisporangia, and in possessing deep spermatangial conceptacles of *verrucosa* type. Yamamoto (1991) subsequently reported the presence of *C. babae* in the Philippines based on his observation of bisporangia in the Philippine specimens. Despite being slightly larger in pustule size, the Philippine specimens were similar to the type specimens in terms of external morphology, cellular structures and reproductive organs. The occurrence of similar red algal parasites with some anatomical variations had been reported in other Southeast Asian countries including Malaysia (Yamamoto & Phang, 1997), Indonesia (Gerung et al., 1999), and Thailand (Terada et al., 1999). Until recently, there was no further investigation carried out to clarify the taxonomic conundrum of these parasitic taxa since their original taxonomic descriptions.

The application of molecular data has addressed a number of phylogenetic issues when morphological and anatomical observations alone are insufficient to delineate species boundaries (Tan et al., 2013; Poong et al., 2013), especially in resolving the relationships between the red algal parasites and their hosts (Zucarello et al., 2004; Kurihara et al., 2010; Ng et al., 2013, 2014). Considering the unique biology of the red algal parasites (Goff & Coleman, 1987, 1995), molecular characterisation of the parasites is more reliable with a multi-loci approach using markers from different genomic compartments (Kurihara et al., 2010; Ng et al., 2013, 2014). Using molecular tools, Ng et al. (2013) identified the Malaysian parasitic red alga on *G. salicornia* as *C. babae* despite the anatomical variations observed. In a subsequent study, Ng et al. (2014) transferred *C. babae* to the genus *Gracilaria* as the parasite was placed in the *Gracilaria* sensu stricto clade in the molecular phylogenies based on the plastid, mitochondrial and nuclear markers.

Gracilaria salicornia has been recorded in the Singapore waters (Lee et al., 2009), but the occurrence of parasitic red algae on *G. salicornia* remains undocumented in Singapore. The presence of this parasite is anticipated, since the distribution of its host species is within the range where a similar parasite has been previously reported (Yamamoto & Phang, 1997; Terada et al., 1999). This paper provides the first formal record of *G. babae* from Singapore. The Singaporean samples collected from Ubin Island were compared against our previous collections of *G. babae* from Japan (Okinawa) and southeast Johor, Malaysia (Che Kamat Island and Ramunia Bay). We aimed to characterise the Singaporean *G. babae* growing on *G. salicornia* with the DNA sequences of nuclear ITS and LSU, mitochondrial *cox*1 and plastid *rbc*L. Morphological and anatomical features of the Singaporean parasite are also described. The utility of haplotype network analyses in inferring the relationships between the red algal parasite and its host, as well as that among the parasite individuals was demonstrated in this study. The haplotype networks were congruent with the phylogenies of *G. babae* and *G. salicornia* inferred from the sequences of the ITS, LSU, *cox*1 and *rbc*L from a previous study by Ng et al. (2013), in which ITS proved useful in discriminating the parasites from its host.

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MATERIAL AND METHODS

Sample collection and processing. Individuals of parasitic red alga were observed on *G. salicornia* collected from several localities around Ubin Island (OBS Camp 1, Changi, Ketam Island, and the intertidal zones between OBS Camp 1 and Camp 2) in Singapore during the Johor Straits Biodiversity Workshop. Treatment of algal samples, sectioning and staining, DNA extraction and PCR amplification procedures are as described in Ng et al. (2013). Voucher specimens were deposited in the Lee Kong Chian Natural History Museum, National University of Singapore.

Sequence alignment and analyses. The sequences of four genetic markers (ITS, LSU, *cox*1 and *rbc*L) for the Singaporean *G. salicornia* and its red algal parasite were obtained (Table 1). All datasets were aligned using ClustalX2 (Larkin et al., 2007), with the default gap extension/opening parameters and the alignments were trimmed with BioEdit v7.0.5.3 (Hall, 1999). For all genetic markers examined, at least two parasite individuals from each locality were sequenced, and in each case, all sequences were identical. Only one representative sequence for each marker from each population was deposited in GenBank and included in the analyses. The K2P-corrected mean distances, excluding gaps and ambiguities for *G. salicornia* and its parasite from different populations were computed with PAUP* v4.0b10 (Swafford, 2002) for all genetic markers tested. The sequences of *G. salicornia* and *G. babae* from Japan and Malaysia generated from our previous study (Ng et al., 2013) were also included in the analyses (Table 1).

The molecular analyses undertaken by Ng et al. (2013) provided a baseline understanding in the genetic features of *G. babae* parasitic on *G. salicornia*. *Gracilaria babae* is very closely related to *G. salicornia* in having *cox*1 and *rbc*L sequences identical to those of *G. salicornia*, and is only distinguished from *G. salicornia* by the LSU and ITS sequences. A haplotype network is a representation of the genealogical relationships among haplotypes, which allows the incorporation of non-bifurcating genealogical information associated with population level divergences (Clement et al., 2000). Considering (1) the minimal sequence diversity for intraspecific haplotype comparison and poor performance of phylogenetic inference using standard tree construction methods (Crandall & Templeton, 1993; Zuccarello et al., 2002) with LSU, *cox*1 and *rbc*L, and (2) the promising use of the this analysis at 95% connection limit for differentiating species (Hart & Sunday, 2007) which may be applicable to ITS, haplotype network analyses are more appropriate to consider the relationship among the parasites and that between the host-parasite association. Haplotype networks inferred for all four markers using a statistical parsimony framework were calculated for *G. salicornia* and its red algal parasite as implemented in TCS v1.2.1 (Clement et al., 2000). Gaps were treated as missing data, and the connection limit was set to 95%. Alignments used in the analyses after trimming of the sequences are available upon request to the authors.

RESULTS

Morphological and anatomical observations. The parasite *Gracilaria babae* was found on *G. salicornia* collected in various habitats spanning the intertidal zones (Fig. 1), ranging from moderately exposed to exposed sandy and rocky habitats to muddy estuarine habitats. The Singaporean parasite has a small mushroom-like appearance, up to 2 mm high from the interface with the host to the top, and up to 3 mm wide. The colour of the parasite is similar to that of the host, olive black in the unaided eye (Fig. 1). The parasite pustules occurred either singly or in clusters, seemingly without any apparent pattern on any part of the host thallus. They were prevalent on the thallus of *G. salicornia* with heavy epiphytic load, and some parasites even had epiphytes growing on them (Fig. 2A). Sometimes the parasites grew into extensive tissue masses along the axis of the host thallus (Fig. 2B), which may have resulted in bending of the host thallus in some instances (Fig. 2C, arrow). The presence of parasites did not seem to have much effect on the fecundity of host species, as these parasites were also observed on host plants at post-fertilisation stage.

The pigmented outer cortical cells measured up to 12 µm long by 5 µm wide, whereas the non-pigmented medullary cells reached 300 µm in diameter in the centre of the pustule with the cell size diminishing towards the cortex. A demarcation between the tissue of the parasite and its host was not observed. There were no rhizoids penetrating into the host tissue (Fig. 2D). The female gametophyte of the parasite is easily recognised at post-fertilisation stage, with cystocarps present over the entire surface of a parasite pustule, giving the pustule a bumpy appearance (Fig. 2A, arrow). Cystocarps are dome-shaped, up to 425 µm high and 530 µm wide (Fig. 2E). Cystocarp structure is similar to that originally described for *G. babae*. Carposporangia uninucleate, up to 15 µm long and 11 µm wide. Cruciately divided tetrasporangia (Fig. 2F) formed at the periphery of the parasite pustule, and these measured up to 28 µm high and 12 µm wide. Spermatangia were present in deep conceptacles (Fig. 2G) up to 90 µm deep, and were always together with cystocarps on the same pustule. Parasite pustules with only spermatangial conceptacles or only cystocarps were also observed.

Molecular analyses. The DNA sequences of all genetic markers were obtained for almost all of the samples of Singaporean *G. salicornia* and its parasite from different localities, except for the nuclear markers (ITS and LSU) for *G. salicornia* from Changi. All collections of the Singaporean parasite were identical at multiple loci.

The alignment of ITS sequences was trimmed to 1229 sites with the inclusion of some gaps. The haplotype analysis under statistical parsimony split the samples into two unconnected networks corresponding to *G. salicornia* and its parasite, *G. babae* at 95% probability threshold (Fig. 3A). The *G. salicornia* haplotype network contained four haplotypes (IH1–IH4), while the *G. babae* haplotype network comprised of five haplotypes (IP1–IP5) linked by nine possible missing

Table 1. Isolates of *Gracilaria babae* and *G. salicornia* used in this study.

Taxa	Collection locality/date	Voucher	Isolate	GenBank accession numbers and haplotypes							
				ITS		LSU		***cox*1**		***rbc*L**	
G. babae (Yamamoto) Ng et al.	Ramunia Bay, Malaysia/15 March 2012	PSM 12481_UMSS 0632	90P	KC209011	IP3	KC209036	LP2	KC209004	C2	KC209054	R2
	Che Kamat Island, Malaysia/16 March 2012	PSM 12482_UMSS 0635	83P	KC209010	IP4	KC209034	LP3	KC208999	C2	KC209052	R2
	Bise, Okinawa, Japan/10 July 2010	PSM 12276_UMSS 0351	38P	KC209012	IP1	KC209041	LP1	KC203995	C1	KC209045	R1
	Bise, Okinawa, Japan/10 July 2010	PSM 12276_UMSS 0352	71P	JQ362438	IP2	KC209040	LP1	JQ694686	C1	JQ694702	R1
	OBS Camp 1, Singapore/26 October 2012	JS 3172	128P	KF831107	IP5	KF831114	LP2	KF831100	C2	KF831122	R2
	Changi, Singapore/28 October 2012	JS 3238	139P	KF831108	IP5	KF831115	LP2	KF831101	C2	KF831123	R2
	Ketam Island, Singapore/29 October 2012	JS 3275	130P	KF831109	IP5	KF831116	LP2	KF831102	C2	KF831124	R2
	Between OBS Camp 1 and Camp 2, Singapore/29 October 2012	JS 3274	132P	KF831110	IP5	KF831117	LP2	KF831103	C2	KF831125	R2
G. salicornia (C. Agardh) Dawson	Ramunia Bay, Malaysia/15 March 2012	PSM 12481_UMSS 0632	90H	KC209018	IH2	KC209023	LH2	KC209002	C2	KC209047	R2
	Che Kamat Island, Malaysia/16 March 2012	PSM 12482_UMSS 0635	83H	KC209009	IH3	KC209024	LH2	KC209001	C2	KC209050	R2
	Bise, Okinawa, Japan/10 July 2010	PSM 12276_UMSS 0352	71H	KC209016	IH1	KC209029	LH1	KC208994	C1	KC209048	R1
	OBS Camp 1, Singapore/26 October 2012	JS 3172	128H	KF831104	IH2	KF831111	LH2	KF831096	C2	KF831118	R2
	Changi, Singapore/28 October 2012	JS 3238	139H	-	-	-	-	KF831907	C2	KF831119	R2
	Ketam Island, Singapore/29 October 2012	JS 3275	130H	KF831105	IH2	KF831112	LH2	KF831908	C2	KF831120	R2
	Between OBS Camp 1 and Camp 2, Singapore/29 October 2012	JS 3274	132H	KF831106	IH4	KF831113	LH2	KF831909	C2	KF831121	R2

Fig. 1. Different habitats of *Gracilaria salicornia*. A, Moderately exposed sandy habitat; B, Exposed sandy habitat; C, Exposed rocky habitat; D, Muddy estuarine habitat.

haplotypes (see Table 2 for the variation sites for each haplotype). The K2P-corrected genetic distance between *G. babae* and *G. salicornia* was 1.771–2.714%, while that between the *G. babae* haplotypes was 0–0.752%.

The partial LSU sequences yielded an alignment of 481 sites, including a few introduced gaps. The DNA sequences of LSU for the Singaporean *G. salicornia* and *G. babae* are identical to those of their Malaysian counterparts collected in Johor, Malaysia. The parsimony haplotype network analysis recovered five haplotypes of *G. salicornia* (LH1 and LH2) and *G. babae* (LP1–LP3) (Fig. 3B). LSU did not show a clear differentiation between *G. salicornia* and *G. babae*, as the two clusters were linked only by a mutational step (for the variation sites for each haplotype, see Table 3). The K2P-corrected genetic distance between *G. babae* and *G. salicornia* was 0.209–1.049%, while that between the *G. babae* haplotypes was 0.209–0.418%.

The alignment of the partial *cox*1 and *rbc*L sequences was 1015 bp and 1118 bp each. The *cox*1 and *rbc*L sequences of the parasite isolates were identical to those of their host species, *G. salicornia*. These sequences of Singaporean *G. salicornia* and *G. babae* were identical to those of their Malaysian counterparts sampled in Johor, Malaysia. Only two haplotypes were revealed from the *cox*1 (Fig. 3C) and *rbc*L (Fig. 3D) statistical parsimony networks of *G. salicornia* and its parasite based on their geographical origin. The haplotype C1 differed from the haplotype C2 by 10 mutational steps (for the variation sites for each haplotype, see Table 4). On the other hand, the haplotype R2 differed from the haplotype R1 only by two mutational steps with a transition of thymine to cytosine at position 173, and a transversion of guanine to cytosine at position 407.

DISCUSSION

Yamamoto (1986, 1991) provided detailed descriptions for *Gracilaria babae*, the parasitic red alga growing on *G. salicornia*, from Japan and the Philippines. Several parasitic taxa referable as *G. babae* found on *G. salicornia* described from Southeast Asia displayed discernible anatomical variations from the type specimen (Table 5). The Singaporean parasites found on *G. salicornia* are a reasonable match to the Japanese and Philippines *G. babae*, despite the smaller stature and the occurrence of tetrasporangia (Table 5). The parasites from Singapore are also similar to the Malaysian and Thai parasites in terms of pustule size as well as the occurrence of tetrasporangia (Table 5). In contrast to the distinct host-parasite demarcation observed in Malaysian

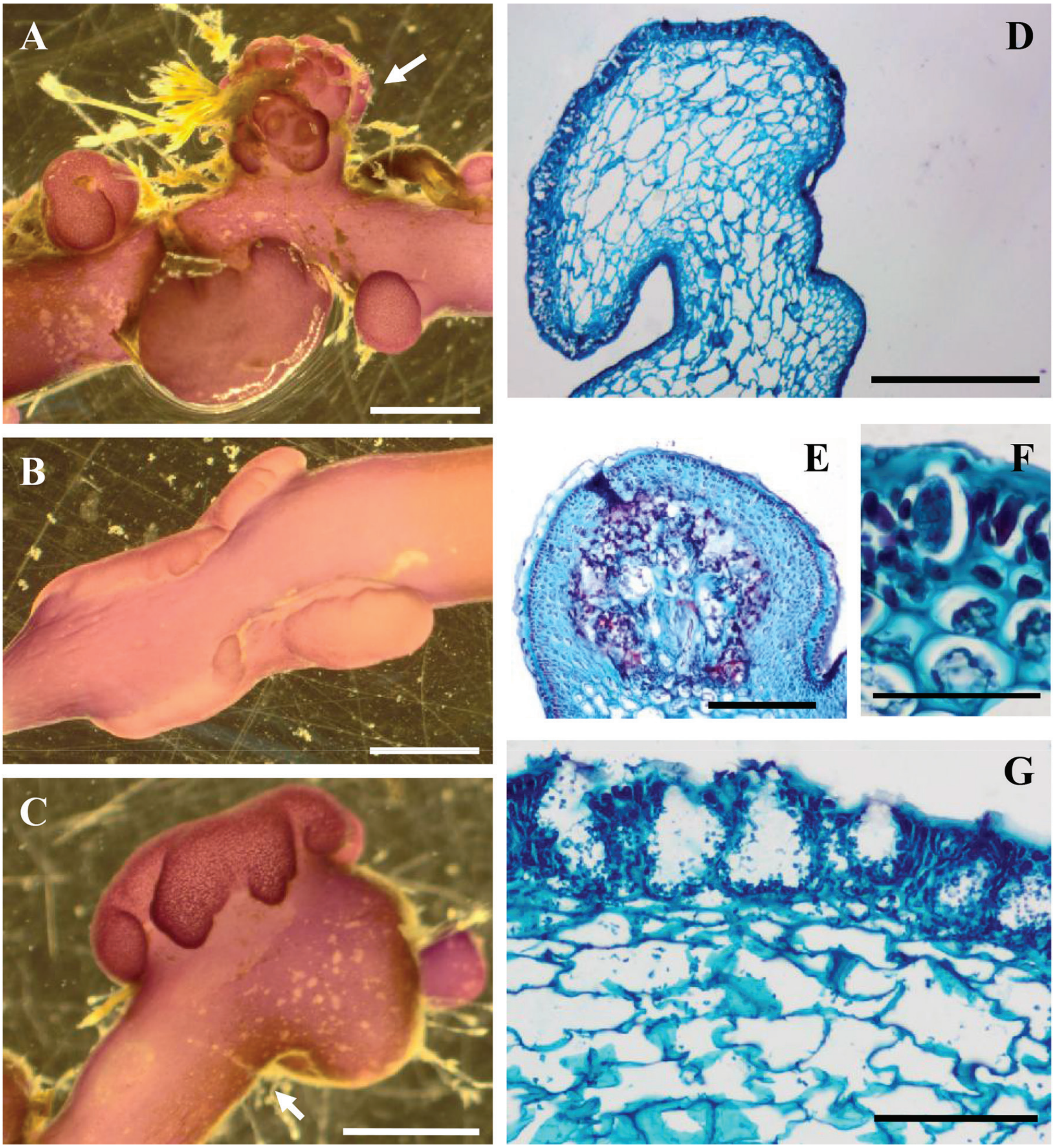

Fig. 2. *Gracilaria babae* growing on *G. salicornia*. Wet habit of formalin-preserved material: A, Heavy epiphytic load on the thallus of both *G. salicornia* and its parasite, arrow indicates female gametophyte of parasite; B, Extensive tissue proliferation of the parasite along the axis of host thallus; C, Bending of host thallus as a result of extensive parasite tissue proliferation (arrow); Sections prepared using paraffin method: D, Continuous connection between the medullary tissue of the parasite and the host; E, Cystocarp of the parasite; F, A tetrasporangium; G, Deep spermatangial conceptacles at the periphery of parasite pustule. Scale bars = 2 mm [A–C]; 500 µm [D]; 200 µm [E]; 50 µm [F]; 100 µm [G].

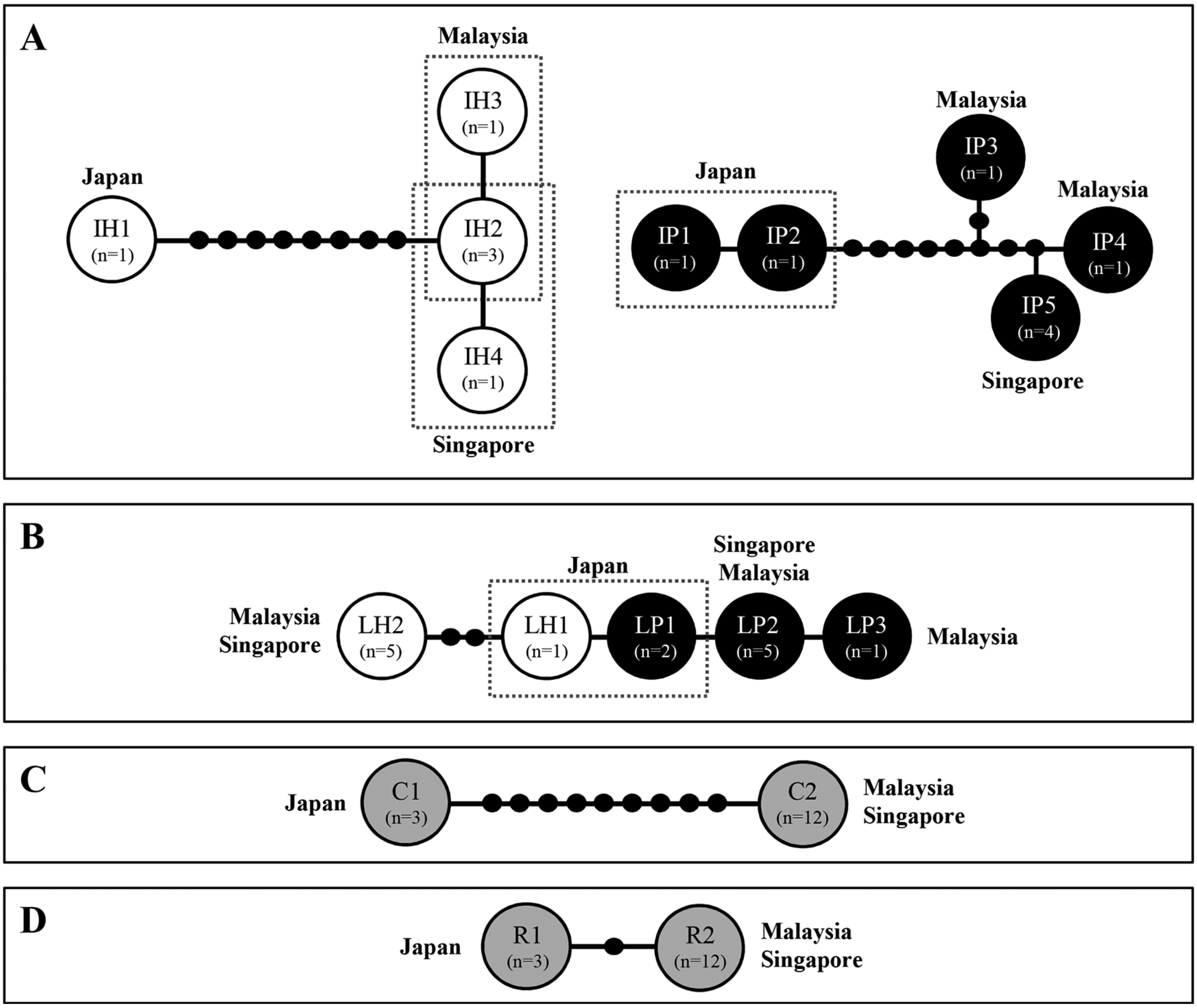

Fig. 3. TCS parsimony networks for: A, ITS; B, LSU; C, *cox*1; and D, *rbc*L sequences of *Gracilaria salicornia* and its parasite *G. babae*. Circles depict genotypes: white circle corresponds to the genotype of *G. salicornia*; black circle corresponds to the genotype of *G. babae*; and grey circle corresponds to the genotype shared by both the host and its parasite; 'n' in the circle represents number of individual. Each connecting line represents one substitution; each small circle represents a missing intermediate sequence.

Table 2. Variation sites for the ITS haplotypes of *Gracilaria salicornia* and its parasite, *G. babae*.

Haplotype	**Variation sites in DNA sequence**										
	19	**60**	**156**	**241**	**702**	**705**	**787**	**944**	**960**	**1048**	**1113**
IH1	A	C	A	G	C	T	A	T	G	G	A
IH2	A	C	T	A	A	C	G	A	A	C	G
IH3	G	C	T	A	A	C	G	A	A	C	G
IH4	A	G	T	A	A	C	G	A	A	C	G

Haplotype	**Variation sites in DNA sequence**												
	16	**151**	**202**	**242**	**245**	**259**	**465**	**499**	**504**	**683**	**773**	**897**	**993**
IP1	A	A	T	G	T	G	C	C	A	T	A	G	T
IP2	A	A	T	G	T	G	C	C	G	T	A	G	T
IP3	G	G	G	A	G	G	T	T	G	G	A	G	T
IP4	G	A	G	A	G	A	C	T	G	G	A	A	C
IP5	G	A	G	A	G	G	C	T	G	G	G	A	C

Table 3. Variation sites in different haplotypes of partial LSU sequences in *Gracilaria babae* from Singapore.

Haplotype	**Variation sites in DNA sequence**					
	386	**415**	**441**	**443**	**445**	**473**
LH1	C	G	T	T	T	A
LH2	C	G	T	A	A	T
LP1	C	G	C	T	T	A
LP2	C	A	C	T	T	A
LP3	G	A	C	T	T	A

Table 4. Variation sites in different *cox*1 haplotypes of *Gracilaria babae* from Singapore.

Haplotype	**Variation sites in DNA sequence**									
	241	**253**	**433**	**472**	**511**	**584**	**598**	**700**	**820**	**925**
C1	A	G	G	A	G	A	T	T	A	T
C2	G	A	A	G	T	G	C	C	C	C

Table 5. Comparison of *Gracilaria babae* parasitic on *G. salicornia* reported from Japan and some Southeast Asian countries.

Locality	**Japan**	**Philippines**	**Malaysia**	**Thailand**	**Singapore**
References	Yamamoto (1986)	Yamamoto (1991)	Yamamoto & Phang (1997)	Terada et al. (1999)	This study
Overall pustule size	Up to 3 mm high, 4.5 mm in diameter	Up to 3.5 mm high, 5 mm in diameter	Up to 3 mm high, 3 mm in diameter	Up to 3 mm high	Up to 2 mm high, 3 mm in diameter
Cortical cell size	7.2–9.6 µm high, 5.6–9.6 µm wide	8–9.5 µm high, 5.5–9.5 µm wide	Up to 12 µm high, 5 µm wide	Up to 15 µm high, 5 µm wide	Up to 12 µm high, 5 µm wide
Medullary cell size	Up to 560 µm wide	Up to 450 µm wide	Up to 140 µm wide	m. d.*	Up to 300 µm wide
Spermatangial conceptacle	*verrucosa* type, up to 50 µm deep, 40 µm wide	*verrucosa* type, up to 80 µm deep, 60 µm wide	*verrucosa* type, up to 72 µm deep	*verrucosa* type, 50–90 µm deep	*verrucosa* type, up to 90 µm deep
Sporangium	Bisporangium, up to 50 µm high, 20 µm wide	Bisporangium, up to 44.5 µm high, 22.2 µm wide	Tetrasporangium	Tetrasporangium	Tetrasporangium, up to 28 µm high, 12 µm wide
Cystocarp	Up to 540 µm high, 700 µm in diameter	Up to 600 µm high, 750 µm in diameter	Up to 560 µm high, 550 µm in diameter	m. d.*	Up to 425 µm high, 530 µm in diameter
Host-parasite demarcation	Not observed	Not observed	Observed	Not observed	Not observed

*m.d., missing data.

specimens, an almost continuous medullary tissue between the parasite and the host were encountered in the Singaporean and Thai taxa. A previous study by Ng et al. (2013) showed that emphasis on the diagnostic value of bisporangia for *G. babae* is superfluous, as the Malaysian parasite which has tetrasporangia was shown to be conspecific with the Japanese *G. babae* based on the molecular analyses. We recognised the parasitic red algal taxa growing on *G. salicornia* from Japan, Philippines, Thailand, Malaysia and Singapore as *G. babae*, featuring pigmented parasitic red alga that has no rhizoids penetrating into the host tissues, with reproductive structure that was similar to that of *Gracilaria* – deep spermatangial conceptacles and the presence of tubular filaments linking gonimoblast and pericarp in the cystocarps.

All parasite populations from Singapore were represented only by a common association of haplotypes belonging to different genomic compartments. The Singaporean *G. babae* examined were characterised by the ribotype IP5-LP2, mitotype C2 and chlorotype R2 (Fig. 3). The lack of nucleotide variations observed within the Singaporean *G. babae* from several localities which spanned different intertidal habitats ranging from rocky shores to muddy areas indicated the occurrence of high gene flow between populations. In view of the close proximity (~6 km) from which the samples of *G. babae* were collected, spore dispersal is possible within the sampling range in this study, evident by the high frequency of fertile parasite individuals at all sites. Ubin Island is a popular destination for leisure and recreation (Henderson, 2000). The continued anthropogenic dispersals resulting from the ferrying of tourists from Singapore mainland to Ubin Island across the Eastern Straits of Johor are likely to contribute to the regional genetic homogeneity of *G. babae*. The occurrence of only a single haplotypes association for the Singaporean *G. babae* may also be a result of uneven sampling. Extensive sampling in other coastal areas in Singapore would reveal if *G. babae* populations are homogenous across the country.

Each of the LSU, *cox*1 and *rbc*L haplotype for Singaporean parasite is identical to that of Malaysian *G. babae*, corroborating the identity of the Singaporean parasite growing on *G. salicornia* as *G. babae*. The different ITS haplotypes found between the Singaporean and Malaysian material could be attributed to the rapid mutation rate of the spacer region. This spacer region was shown to provide good phylogenetic resolution at and below the species level in red algae (Goff et al., 1994; Van Oppen et al., 1995; Hu et al., 2009). The results from this study clearly indicated that ITS is a good marker candidate to resolve inter-population relationships in *G. babae* and those of other algal parasites as well. Our molecular data echoed with a previous suggestion (Ng et al., 2013) that the red algal parasite growing on *G. salicornia* should be recognised as *G. babae*.

The analyses of genetic divergence between *G. salicornia* and its parasite based on a multi-loci approach confirm the occurrence of *G. babae* in Singapore. We have documented the presence of *G. babae* in Singapore for the first time. The results from this study highlight the need for further sampling and examination of this parasite from more regions to help complete the picture of *G. babae* distribution pattern.

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