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Morphometric and molecular analysis of *Gracilaria salicornia* and its adelphoparasite in Thailand

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ABSTRACT: The red seaweed, *Gracilaria salicornia* and its adelphoparasite were collected from eleven study sites along the east and west coasts of the Gulf of Thailand. Eight morphometric characters of *G. salicornia* and seven of the parasite were examined. Sets of the multivariate data of the host and the parasite were analysed by the discriminant function in combination with a clustering procedure. The analysis showed that the populations of *G. salicornia* and the adelphoparasite could be divided into two groups, but the degree of separation was low ($p = 0.05$, $n = 39$). This close relation among populations of the algal host and of the parasite grown at different habitats was confirmed by DNA-fragment polymorphism using the random amplified polymorphic DNA technique. Twelve out of the 20 screened primers gave polymorphism. The DNA fingerprints were visually analysed and clustered using the TFPGA program. Differences in the data sets were tested with the UPGMA program which showed identity values close to one, and were in agreement with the analysis of the discriminant function. In addition, this study showed that differences in external appearance may depend on environmental factors in the habitat, particularly high seawater turbidity. Parasite colour was closely related to populations collected from different locations, indicating that all plants of the parasite were possibly the same species.

KEYWORDS: agarophyte, morphology, parasitic red seaweed, RAPD

INTRODUCTION

The red seaweed *Gracilaria salicornia* (C. Agardh) Dawson is of uncertain taxonomic status [1] although it is of interest commercially. Several morphologically similar species have been recognized [2,3]. In 1986 Xia [2] pointed out that several external features, especially the presence of a main axis and constrictions, were not constant. Thus Xia combined all related species as synonyms under *G. salicornia* based on the external features. This species is one of the most common red seaweeds in Thailand and is widely distributed along the coast of the Gulf of Thailand and the Andaman Sea [4]. Plants of this species are generally found with different external morphological features and some plants contain a parasitic red alga called adelphoparasite, one of the marine partially endophytic algae. There have been several reports of adelphoparasites in the red, green, and brown seaweeds [5–7]. Goff et al [8] reported that nearly 15% of all known red algae genera occur only as parasites of other red algae. They also suggested that the majority of the red algal parasites are very closely related to their host. The parasitic red seaweed not only causes morphological aberrations on their hosts but also reduces the value of its host as food [5,7,9].

Adelphoparasites growing on species of *Gracilaria* have been found along the coast of various regions of Asia [10–12]. In particular, the parasitic red alga has been found in *G. salicornia* from Malaysia [13] and Thailand [12], and in *G. edulis* from Indonesia [11]. The adelphoparasite genera *Gracilariophila* and *Congracilaria* have been observed in *G. salicornia* found in the Pacific region [14]. However, there have been few studies on *G. salicornia* and the adelphoparasite in regions around Thailand and no reports on the morphometric analysis of Thai specimens. Hence the present study was done to examine morphological and molecular differentiation in *G. salicornia* and the adelphoparasite growing in different habitats.

MATERIALS AND METHODS

Study sites, field sampling, and preparation of the algal samples

Sampling was conducted during the dry and monsoon seasons along the east and west coasts of the upper Gulf of Thailand. Study sites were set up at 11 locations: AnS (Ang Sila), Sms (Samaesan), SRc (Sri Racha harbour), KoC (Ko Sichang, Chonburi) BaP (Ban Phe, Rayong), LaT (Laem Tien), LaS (Laem

Sok), AoC (Ao Cho, Trat), TaM (Ta Mong Lai) and Wak (Haad Wanakorn) in Prachuap Khiri Khan, and TWL (Haad Thung Wua Lean, Chumporn). Samples were collected randomly by hand in shallow waters. The study sites were characterized as exposed (Sms, LaS, TaM, Wak, and TWL), semi-exposed (KoC, SRc, LaT, and AoC), and sheltered environments (AnS and BaP). Samples were washed to remove sand and mud, and divided into two groups. One group of algal plants was washed in filtered seawater to remove all epiphytes and debris. Individual plants were kept frozen at −80 °C prior to DNA extraction. The other group was washed to remove sand and mud and then preserved in a solution of 4% formaldehyde with seawater and eventually as dried specimens on herbarium sheets.

Morphometric examination

All microscopic mounts from specimens preserved in the formaldehyde solution or from herbarium sheets were made using 50% corn syrup (Karo Syrup, Corn Products, Inc.) solution in distilled water containing a trace of phenol. Transverse sections of the specimen were prepared by hand, stained with 1% aniline blue, and mounted in 25% Karo Syrup. Pieces of the sections were examined under an optical microscope (Eclipse E600, Nikon) at 10, 20, and 40 × magnification.

The morphometric examination was done on 30 samples of each host and its adelphoparasites. Branch segments were measured using a Vernier calliper. The microscopic transverse section was measured using a stage micrometer. The morphological characters used in the analysis were (1) diameter of segment, (2) size of cortical cell, (3) number of cortical cell layers, (4) thickness of cortical layer, (5) diameter of medullar cell, (6) number of medullary layers, (7) diameter of medullary layers, (8) width of intercellular space, (9) total height of parasite, (10) length of stipe, (11) height of cap, and (12) diameter of cap. Eight characters (1–8) were used for *G. salicornia* and seven characters (2, 5, 8, and 9–12) were used for the adelphoparasite. The relations between the algal groups was done using canonical discriminant function analysis. The data set was clustered using the PAST multivariate statistical programs.

Collection of environmental data and laboratory analyses

At the study site salinity, turbidity, orthophosphate, and total dissolved inorganic nitrogen (ammonia + nitrite+nitrate) were determined every working day as one-point measurements. Salinity of surface seawater was measured using a refracto-salinometer. Seawater turbidity was determined at 420 nm with a spectrophotometer (HACH model DR/2010). Ammonia-nitrogen, nitrite-nitrogen, nitrate-nitrogen, and orthophosphate were examined according to Ref. 15.

DNA extraction

DNA was extracted from 30 samples of each host and the adelphoparasites from each locality. The frozen samples (250 mg) were ground in liquid nitrogen using mortar and pestle until pulverized. The frozen powder was then placed in an Eppendorf tube (1.5 ml) and 1.5 ml of extraction buffer (0.1 M Tris-HCl, 50 mM EDTA, 0.5 M NaCl, pH 8.0) was added. The mixture was swirled (gently and occasionally in vortex mode) while being incubated in a 65 °C water bath for 45 min. It was then centrifuged at 13 000 rpm for 15 min at 4 °C. The clear supernatant (1.3 ml) was placed in a new tube to which 10 µl ribonuclease A was added before being incubated at 37 °C for 30 min. It was then placed on ice for 30 min. The mixture solution was lightly swirled 2–3 times prior to incubation for 24 h at −20 °C. Thereafter, the mixture solution was centrifuged at 15 300 g for 15 min, at 4 °C. The DNA was then precipitated overnight with 700 µl of cool (−20 °C) isopropanol and washed three times with 1 ml of cool (−20 °C) 70% ethanol. The pellet DNA was air-dried for 10 min, resuspended in 50 µl TE buffer, and kept at −20 °C. The quantity and quality of the extracted DNA was determined spectrophotometrically. Three samples of the extracted DNA that gave high yield were selected for random amplified polymorphic DNA (RAPD) analysis.

RAPD analysis

Screening for variable RAPD markers was conducted using 20 primers. Group 1 primers were OPA1, OPA10, OPA11, OPK7, Group 2 consisted of the primer given in Ref. 16, and the remaining 15 primers were Group 3. Polymerase chain reaction amplifications of all DNA was performed in a final volume of 25 µl containing 2.5 µl of 10 × *Taq* DNA polymerase buffer, 0.2 mM of dNTP (dATP, dGTP, dCTP, dTTP), 1 µl of *Taq* DNA polymerase, 10 pmol of the primers, 15, 20, 25 ng of genomic DNA and 1.0, 1.5, 2.0, 2.5, and 3.0 mM $MgCl_2$.

Amplification was performed at 95 °C for 2 min for Group 1 (3 min for the others), 45 cycles of denaturation at 94 °C for 1 min for Group 1 (30 s for the others), annealing for 1 min at different temperatures depending on the Group 1 primer (30 s at 45 °C for Group 2, 30 s at 34 °C for group 3), extension at 72 °C

for 2 min, and a further final extension at 72 °C for 10 min. The reactions were stopped at 4 °C.

Between 2 µl and 25 µl of the reaction products were separated by electrophoresis through 1.7% agarose gels and stained with ethidium bromide. Agarose gels were photographed with a Polaroid camera over a UV transilluminator.

RAPD data analysis was done using 3 replicates per DNA sample of each alga after binary modification of the data. The calculation of the similarity index [17] was done using $F = 2X_{1,2}/(X_1+X_2)$, where F is the estimate of similarity, $X_{1,2}$ is number of DNA fragments which are identical in the two samples, and X_i is the total number of DNA fragments from sample i. $F = 1$ means that the two samples are similar. If $F = 0$ the two samples are totally dissimilar. The similarity was also examined using a statistical program of Tools for Population Genetic Analyses (TFPGA) and the clustering was carried out using the unweighted pair group method using arithmetic averages (UPGMA).

RESULTS AND DISCUSSION

In 2005 and 2006, specimens of *G. salicornia* and their adelphoparasites were found in all 11 study sites. However, in 2007 the specimens were obtained from only 10 sites, as the alga could not be found at KoC.

The salinities of the seawater at the study sites ranged from 12–35‰ (Table 1). In most study sites, the seawater showed high concentration of dissolved inorganic nitrogen (DIN), with the highest concentration of 1.6 mg/l at BaP during the 2006 dry season and at AnS in 2007. The concentration of orthophosphate of the seawater was highest (0.2 mg/l) in 2007 at the AnS site. Alkalinity of the seawater varied from 37.0–59.5 mg $CaCO_3$/l during the 2005 monsoon season. During the dry seasons in 2006 and 2007, seawater alkalinity varied from 13.5–70.5 mg $CaCO_3$/l and 79.5–98.5 mg $CaCO_3$/l, respectively. The seawater turbidity was very high in most of the sampling sites, particularly at AoC and LaS where the maximum values were, respectively, 1818 NTU (in 2006) and 1398 NTU (in 2007).

Table 1 Characteristics of seawater collected from 11 study sites.

Parameters	mean (range), $n = 33$		
	2005	2006	2007
DIN (mg/l)	0.2 (0.1–0.9)	0.5 (0.1–1.6)	0.6 (0.2–1.6)
orthophosphate (mg/l)	0.01 (0.00–0.02)	0.03 (0.02–0.11)	0.04 (0.00–0.20)
alkalinity (mg $CaCO_3$/l)	51 (37.0–59.5)	37.3 (13.5–70.5)	89.2 (79.5–98.5)
turbidity (NTU)	118 (3.6–953)	283 (5.5–1818)	308 (9.1–1398)
pH	8.1 (7.9–8.4)	7.4 (6.7–7.8)	8.4 (7.7–8.9)
salinity (‰)	29 (12–34)	32 (28–35)	33 (29–35)

Morphometric analysis of *Gracilaria salicornia*

The algal plants are succulent and prostrate forming a rough entangled mass attached on small pebbles, shells, and other seaweed with disc-like holdfast (Fig. 1l,m) with or without rhizoid (Fig. 1n,o). Some also show entangled branches forming from a rigid disc-like holdfast (Fig. 1p) and conjugated branches (Fig. 1q). Variations in gross morphology of *G. salicornia* are given in Table 2 and Table 3.

In different environments, the algal thalli showed a similar branching, di- or trichotomously, or irregularly branching, and some branches forming cylindrical segments. Branches were both constricted and not constricted at the base. Some were slightly constricted at the apical portion but not constricted at the base. Most of the collected plants had a root like rhizoid, except for the samples from Ang Sila (AnS). This study showed a distinct morphological variation of *G. salicornia* from different habitats.

The discriminant analysis showed a close relationship among the algal populations collected from different habitats and seasons. The thallus has a cylindrical segment which can be either elongated (KoC, AnS) or robust (other sites). The analysis based on basal branches divided the algae into two groups: (1) branches which were slightly constricted or not constricted at the base (KoC, SRc, Sms, BaP, AoC, Wak), and (2) branches at terminal portion with marked constriction, slight constriction or no constriction at the lower portion (other sites).

Based on eight characters (1–8), the discriminant analysis showed the distribution of the samples with a low degree of separation (Fig. 2a). The clustering divided specimens of *G. salicornia* into group 1 (LaS, LaT, TaM, TWL, AoC, AnS, SRc), and group 2 (KoC, Wak, Sms, BaP) (Fig. 2b). The collected samples showed a significant correlation with different seasons ($p = 0.05$, $n = 39$). This may explain why the algal morphology was site dependent.

Plants growing in a sheltered environment in sampling sites AnS and BaP had a yellow green or brown

Fig. 1 External appearance of *Gracilaria salicornia* collected from (a) KoC (b) AnS (c) SRc (d) Sms (e) BaP (f) LaS (g) AoC (h) LaT (i) TaM (j) Wak (k) TWL; (l) habit of holdfast (m) disc like holdfast (arrow) (n,o) rhizoids (arrows) (p) entangled branches forming from disc like holdfast (arrow) (q) conjugated branches (arrow).

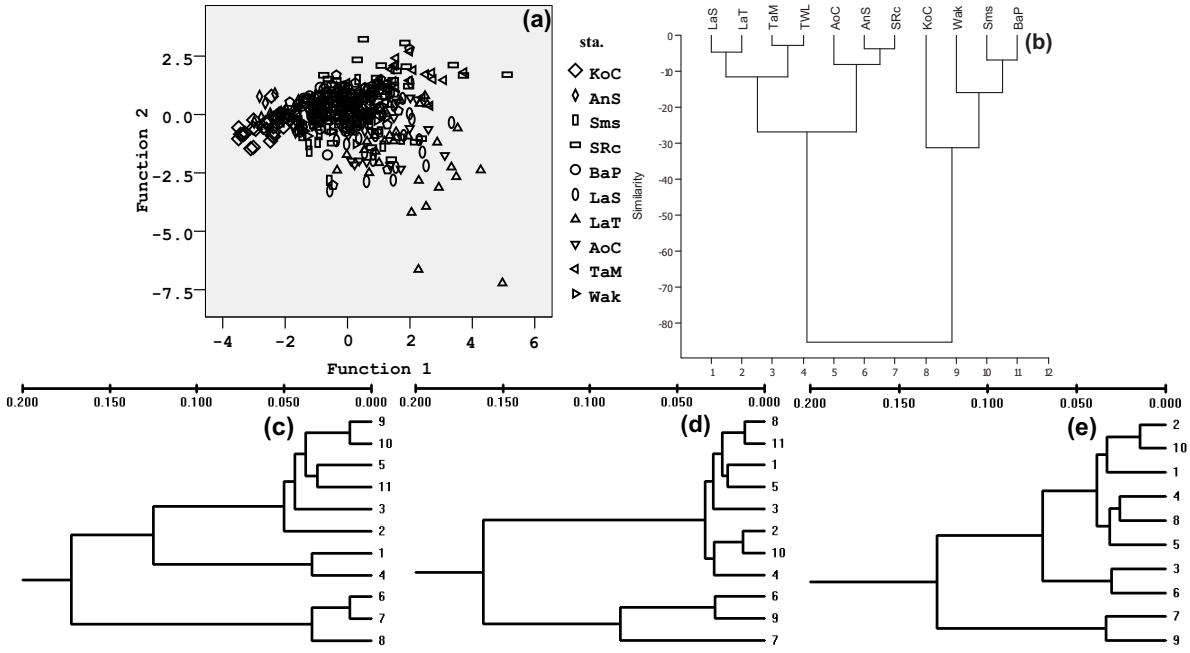

Fig. 2 (a) Relationship among specimens of *Gracilaria* collected from different environment as discriminant function of eight morphological characters. (b) Similarity of the *Gracilaria salicornia* samples based on eight morphological characters. Phylogenetic tree indicating relation in populations of *G. salicornia* collected from different habitats in (c) 2005 (d) 2006 (e) 2007. 1 = KoC, 2 = AnS, 3 = SRc, 4 = Sms, 5 = BaP, 6 = AoC, 7 = LaS, 8 = LaT, 9 = TaM, 10 = Wak, 11 = TWL.

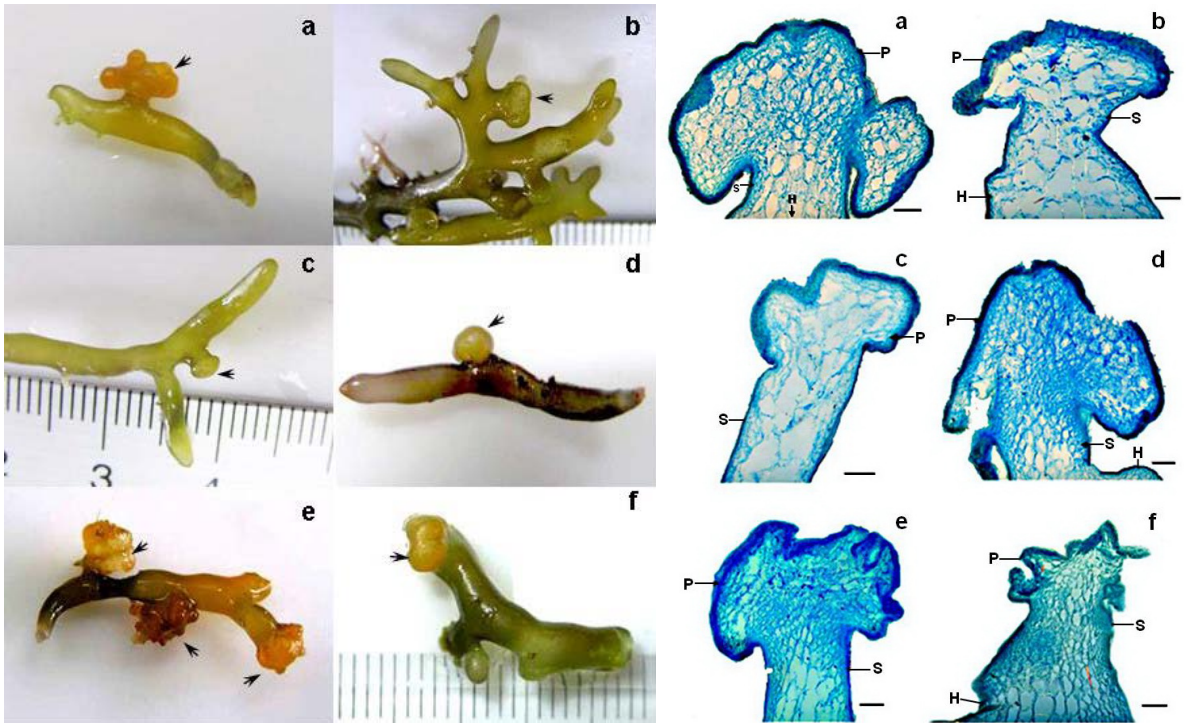

Fig. 3 External appearance of the adelphoparasite in *Gracilaria salicornia* (left panel); transverse sections of the adelphoparasite collected from different habitats (right panel): (a) Sms (b) AoC (c) LaS (d) TaM (e) Wak (f) TWL. H = host, P = parasite, S = stalk. Scale bars = 250 μm.

thallus. In the AnS specimens (Fig. 1b), branches were elongated and cylindrical with blunt tips in which the upper parts of each branch were clearly constricted, while the lower parts were slightly constricted or not constricted at each node. Notably, the AnS specimens had no rhizoid and each branch was attached with root-like discs as holdfast or rigid root-like holdfasts, which is different from the BaP plants (Fig. 1e). The BaP plants grow only in abandoned cement tanks established along the seashore that are always submerged during low tide and in rather clear seawater. Moreover plants of the BaP were generally found along the upper part of the cement tanks, near the surface of the seawater. The thallus was dark green or yellowish orange in colour and was entangled with the green seaweed *Caulerpa racemosa*. The thallus of the BaP specimen was succulent and rigid and was attached to the substrate with a rhizoid. It had a semi-erect branch with cylindrical segments which were blunt at the tips and slightly constricted or not constricted at each node. These were clearly different from the AnS plants, although these two collecting sites were in similar sheltered environments. The AnS site was located inland near a mangrove area with a small canal connection to the sea. The BaP site was sheltered in the abandoned cement tanks established along the seashore. The differences in these collecting sites may be the cause of their changes in external feature[3].

KoC, SRc, LaT, and AoC had semi-exposed environments with rather turbid to very turbid seawater. The samples were mostly dark brown or brownish green. The KoC plants (Fig. 1a) had cylindrical branches while the SRc plants (Fig. 1c), branches formed robust segments. The external appearance of the SRc plant resembles the specimens found at LaT and AoC. However, we noted that branches of the LaT plants (Fig. 1h) were conjugated (Fig. 1q), and their branches formed segments with apex protuberance, which were not found in other samples from AoC, KoC, and SRc.

Sms, LaS, TaM, Wak, and TWL had turbid water. The external features appeared succulent and the plants were attached to the substrate with a root-like rhizoid. However, with the LaS (Fig. 1f) and TaM (Fig. 1i) plants, branches formed segments with a protuberance at the apex. Upper branches were constricted while lower branches were slightly constricted or not constricted at each node, which was similar to the plants collected from LaT. Plants collected from

Table 2 Comparative external feature of *Gracilaria salicornia* collected from different sites.

Site	external feature	branch feature	substrate	rhizoid	branch diameter (mm)	branch base	branching
KoC	ef1	bf1	sbs1	r1	0.90–1.95	brb1	br1
AnS	ef1	bf1	sbs2	r2	0.80–1.90	brb2	br1
SRc	ef2	bf1	sbs2	r3	2.20–3.85	brb3	br1
Sms	ef2	bf2	sbs1	r1	1.30–2.85	brb3	br2
BaP	ef2	bf2	sbs3	r1	1.20–3.20	brb3	br2
LaS	ef2	bf3	sbs4	r1	1.80–3.35	brb4	br3
AoC	ef2	bf2	sbs4	r1	1.50–3.85	brb1	br4
LaT	ef3	bf4	sbs4	r3	2.25–3.80	brb5	br5
TaM	ef3	bf4	sbs5	r1	2.05–3.75	brb5	br5
Wak	ef4	bf5	sbs4, sbs5	r1	0.95–3.75	brb1	br6
TWL	ef2	bf4	sbs4, sbs5	r1	1.80–3.70	brb6	br7

ef1 = thallus prostrate forming a rough entangled mass; ef2 = thallus succulent, prostrate forming a rough entangled mass; ef3 = thallus succulent prostrate to semi-erect up to 6 cm high, forming entangled mass; ef4 = thallus succulent prostrate to semi-erect, forming a rough entangled mass; bf1 = branches having elongate or cylindrical segment with blunt tips; bf2 = semi-erect branch forming cylindrical segments; bf3 = branches having elongate or cylindrical segment with protuberance at apex; bf4 = branches forming cylindrical segments with protuberance at apex; bf5 = branches having cylindrical segment with blunt tips; sbs1 = small pebble, shell and other seaweed; sbs2 = small pebble, shell; sbs3 = abandoned cement tanks along the sea shore; sbs4 = gravel, shells; sbs5 = rock; r1 = present; r2 = absent (but root like disc holdfast present); r3 = present (and root like disc holdfast); brb1 = slightly constricted or not constricted; brb2 = upper part of each branch clearly constricted, lower part slightly constricted or not constricted at each node; brb3 = slightly constricted or not constricted at each node; brb4 = clearly constricted at the upper part of branch but slightly constricted or not constricted at the branch base; brb5 = markedly constricted at upper portion, but slightly constricted or not constricted at the lower portion; brb6 = markedly constricted at upper portion, but slightly constricted at the lower portion; br1 = each segment with di- or tri-chotomously or irregularly branching; br2 = segments with di- or tri-chotomously or irregularly branching with blunt at tips; br3 = segments with dichotomously or irregularly branching with blunt at tips; br4 = branch segments with di- or tri-chotomously or irregularly branching with blunt at the tips; br5 = branch segments with di- or tri-chotomously or irregularly branching; br6 = branch segments with dichotomously or irregularly branching with blunt at the tips; br7 = branch segments with dichotomously or irregularly branching.

Table 3 Transverse section and reproductive structure of *Gracilaria salicornia* collected from different habitats.

Site	cortical layer		medullary layer			sporangium	spermatangium			cystocarp					
	cell layer	cell size	cell size	diameter (μm)	Intercellular space (μm)	type	type	width (μm)	height (μm)	shape	base of cystocarp	diameter (mm)	height (mm)	absorbling filament	pericarp layer
KoC	1–2	S	L	158–416	8–13	–	–	–	–	–	–	–	–	–	–
AnS	2	S	L	133–359	4–8	t	v	24–33	28–55	sh1	bc1	0.70–1.04	0.56–0.84	abf1	13–17
SRc	2	S	L	216–509	4–8	t	v	25–35	30–66	sh1	bc1	1.00–1.54	0.66–1.02	abf1	15–18
Sms	2	S	L	150–434	4–8	t	v	25–33	33–55	sh1	bc2	1.24–1.34	1.80–0.96	abf2	15–17
BaP	2	S	L	150–509	4–8	t	v	24–34	36–68	sh2	bc1	0.96–1.40	0.80–1.04	abf2	12–18
LaS	2	S	L	175–409	8–13	t	v	25–30	25–70	sh2	bc1	0.76–1.08	0.32–0.88	abf3	15–18
LaT	2	S	L	325–442	8–13	–	–	–	–	sh1	bc1	0.78–1.28	0.40–0.80	abf1	16–18
AoC	2	S	L	208–500	4–8	t	v	25–34	25–50	sh1	bc2	0.96–1.38	0.50–1.08	abf2	14–18
TaM	1–2	S	L	216–692	4–8	t	v	22–38	35–62	sh1	bc1	0.80–1.34	0.50–1.20	abf2	14–17
Wak	1–2	S	L	258–500	8–13	t	v	24–30	30–64	sh1	bc1	0.98–1.60	0.70–1.24	abf1	13–18
TWL	2	S	L	225–434	8–13	t	v	25–34	33–50	sh1	bc2	0.62–1.56	0.40–0.98	abf1	15–17

– = reproductive structure not found; S = small; L = large; t = tetrahedral sporangia; v = verrucosa type spermatangia; sh1 = spherical or dome shaped without rostrum; sh2 = spherical or dome shaped, slightly rostrate or non rostrate; bc1 = slightly constricted or not constricted; bc2 = slightly constricted; abf1 = long, connect to pericarp; abf2 = long, connect to or not to pericarp; abf3 = not connect to pericarp.

Sms (Fig. 1d) resemble the specimens collected from Wak (Fig. 1j) and TWL (Fig. 1k). The appearance of branching and base constriction of each segment did not obviously vary with the environment as had been mentioned in previous reports[2,3,18]. The branching pattern of the algae varied with the environment. This study was in agreement with the previous report on *G. salicornia* collected from the coast of China[2]. The author suggested that branching of *G. salicornia* depends on the environment while the forms of male and female reproductive organs are distinct and do not change with environmental factors. This had been

confirmed in a laboratory culture of *G. salicornia*[19]. Several studies have been reported on variations in morphology of *G. salicornia*[2,3].

Morphometric analysis of the adelphoparasite

We observed the adelphoparasite on the *Gracilaria* collected from the sites at LaS, AoC, Sms, TaM, Wak, and TWL (Fig. 3 left panel). The specimens of *G. salicornia* from the sampling sites at LaT, BaP, AnS, KoC, SRc were found to be without adelphoparasites. The adelphoparasite showed external features and distribution, which varied depending on the environment of each study site. Morphometric analysis of the adelphoparasite is shown in Table 4 and Table 5. Transverse sections of the thallus (Fig. 3 right panel) showed the parasite cell without penetrating rhizoids into the host tissue and the boundary tissue between the host and parasite is therefore indistinguishable.

The discriminant function (Fig. 4a) showed a low degree of separation of the samples suggesting that the parasites collected from different study sites constituted a similar species. According to the data set analysed using the program PAST for multivariate statistics, the clustering based on all seven characters (2, 5, 8, and 9–12) divides the specimens of the adelphoparasite into two groups: (1) specimens of Sms, LaS, and AoC, and (2) specimens of TWL, TaM, and Wak (Fig. 4b).

The adelphoparasite was found in six out of 11 sites. Five of these (Sms, LaS, Wak, TaM, and TWL) had a mostly exposed habitat and one (AoC) was semi-exposed. The samples did not exhibit significant seasonal variation ($p = 0.05$, $n = 39$). The adelphoparasite colour sometimes was similar to that of the host (Fig. 3 left panel) e.g., at the AoC, LaS, and TaM sites. The colours differed at the other sites. Notably, the parasite was the same colour as the algal host in rather high turbidity waters but not in lower turbidity waters. The occurrence of the adelphoparasite showed a positive correlation to turbidity of the seawater ($r = 0.510$, $p < 0.05$). The variation in morphology of the parasite may be a response to the changing environment at each study area.

Appearance of parasites of the same colour as the algal host has been reported in *Congracilaria*[10,14] while a parasite of a different colour from the algal host has been reported in *Gracilariophila*[14]. In this study, distribution of the adelphoparasite was different between the sampling areas in the east and the west coast of the Gulf of Thailand. On the east coast, the adelphoparasite was found in abundance during the hot season while on the west coast the adelphoparasite was found in abundance during the rainy season.

Parasites exhibit thalli with a mushroom-like appearance in general, with a smooth or rather smooth surface, and with a short or long stalk (or stipe). This parasite has no rhizoid penetrating into the host tissue, and the border between host and parasite tissue has no zone of comparatively small cells as was found in previous reports[12,14]. The thallus has a dome-like cystocarp that is slightly constricted or not constricted at the base. The pericarp has 8–19 layers (usually 10–16 layers) except for the specimens collected from LaS which had 15–19 layers. The parasites generally have fewer layers than the host.

Specimens of the parasites also produce verrucosa type male spermatangia as in the host. This species of adelphoparasite is similar in external morphology to *Congracilaria*[14]. However, the Thai parasite has tetrasporangia, while *Congracilaria* has bisporangia. These results are similar to those of Terada et al[12] who studied adelphoparasites on *G. salicornia* collected from fishponds in Trat and Rayong in May 1997. They also reported that the adelphoparasite is similar to *Gracilariocolax*, but with monosporangia. In this study, all parasite specimens are different in morphology from the one reported in Malaysia. The Malaysian parasite has no stipe or a very short stipe and consists of small cells in the medulla, and a zone of small cells between host and parasite[13]. The Thai species is mushroom-like in shape with stipe and without penetrating rhizoid which is similar to the parasite found in *G. edulis* collected from Indonesia[11]. However, the Indonesian parasite has a zone of small cells between host and parasite, but this is absent in the Thai species. In our study, even though some of the Thai parasites are the same colour as their hosts, they are similar to *Gracilariophila*. The male plant has superficial spermatangia with penetrating rhizoid between host and parasite[20].

Molecular analysis

The yield of DNA extracted from all samples (300–350 mg/sample) ranged from 34 to 558 µg/µl and the purity (A_{260}:A_{280}) ranged from 1.164 to 1.662. Of 20 random primers screened, twelve primers, OPA10 (GTGATCGCAG), OPA11 (CAATCGCCGT), OPK7 (AGCGAGCAAG), Meyer and Mitchell (GAGGGTGGCGGTTCT), primer 2 (GGGCATCACC), primer 3 (AGCCAACTTG), primer 5 (TGTCAGCAAA), primer 7 (GCTGCTACAA), primer 9 (CGGGTCAACG), primer 11 (CCACATCCAA), primer 14 (TGGAGTGATC), and primer 15 (GAAGACGAAC) generated reproducible polymorphic DNA bands from the samples.

Table 4 External features of adelphoparasite in *Gracilaria salicornia* collected from different habitats.

Sites	external appearance	total height (mm)	length of stipe (mm)	height of cap (mm)	diameter of cap (mm)
Sms	mushroom-shaped having slightly undulate cap with a short stipe, thallus orange, different from host	1.08–5.46	0.34–3.08	0.44–2.38	1.08–5.64
LaS	mushroom-shaped frond having smooth cap with a short stipe, thallus greenish yellow or dark-brown, resemble to host	1.00–3.12	0.30–1.96	0.50–1.96	1.00–4.08
AoC	frond having smooth or slightly undulate cap with a short stipe, thallus greenish yellow or dark-brown, resemble to host	1.20–3.16	0.32–1.94	0.60–2.18	0.90–3.58
TaM	mushroom-shaped frond having smooth cap with a short stipe, thallus yellow-green, resemble to host	1.28–2.68	0.42–1.72	0.52–1.74	1.26–3.90
Wak	frond having smooth or slightly undulate cap with a short stipe, thallus orange, different from host	1.72–2.40	0.34–1.52	0.78–1.92	0.98–3.00
TWL	frond having smooth or slightly undulate cap with a short stipe, thallus brownish orange, different from host	1.56–2.86	0.50–1.48	0.74–1.76	1.36–2.56

Table 5 Transverse section and reproductive structure of adelphoparasite in *Gracilaria salicornia* collected from different habitats.

Site	cell	cell	intercellular	sporangium	spermatangium			cystocarp					
	cortex (μm)	medullar (μm)	space (μm)	type	type	width (μm)	height (μm)	shape	base of cystocarp	width (mm)	height (mm)	absorbling filament	pericarp layer
Sms	2.8–6.3	81.3–241.7	4.2–8.3	t	v	25.0–50.0	37.5–80.6	sh1	bc1	44–80	32–54	1 (0)	8–11
LaS	4.2–7.6	106.3–270.8	4.2–16.7	t	–	–	–	sh1	bc1	50–56	24–30	1 (0)	15–19
AoC	3.5–7.6	102.1–312.5	4.2–16.7	t	v	25.0–37.5	34.7–93.1	sh1	bc1	42–54	28–36	1 (0)	9–16
TaM	3.5–7.6	72.9–120.8	4.2–8.3	t	v	25.0–33.3	35.4–63.9	sh1	bc1	40–68	22–58	1 (0)	10–16
Wak	4.2–6.9	72.9–147.9	4.2–16.7	t	v	25.0–33.3	40.3–68.1	sh1	bc1	50–100	38–70	1 (0)	10–15
TWL	3.5–6.9	68.8–97.9	4.2–8.3	–	v	25.0–34.5	34.7–63.9	sh1	bc1	48–66	28 –44	1 (0)	10–16

– = not found; t = tetrahedral sporangia; v = verrucosa spermatangia; sh1 = spherical or dome shaped without rostrum; bc1 = slightly constricted or not constricted; 1 (0) = present or absent.

Polymorphism analyses of *Gracilaria salicornia*

In the specimens collected during the monsoon season in 2005 and dry season in 2006 and 2007, the polymorphic DNA bands were generated by primer OPA10, OPA11, primer 2, and primer 9 at 200–2000 bp. The primers of Meyer and Mitchell and OPK7 generated the DNA bands at 200–3000 bp. However, primers 5, 7, 11, and 15 generated the DNA bands at 300–1500, 300–2000, 300–3000, and 100–2000 bp, respectively. Primer 3 generated the DNA bands at 100–2000 bp in the specimens collected during the dry season in 2006 and 2007, while not in the specimens collected in the monsoon season of 2005. Primer 14 generated the DNA bands at 200–2000 bp in the monsoon season specimens collected

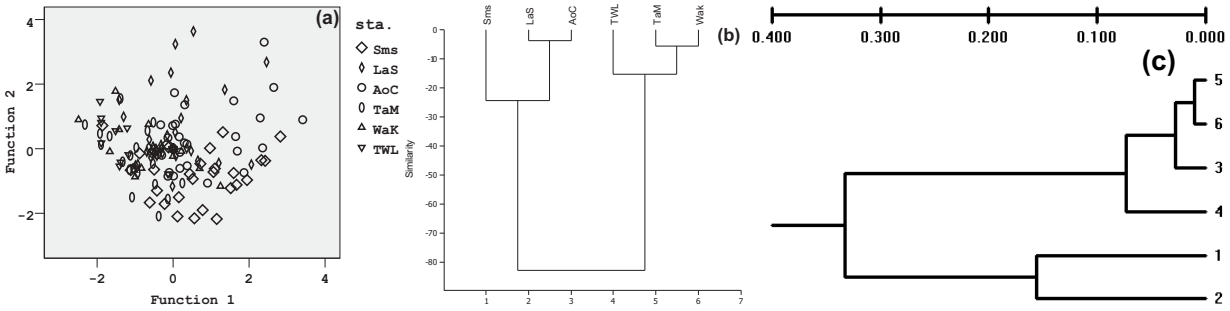

Fig. 4 (a) Relationship among specimens of the adelphoparasite on *G. salicornia* collected from different environments as a discriminant function of seven morphological characters; (b) similarity of the adelphoparasite collected from different habitats based on seven morphological characters; (c) phylogenetic tree indicating relations in populations of the adelphoparasite collected from different habitats: 1 = AoC in 2006, 2 = TaM in 2005, 3 = Wak in 2006, 4 = TWL in 2005, 5 = AoC in 2007, and 6 = LaS in 2007.

in 2005 and dry season specimens collected in 2006 and 2007. However, the specimens collected from the sites at SRc, BaP, and TWL in 2007 did not generate the polymorphic DNA bands.

All the screened primers that generated reproducible polymorphic DNA bands from the *Gracilaria salicornia* specimens showed a similarity index with the highest value of 0.988 in the specimens of AoC–LaS and TaM–Wak in 2005. The lowest value was 0.764 in the KoS–LaS and Sms–LaS. In 2006 the similarity index had the highest value of 0.989 in the LaT–TWL specimens, and the lowest was 0.814 in the Sms–LaS specimens. Specimens collected in 2007 had the highest similarity index of 0.986 in the SRc–TWL specimens, and the lowest of 0.851 in the Sms–LaT.

The binary data of all twelve primers tested by the TFPGA and UPGMA showed the relationship among *G. salicornia* specimens collected in 2005 and 2006. The phylogenetic tree of the specimens collected in 2005 showed that the population was clustered into three groups: (1) specimens from LaT, LaS, and AoC, (2) specimens from Sms and KoC, and (3) specimens from AnS, SRc, TWL, BaP, Wak, and TaM (Fig. 2c). However, the phylogenetic tree for 2006 showed that the population was clustered into two groups: (1) specimens from LaS, TaM and AoC, and (2) specimens from Sms, Wak, AnS, SRc, BaP, KoC, TWL, and LaT (Fig. 2d).

In addition, the phylogenetic tree of the specimens collected in 2007 showed that the population was clustered into three groups; (1) specimens from Wak and LaT, (2) specimens from LaS and Sms, and (3) specimens from AoC, TaM, BaP, AnS, TWL, and SRc (Fig. 2e).

As above, the phylogenetic tree showed clustering of the alga into 2–3 groups that corresponded to the results obtained from morphometric analysis. Their similarity index was close to 1, showing a relation among the populations of *G. salicornia* collected from different locations. They may be the same or similar species and their difference in morphology might be caused by differences in environment. However, further confirmation must be made by increasing the number of samples.

Polymorphic analyses of adelphoparasite

From the analysis of DNA from the specimens of TWL and TaM in 2005, Wak and AoC in 2006, and LaS and AoC in 2007, polymorphic DNA bands were generated by OPA10, OPK7, primer 2 and primer 11 at 200–1200 bp, while primer 3 and primer 14 generated the DNA bands at 100–1200 bp. Other primers, i.e., Meyer and Mitchell, OPA11, primer 5, primer 7, primer 9, and primer 15 generated the polymorphic DNA bands at 200–1500, 200–1000, 300–800, 100–1500, 100–2000, and 200–2000 bp, respectively. The DNA bands generated by each primer showed a similarity index close to the one with the highest value of 0.990 in the AoC and LaS specimens collected in 2007, suggesting a close relationship among populations of the parasite collected from different locations or environments.

The phylogenetic tree (Fig. 4c) shows that the adelphoparasite populations were clustered into two groups: (1) specimens collected from the site at TaM in 2005 and AoC in 2006, and (2) specimens collected from the site at TWL in 2005, Wak in 2006, and from the sites at LaS and AoC in 2007.

As with the molecular results of their algal host, the adelphoparasites were clustered into two groups, which corresponded to the result of the morphometric analysis. The similarity index of parasites collected from different habitats had values close to one, indicating a close relationship which might constitute the same or similar species despite the wide ranging morphology observed.

The DNA polymorphism analysis between populations of *G. salicornia* collected in 2006 and the adelphoparasite collected in 2005–2006, and the relationship between populations of *G. salicornia* and the adelphoparasite collected in 2007 were performed. The primers that generated reproducible polymorphic DNA bands were primers Meyer and Mitchell (200–1200 bp), OPA10 (200–3000 bp), OPA11 (200–2000 bp), OPK7 (200–3000 bp), primer 2 (200–2000 bp), primer 3 (100–2000 bp), primer 5 (100–2000 bp), primer 7 (100–2000 bp), primer 9 (100–2000 bp), primer 11 (200–3000 bp), primer 14 (100–2000 bp), and primer 15 (200–2000 bp).

The binary data of all twelve primers tested by the TFPGA and UPGMA showed a relationship among *G. salicornia* specimens collected in 2006 and the adelphoparasite collected in 2005–2006 (Fig. 5a). The phylogenetic tree illustrates the relationships among *G. salicornia* and the adelphoparasite, which were clustered into 4 groups: (1) specimens of *G. salicornia* from Sms, BaP, and AoC, (2) AnS, LaT, KoC, SRc, and TWL, (3) LaS, Wak, and TaM, and (4) adelphoparasite from TWL and TaM collected in 2005, and adelphoparasite from Wak and AoC collected in 2006. However, the phylogenetic tree for the samples collected in 2007 showed two groups (Fig. 5b): (1) specimens of *G. salicornia* from Sms, LaS, AnS, LaT, TaM, BaP, Wak, and TWL, and (2) specimens of the adelphoparasite from AoC, LaS, and

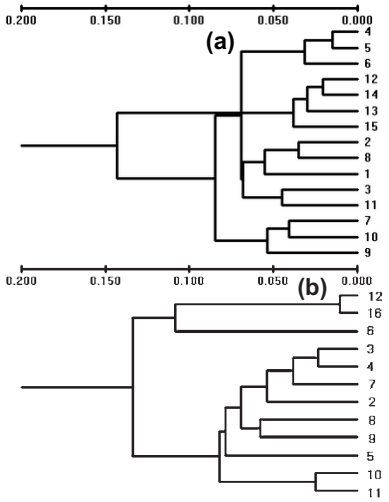

Fig. 5 (a) Phylogenetic tree showing relation among *G. salicornia* collected in 2006 from different habitats at 1 = KoC, 2 = AnS, 3 = SRc, 4 = Sms, 5 = BaP, 6 = AoC, 7 = LaS, 8 = LaT, 9 = TaM, 10 = Wak, 11 = TWL, and the adelphoparasite collected at 12 = AoC in 2006, 13 = TaM in 2005, 14 = Wak in 2006, and 15 = TWL in 2005. (b) Phylogenetic tree of *G. salicornia* and adelphoparasite collected in 2007 from 2 = AnS, 3 = SRc, 4 = Sms, 5 = BaP, 6 = AoC, 7 = LaS, 8 = LaT, 9 = TaM, 10 = Wak, 11 = TWL, and 12 = adelphoparasite from AoC and 16 = adelphoparasite from LaS.

G. salicornia from AoC. The phylogenetic trees of the alga host and the parasite in Fig. 5 were clearly grouped, while the similarity index was close to one. This suggests that the populations of both *G. salicornia* and the parasite had a close relationship.

A close relation between the algal host and the parasite may result from the parasite cell penetration into the algal host, *G. salicornia* and the transfer of their replicated nuclei into the cytoplasm of the host cell[5]. Genomic transfer into the host cell, as shown in the DNA analysis, as had been reported in other adelphoparasites[8,21].

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