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A novel obligate cultivation mutualism between damselfish and *Polysiphonia* algae

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In cultivation mutualisms, farming animals prepare fields for cultivars, enhance their growth and harvest them. For example, in terrestrial ecosystems, plant–herbivore cultivation mutualisms arose between humans and their crops only relatively recently. We discovered an obligate cultivation mutualism between a damselfish and an alga in a coral reef ecosystem. The damselfish, *Stegastes nigricans*, manages algal farms through territorial defence against the invading grazers and through weeding of unpalatable algae. As a result, the algal farms of *S. nigricans* are dominated by one species, *Polysiphonia* sp. We performed an exhaustive survey of algal assemblages inside and outside the territories of five damselfish species around the Ryukyu Islands, Japan, using molecular and morphological characteristics. *Polysiphonia* sp. 1 grew exclusively inside the farms of *S. nigricans*, and never elsewhere. Since only *Polysiphonia* sp. 1 is harvested and consumed by the damselfish as a staple food, this interdependent relationship is an obligate cultivation mutualism. This is the first record of an obligate plant–herbivore cultivation mutualism in a marine ecosystem. Our data also suggest that three other *Polysiphonia* species are facultatively mutual with, commensal with, or parasitic on other damselfish species.

Keywords: obligate cultivation mutualism; territorial damselfish; *Polysiphonia* algae; coral reef

1. INTRODUCTION

Cultivation mutualisms between humans and their crops have evolved through 'proto-domestication' in which humans use and select plants intruding on human-disturbed habitats (Rindos 1984). In marine ecosystems, some herbivorous damselfishes and limpets maintain proto-domesticated algal assemblages (i.e. algal farms) by excluding grazers and cultivating distinct crop assemblages (Branch 1981; Ceccarelli *et al.* 2001). Some limpets on intertidal rocky shores in South Africa and the west coast of North America have evolved facultative cultivation mutualisms with species-specific but ubiquitous algae (Branch 1981). Similarly, the damselfish *Stegastes nigricans* has been shown to maintain a monoculture of a filamentous red-alga, *Polysiphonia* sp., by excluding invading herbivores (Hata & Kato 2004). In addition, they remove less-digestible competitive algae from their algal farms (Hata & Kato 2002). This intensive management by *S. nigricans* results in selection for fast-growing palatable algae. Cage experiments that exclude a territory-holding damselfish as well as all herbivores have shown that in the absence of weeding by the fish, the *Polysiphonia* sp. are overgrown by other algal species within a week (Hata & Kato 2003). When only *S. nigricans* was removed, its algal farm was invaded by grazers and denuded of algae in a few days. Thus, intensive management and aggressive territorial defence allow the fish to maintain a monoculture of *Polysiphonia* sp. on which it feeds as staple food (Hata & Kato 2002). We investigated whether the dependence of this alga on the fish is obligatory by determining the occurrence of the alga outside *S. nigricans* algal farms. In addition, we investigated whether other *Polysiphonia* spp. algae have species-specific relationships with damselfishes and assessed the phylogenetic relationships among *Polysiphonia* spp. algae that are cultivated by damselfish.

2. MATERIAL AND METHODS

(a) *Sampling*

We collected *Polysiphonia* spp. algae and related algal species from inside and outside the territories of various damselfish. Whether a site was inside or outside the territories of damselfish was determined after 20 min of observation immediately before sampling. To collect algae exhaustively from outside the territories of fish, we set line transects from the beach to offshore areas, perpendicular to the shoreline, at two, four and ten reef flats around Okinawa Island (26°04–52′ N and 127°38′–128°19′ E), Ishigaki Island (24°19–36′ N and 124°04–20′ E) and Iriomote Island (24°15–25′ N and 123°40–55′ E) respectively, in 2003 and 2004. The average length of the 16 line transects was 477 m (1057 m maximum and 156 m minimum). We set a 1×1 m quadrat outside the territories at 50 m intervals on each transect and scraped all algae and seagrass inside the quadrat into a mesh bag by grazing the entire substratum with a knife. In total, 158 samples were collected from outside damselfish territories. Five territorial herbivorous damselfishes inhabited these study areas: *S. nigricans*, *Stegastes lividus*, *Hemiglyphidodon plagiometopon*, *Dischistodus prosopotaenia* and *Plectroglyphidodon lacrymatus*. Whenever we found these damselfishes along the line transects, we collected all the algae from 7×7 cm quadrats placed inside the territories. *Stegastes nigricans* was found on 14 lines, together with all the other damselfishes except *H. plagiometopon*, which was the sole inhabitant of one site near a river mouth. In total, 53, 18, 9, 19 and 13 samples were collected from the territories of *S. nigricans*, *S. lividus*, *H. plagiometopon*, *D. prosopotaenia* and *P. lacrymatus*, respectively.

We also collected algae outside the damselfish territories in Makaha Beach and Pokay Bay around Oahu Island (21°15–42′ N and 157°38′–158°16′ W) in Hawaii in October 2003, and inside and outside territories of *H. plagiometopon* in a coral reef around Koh Hae Island (7°46′ N, and 98°21′ E) in Thailand in March 2004. The algal samples were immediately preserved in 100% ethanol. In the laboratory, samples were displaced using distilled water, and all *Polysiphonia* algae were sorted under a microscope. Small thalli of *Polysiphonia* algae collected from inside ($n=67$) and outside ($n=28$) territories were classified into 16 species using molecular data. The total biomass of algae in samples collected from damselfish territories and that of *Polysiphonia* species were measured in wet weight.

(b) *Molecular methods*

We extracted total DNA from field-collected, ethanol-preserved algae. A fragment of the 18S ribosomal RNA gene was amplified by PCR using the primers 5′-ACCTGGTTGATCCTGCCAG-3′ and G07 and was directly sequenced using the above two and other four primers (Saunders & Kraft 1994). All the sequences were deposited in the NCBI GenBank database (accession nos. AB219858–AB219930).

(c) *Phylogenetic analyses*

Maximum-parsimony (MP) and maximum-likelihood (ML) analyses were conducted using PAUP* v. 4.0b10; Bayesian

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inference (BI) was conducted using MrBayes v. 3.0b4. The MP analyses employed the heuristic search option with TBR (tree bisection and reconnection) branch swapping and 1000 random-taxon-addition replicates, identifying the 60 most parsimonious trees of length 468 steps, C.I. $=0.607$ and R.I. $=0.804$. Heuristic MP bootstrap analysis consisted of 1000 pseudoreplicates with 10 random-taxon-addition replicates per pseudoreplicate. The likelihood ratio test implemented in ModelTest v. 3.06 found that the $\mathrm{TrN}+\Gamma+\mathrm{I}$ model best fits the sequence data, and this model was employed in a heuristic ML analysis. A heuristic search with 10 random-taxon-addition sequences and TBR branch swapping was performed. BI was carried out based on the model of $\mathrm{GTR}+\Gamma+\mathrm{I}$ with 1 000 000 generations, sampling every 100 generations. The first 100 samples were discarded as burn-in.

3. RESULTS AND DISCUSSION

Our field collections revealed four *Polysiphonia*, specialized to specific damselfish species (figure 1; Fisher's exact test, all $p<0.001$; figure 2). These four *Polysiphonia* species were morphologically distinguished from 21 species known from Japan (Yoshida 1998) in having four pericentral cells, ecorticated fronds and rarely branched erect axes (figure 1). This indicates that these *Polysiphonia* species have never been found as free-living forms, and thus, we called the algal species, *Polysiphonia* spp. 1–4. *Polysiphonia* sp. 1, which was always dominant in the algal farms of *S. nigricans*, was encountered only inside the farms of *S. nigricans* and never outside them, irrespective of intense sampling (figure 1). This suggests that only *S. nigricans* can provide *Polysiphonia* sp. 1 with the exposed sunny habitat, where grazing pressure is moderate and competitive algae are weeded out. In this way, *Polysiphonia* sp. 1 is obligately dependent on *S. nigricans*. The damselfish manages its algal farm dominated by *Polysiphonia* sp. 1 and feeds exclusively in the farm (Hata & Kato 2002, 2004), suggesting that the fish depends on *Polysiphonia* sp. 1 for staple food. Therefore, this interdependent relationship between *S. nigricans* and *Polysiphonia* sp. 1 is an obligate cultivation mutualism (table 1). We found that another damselfish, *H. plagiometopon*, had a 'semicultivated' (Harris & Hillman 1989) *Polysiphonia* species. Algal farms of this fish species were always dominated by *Polysiphonia* sp. 3 (figure 1). However, *Polysiphonia* sp. 3 also inhabited the algal farms of other damselfishes and was found to occur outside damselfish farms. This association represents a facultative cultivation mutualism, in which the fish depends on the alga, but the alga does not necessarily depend on the fish (table 1).

Polysiphonia species that correspond to 'weeds' (Harlan 1992) in terms of human cultivation were also encountered. *Polysiphonia* sp. 2 and 4 were found inside the algal farms of *P. lacrymatus* and *D. prosopotaenia*, respectively. These algae were rare outside the territories of damselfish, but did not dominate the farms (figure 1). These algae are obligately associated with specific fish, whereas the fish do not necessarily depend on the algae for staple food. Damselfishes manage their farms in a range of intensities (table 1), as both monocultures and mixed cultures (Hata & Kato 2004). Only in intensive farming systems, damselfish seem to have evolved obligate cultivation mutualisms, such as for *S. nigricans*. *Plectroglyphidodon lacrymatus* and *D. prosopotaenia*, which maintain mixed-culture farms by management without weeding, appear to engage only in facultative cultivation mutualism. *Stegastes lividus* did not have any species-specific algae in its mixed-culture farm. On the other hand, the

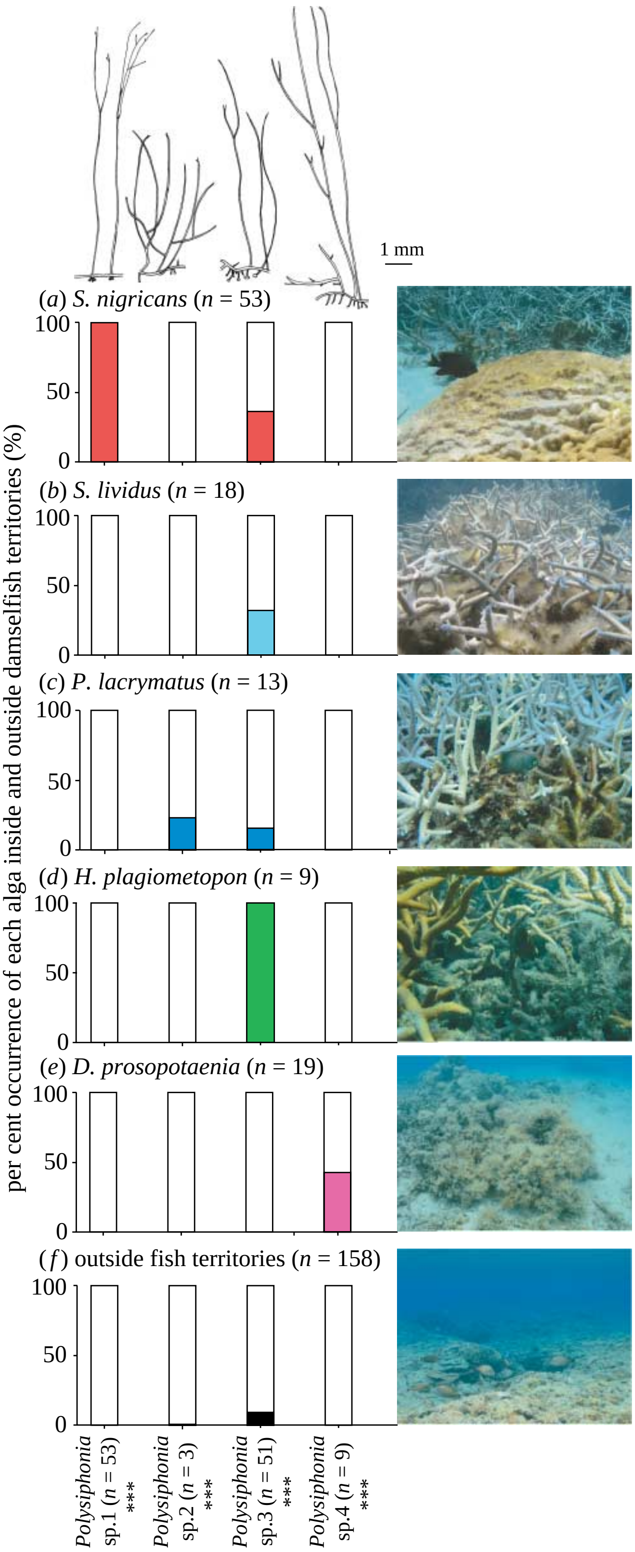

Figure 1. Percent occurrence of four *Polysiphonia* spp. algae inside and outside the territories of the damselfishes (*a*) *Stegastes nigricans*, (*b*) *S. lividus*, (*c*) *Plectroglyphidodon lacrymatus*, (*d*) *Hemiglyphidodon plagiometopon* and (*e*) *Dischistodus prosopotaenia*. The probability of occurrence of each algal species among these sites was analysed using Fisher's exact test. $^{***}p<0.001$.

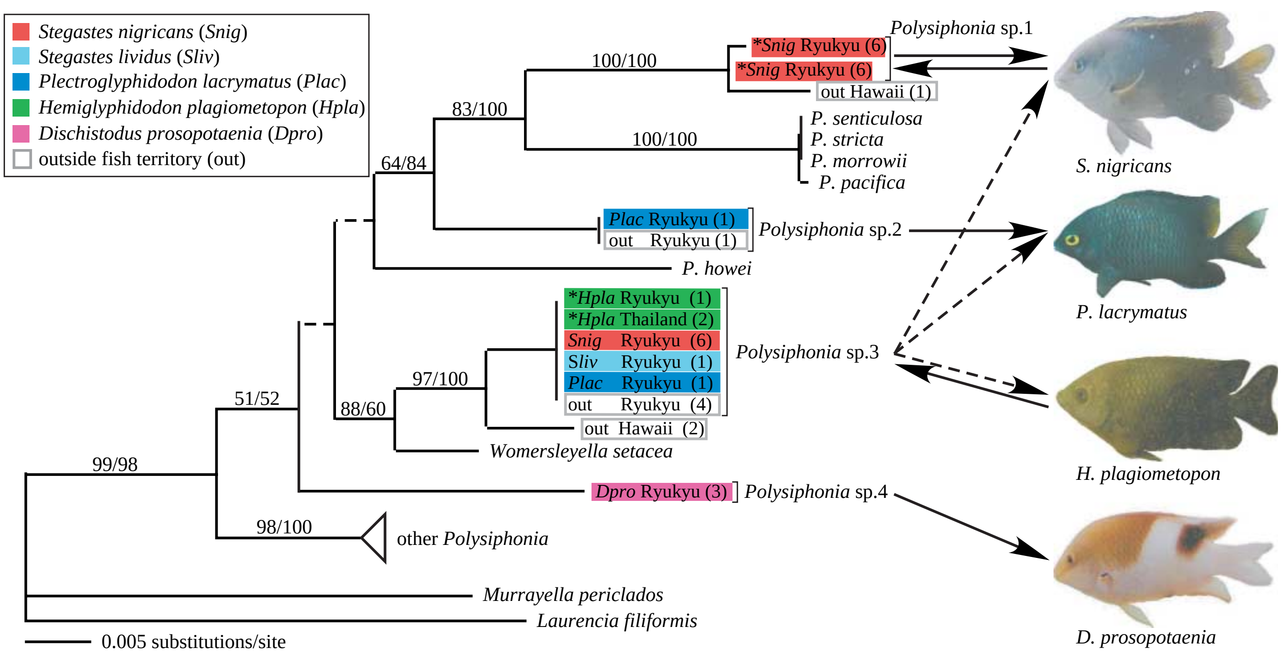

Figure 2. Phylogeny of *Polysiphonia* spp. algae found inside and outside the territories of the damselfishes *Stegastes nigricans*, *S. lividus*, *Plectroglyphidodon lacrymatus*, *Hemiglyphidodon plagiometopon* and *Dischistodus prosopotaenia*. The association of each alga is denoted by the abbreviation and colour of its damselfish host species and by collection site. An asterisk denotes the dominance of the alga in samples (representing more than 50% of the biomass). Numbers in parentheses indicate the number of DNA samples. Data for unshaded species denote citations from the NCBI GenBank. The tree was obtained using ML method, with a log-likelihood score of 4971.063. Branches that collapse in MP, ML and/or BI trees are presented as dotted lines. Nodal support is assessed by bootstrap values of MP and posterior probabilities of BI (above branches, MP/BI, respectively). Solid and broken arrows indicate obligate and facultative associations, respectively.

Table 1. Algae that inhabited the algal farms of damselfishes and their relationships with damselfishes.

attributes	*Polysiphonia* sp. 1	*Polysiphonia* sp. 3	*Polysiphonia* sp. 2 and 4
habitat	only algal farms of *S. nigricans*	mainly algal farms of *H. plagiometopon*	only algal farms of *P. lacrymatus* (sp. 2) or *D. prosopotaenia* (sp. 4)
dependence of algae on fish	obligate	facultative	obligate
intensity of farming by fish	intensive	intensive	extensive
dependence of fish on algae	obligate	obligate	partial
type of interaction	obligate cultivation mutualism	facultative cultivation mutualism	commensalism
status of algae	cultivated	semicultivated	weed

Polysiphonia species that are found exclusively symbiotically with specific damselfishes are not monophyletic (figure 2), suggesting that the adaptations of these algae to damselfishes originated independently.

Cultivation mutualisms have also evolved between fungi and terrestrial invertebrates, i.e. ants, termites and bark beetles (Vega & Blackwell 2005), and a salt marsh snail (*Littoraria irrorata*; Silliman & Newell 2003). However, only high-attine ants, termites and ambrosia beetles occur in obligate cultivation mutualisms with an obligate cultivar (Mueller *et al.* 2005). In these obligate mutualisms, most farming insects transplant inocula of fungi from their natal gardens to new colonies, and thus cultivars are transmitted vertically (Mueller *et al.* 2005). In contrast, the marine cultivation mutualism is analogous to the ancestral fungus–termite mutualism in which termites acquire cultivars horizontally via wind-dispersed spores from other colonies (Aanen *et al.* 2002; Korb & Aanen 2003). In the alga–damselfish mutualism, algal farms of *Polysiphonia* sp. 1 are mostly transmitted by *S. nigricans* from generation to generation (Lee & Barlow 2001). When colonizing a new habitat, *S. nigricans* may use water-borne spores and/or fragments of *Polysiphonia* sp. 1 dispersed from other algal farms. In fact, some *Polysiphonia* species have a high capacity for dispersal by spores (Rindi & Cinelli 2000) or fragments (Eriksson & Johansson 2005), and inside algal farms, both sexual and asexual spores of *Polysiphonia* sp. 1 were observed. Additionally, inside artificial cages that excluded all herbivores, *Polysiphonia* sp. 1 newly colonized even outside *S. nigricans* territories, although they were soon overgrown by competitive macroalgae. This experiment showed a high supply of recruits of *Polysiphonia* sp. 1 in reefs inhabited by *S. nigricans* (Hata & Kato 2003).

In the terrestrial cultivation mutualisms mentioned earlier, farming insects harvest decomposition products that originate from plant remains. In the damselfish–*Polysiphonia* cultivation mutualism, however, the damselfishes harvest photosynthate from algae cultivated on a sunlit substratum. Thus, this is the second example of an obligate cultivation mutualism between plant and herbivore, preceded by the crop–human cultivation mutualism, and the first example in a marine ecosystem.

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