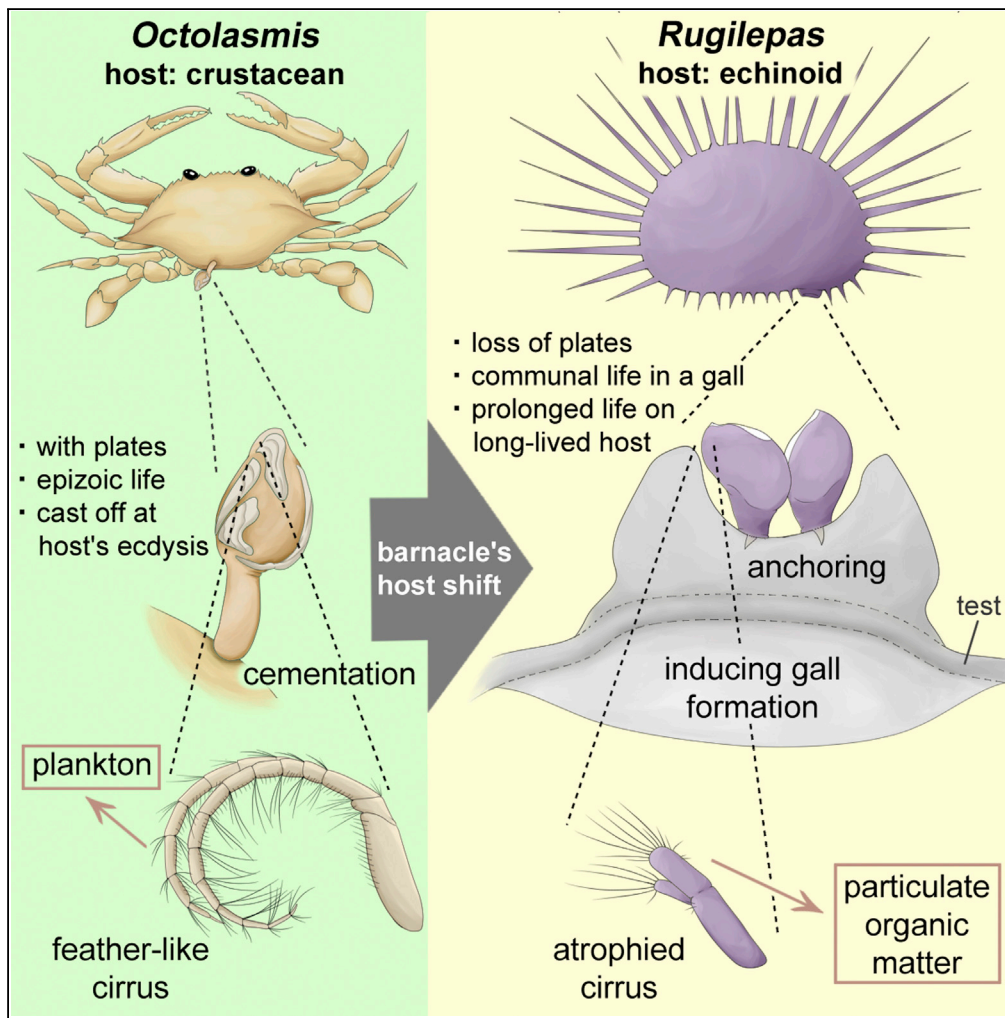


Article

Shift of Feeding Mode in an Epizoic Stalked Barnacle Inducing Gall Formation of Host Sea Urchin



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HIGHLIGHTS

Rugilepas is the first stalked barnacle that induces gall formation on echinoids

The epizoic barnacle feeds on particulate organic matter by using atrophied cirri

The obligate association with echinoids derived from epizoic life on crustaceans

The host shift caused changes of morphology, attaching device, and feeding mode

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Article

Shift of Feeding Mode in an Epizoic Stalked Barnacle Inducing Gall Formation of Host Sea Urchin

Luna Yamamori^{1,2,*} and Makoto Kato¹**SUMMARY**

Among diverse stalked barnacles, *Rugilepas pearsei* (Thoracica: Cirripedia: Arthropoda) is a rare unique species that is associated with echinoids and has highly atrophied cirri. We rediscovered the barnacle for the first time from description and verified that the barnacles live obligately in half-open galls formed on the test of the sea urchin *Echinothrix diadema* (Diadematidae: Echinodermata). A molecular phylogenetic analysis demonstrated that the obligate association with echinoids derived from epizoic life on crustaceans. A stable isotope analysis suggests that the barnacle feeds on particulate organic matter (POM) without parasitizing the host echinoids. These findings suggest that the host shift caused losses of plates and feather-like cirri, changes in the attachment device from cementation to anchoring, and a shift in feeding mode from filter feeding to POM collection. The barnacle's epizoic, superficially sub-endozoic, communal life in stout but narrow galls causes repetitive reproduction at the cost of reduced growth.

INTRODUCTION

Most stalked barnacles (Thoracica: Cirripedia) are sessile suspension feeders that live attached to hard substrata or the exoskeletons/epithelia of diverse marine animals (Darwin, 1851), but some of these cirripedes parasitize their host animals such as annelids (Day, 1939) and sharks (Johnstone and Frost, 1927; Rees et al., 2014) by embedding a root-like organ into the host body. Other epizoic barnacles living on jellyfish (Pagès, 2000) and sea anemones (Yusa et al., 2001) have different parasitic feeding modes that are facilitated by shifting their attachment and feeding devices.

Rugilepas pearsei (Microlepadidae: Lepadiformes) is a rare naked stalked barnacle with atrophied cirri, and it lives in a symbiotic relationship with echinoids (Grygier, 1991), but its feeding habit is poorly understood. Because *R. pearsei* has not been recorded since its original description, rediscovery of the barnacle has been kept waiting to answer the questions: how the association with echinoids derived, which prey/substance the barnacle feeds on, and how shift of feeding mode has occurred in a lineage of epizoic suspension feeders. By conducting extensive search for the barnacle, morphological and ecological observations, a molecular phylogenetic analysis, and a stable isotope analysis, we explored the evolutionary trajectory of the echinoid-symbiotic barnacle.

RESULTS AND DISCUSSION**Epizoic Life in Galls on Echinoids**

We rediscovered *R. pearsei* on a coral reef off Okinawa Island, Japan, and verified that it is an obligate semi-endozoic animal living in half-open galls formed on the sea urchin *Echinothrix diadema* (Diadematidae: Echinodermata), which has venom gland at the point of the secondary aboral spine tip (von Reumont et al., 2014). This is the first report of a stalked barnacle inducing gall formation on echinoids.

We performed an extensive search for this barnacle on a coral reef off Manzamo and Bise on Okinawa Island and found it only in galls on *Echinothrix diadema* (Figures 1A and 1B). The rate of parasitism was 9.3% on *Et. diadema* (43 sea urchins examined) and 0% on *Et. calamaris* (56 sea urchins) and other *Echinometra* species (100 sea urchins each). All of the barnacles on *Et. diadema* were found living communally in semi-open galls formed in the interambulacral areas on the oral side of the sea urchin. We found two to four barnacles growing in clumps at the base of each gall. Computed tomography (CT) showed that the side and bottom walls of the gall tests were thickened markedly. The claw-like peduncular attachment organs of the

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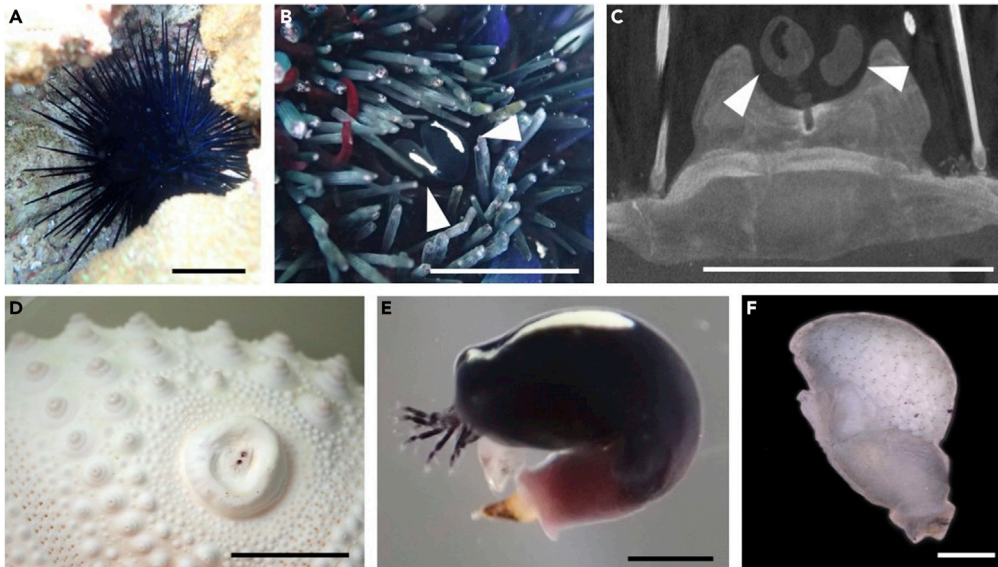


Figure 1. Habit, Morphology, and Phylogeny of *Rugilepas pearsei*

(A) A living individual of *Echinothrix diadema* inhabiting crevice of coral reef.
 (B) Two living barnacles (arrowheads) attached to the base of a semi-open gall formed on the test of the host sea urchin *Echinothrix diadema*.
 (C) Computed tomography (CT) image of a gall inhabited by two barnacle individuals (arrowheads).
 (D) Undersurface of a bleached test of a galled sea urchin.
 (E) Lateral view of a detached individual with extended cirri.
 (F) Lateral view of a brooding individual after carapace removal. Scale bars: 10 cm in (A), 1.0 cm in (B)–(D), and 1.0 mm in (E) and (F).

See also [Figure S1](#) and [Table S1](#).

barnacles were anchored deeply in the thickened basal area of the sea urchin test ([Figure 1C](#)). Compared with other areas of the test, the spines around the galls were highly modified, in that thick primary spines had been replaced with thin, poisonous secondary spines ([Figure 1D](#)). The barnacle color matched that of the host exactly. Galls were surrounded by the secondary spines of the host sea urchin, suggesting that the barnacles defend against predators by their protective coloration and by location of the stout galls on the lower sides of the sea urchin addressed to the hard substratum. Life in such a protected location may have allowed the barnacle to lose its protective plates via evolution ([Figure 1E](#)).

All of the barnacles we found were hermaphroditic adults with short penises. All galls were inhabited by two to four barnacles but no dwarf male, suggesting that reciprocal insemination occurs within each gall. The fact that all the barnacles in a gall are similar in size ([Table S1](#)) suggests that a few cypris larvae have settled almost simultaneously. Eggs or stage I nauplius larvae were brooded inside the mantle sac cavities of all barnacles collected ([Figure 1F](#)). The larvae were tadpole-like with tapering tails and pairs of long frontolateral horns alongside the bodies. These observational data suggest that the barnacles copulate with each other within a single gall and continue to reproduce at the cost of reduced body growth within the confines of the gall. The prolonged communal life on long-lived host contrasts with short life of crustacean-epizoic barnacles, which are cast off along with the exuvia at hosts' ecdysis ([Kobayashi and Kato, 2003](#)).

Feeding Habit of the Epizoic Barnacle in a Gall

Although the cirri were greatly atrophied, they were actively beating. During beating, the cirri protruded only slightly from the capitulum ([Figure 1E](#)); the strokes of individual cirri were not rhythmical. A stable isotope analysis of the $^{13}\text{C}/^{14}\text{C}$ and $^{14}\text{N}/^{15}\text{N}$ ratios of the barnacles and host sea urchins showed that they belonged to different food chains. Superimposition of the data onto the community-level datasets collected from coral reef ecosystems off Ishigaki Island and Palau ([Yamamuro et al., 1995](#)) suggested that the sea urchin feeds on corals and that the barnacle feeds on particulate organic matter (POM), partially decomposed detritus in water, without parasitizing the host echinoids ([Figure 2](#)).

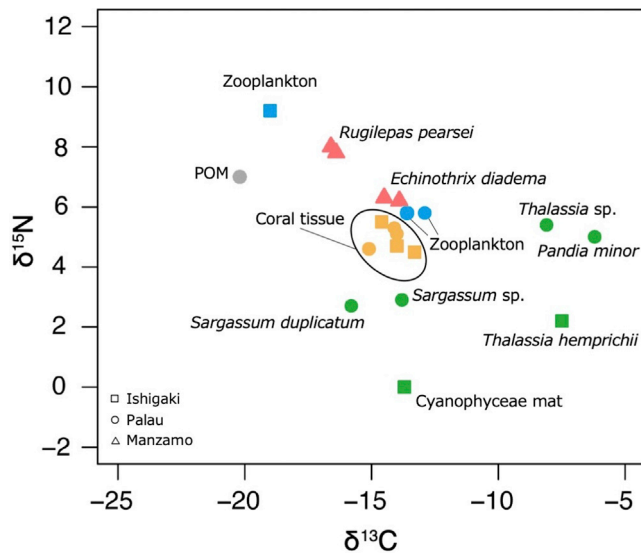


Figure 2. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ Values of *Rugilepas pearsei* and Its Host Sea Urchin, *Echinothrix diadema*, Collected off Manzamo, Okinawa Island (This Study), and of Diverse Organisms and Organic Materials Collected in the Ishigaki and Palau Coral Reef Ecosystems.

Producers are colored green, zooplankton blue, corals orange, benthic consumers red, and particulate organic matter (POM) gray. Superimposition of the data suggests that *Echinothrix diadema* feeds on corals and *Rugilepas pearsei* on POM.

Evolutionary Shift of Feeding Mode

Using the 18S rRNA, 28S rRNA, CO1, and histone 3 (H3) sequences, we performed molecular phylogenetic analyses of *R. pearsei* and related taxa belonging to Heteralepadomorpha and Lepadomorpha (Table S2). An analysis based on all markers combined (18S + 28S + CO1 + H3) (Figure 3) suggests that (1) *R. pearsei* belongs to Poecilasmatidae (Lepadomorpha) and (2) the genus *Rugilepas* has diverged from a group of *Octolasmis* species, most of which are epizoic on crustaceans. Since *Rugilepas* was originally described as Microlepadidae in Heteralepadomorpha, our result also suggested that Heteralepadomorpha is polyphyletic, as suggested by Herrera et al. (2015) and Perez-Losada et al. (2008). The character distribution in the phylogenetic tree suggests that the evolutionary transition involved the following: loss of shell plates, changes in the attachment device from cementation to anchoring, a shift in the feeding mode from filter feeding to POM collection, and a shift in the feeding device from feather-like cirri to atrophied stout cirri. Reduction of shells have occurred several times in epizoic barnacles in Poecilasmatidae, suggesting that epizoic habit eliminated the need for armored body, especially in *Rugilepas* living in armored gall. Although the shift from cementation to anchoring has occurred in coral-epizoic barnacles and shark-parasitic barnacles, cement gland is still functional at least in juvenile stages (Brickner and Høeg, 2010; Rees et al., 2014). Reduction of cirri may have been driven over evolution by the extremely cramped habitat in a narrow adpressed gall, in which filter feeding would be severely hampered. The shortened cirri would function to collect POM and to ventilate mantle cavity to the benefit of the brooded embryos and larvae. In this phylogenetic tree, degradation of cirri appears to have occurred twice: in the POM-feeding *Rugilepas* and in *Koleolepas*, a parasite of sea anemones, suggesting evolutionary flexibility of the filter-feeding mode in Pedunculata.

Although galls on echinoids are rare, several types of galls are formed by myzostomids, gastropods, and copepods (Jangoux, 1987), all of which are internal parasites. The gall formed by *Rugilepas* is unique because (1) it is induced by an epizoic cirripede and (2) the gall inducer is not a parasite of the host. The mechanism by which the barnacle cypris larvae on the sea urchin induce gall formation on the host echinoid remains unclear because echinoderms generally have complex, sophisticated immune systems (Smith et al., 2010).

Limitation of the Study

Because of the difficulties of collecting samples, the phylogenetic tree is constructed from a single specimen.

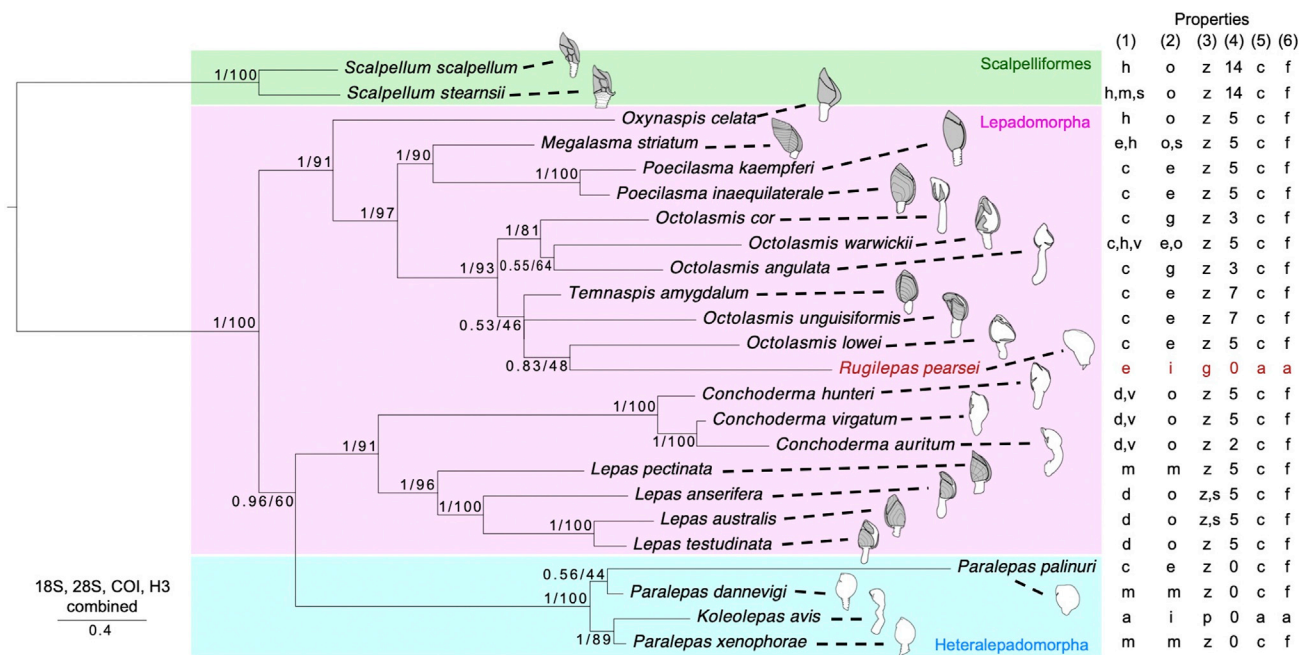


Figure 3. Bayesian Tree of Lepadomorpha and Heteralepadomorpha Species Based on the 18S rRNA, 28S rRNA, CO1, and H3 Gene Sequences
 The numbers at the nodes are (1) Bayesian posterior probability values and (2) maximum likelihood bootstrap support values. Illustration of the ecological habitus (plates are shaded) and the following six morphological/ecological properties of the barnacle species shown adjacent to the species binomials: (1) the dominant attachment host/substratum (a, Anthozoa; c, crustacean; d, driftage matter; e, echinoid; s, abiotic hard substratum; m, Mollusca; h, Hydrozoa; v, vertebrate); (2) attachment site (e, exoskeleton; g, gill; i, inside of host body; m, molluscan shell; o, outside surface; s, spine); (3) habit (g, sub-endozoic in a gall; p, parasitic; s, sessile on abiotic substratum, z, epizoic); (4) number of plates; (5) attachment device (a, anchoring; c, cementing); and (6) morphology of cirri. (a, atrophied; f, feather-like). See also Table S2.

METHODS

All methods can be found in the accompanying [Transparent Methods supplemental file](#).

DATA AND CODE AVAILABILITY

The DNA sequences gained in this study were deposited to GenBank, and the accession numbers are listed in [Table S1](#).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.isci.2020.100885>.

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AUTHOR CONTRIBUTIONS

Conceptualization, L.Y. and M.K.; Investigation, L.Y.; Writing—Original draft, L.Y.; Writing—Review and Editing, L.Y. and M.K.; Funding acquisition, L.Y. and M.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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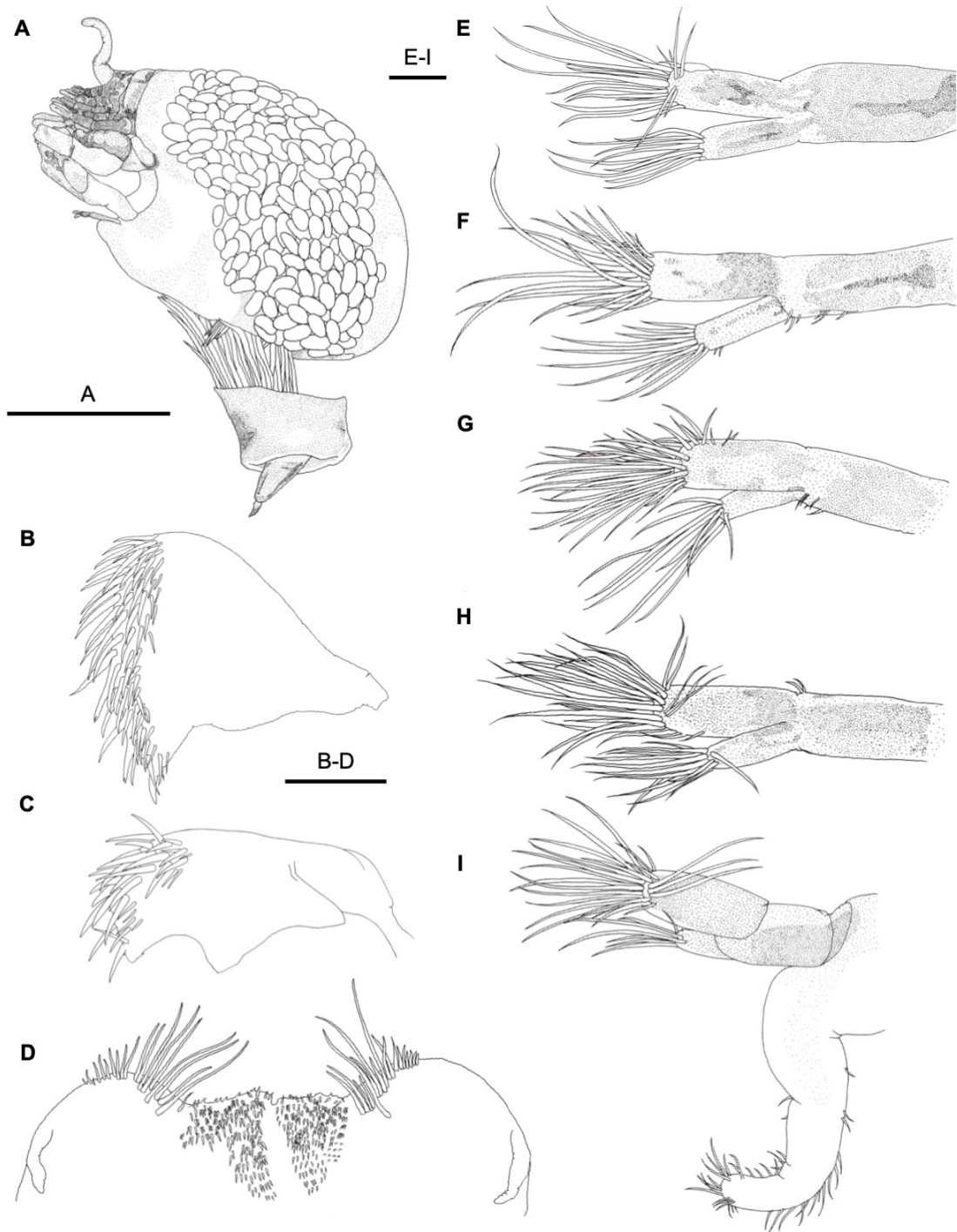
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Supplemental Information

**Shift of Feeding Mode in an Epizoic
Stalked Barnacle Inducing Gall Formation
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Supplemental Figures



FigureS1. Fig 2. A schematic drawing of *Rugilepas pearsei*, related to Figure1. A, Lateral view of the barnacle body with its carapace removed; B, mandible; C, maxillule; D, labrum; E, cirrus I; F, cirrus II; G, cirrus III; H, cirrus IV; I, cirrus V. Scale bars: A, 1 mm; B-I, 0.1 mm.

Table S1. Measurement of each gall and barnacles inside, related to Figure1.

Gall Number	Sea urchin diameter (cm)	Gall diameter (mm)	Number of barnacles in a gall	Capitulum length of barnacle (mm)			
1	9.37	4.5	4	3.41	3.33	3.35	3.44
2	10.9	4.3	2	3.52	3.47		
3	11.1	4.1	3	3.43	3.43	3.31	
4	10.1	4.2	3	3.34	3.54	3.33	

Table S2. A list of lepadomorph species analyzed for genetic sequence of four genes (28S rRNA, 18S rRNA, COI and H3), related to Figure3.

Suborder	Family	Species	Specimen		Genbank #			Locality	References		
			Catalogue #	28S rRNA	18S rRNA	COI	H3				
Scalpelliformes	Scalpellidae	<i>Scalpellum scalpellum</i>	Genbank	EU082307	EU082388	KT209468	EU082347		Høeg et al., 2016		
		<i>Scalpellum stearnsii</i>	Genbank	NA	KF484143	KF484216	KF484288		Chan, 2009		
Lepadomorpha	Lepadidae	<i>Lepas anserifera</i>	Genbank	EU082404	GU993686	EU082363	KY944875		Whitehead et al., 2010		
		<i>Lepas australis</i>	Genbank	EU082324	FJ906777	GU993642	EU082364		Hinojosa et al., 2006		
		<i>Lepas pectinata</i>	Genbank	EU082322	FJ906774	MK308245	EU082362		Hinojosa et al., 2006		
		<i>Lepas testudinata</i>	Genbank	EU082325	EU082406	KC138478	EU082365		Whitehead et al., 2010		
		<i>Conchoderma auritum</i>	Genbank	EU082320	EU082401	KU204353	EU082360		Christensen, 1985		
		<i>Conchoderma hunteri</i>	Genbank	NA	KF484140	KF484213	KF484285		Yamato et al., 1996		
		<i>Conchoderma virgatum</i>	Genbank	EU082402	NA	KC138464	EU082361		Yamato et al., 1996		
		<i>Oxynaspis celata</i>	Genbank	EU082412	NA	KF484231	EU082371		Jones et al., 2000		
	Poecilasmatidae	<i>Megalasma striatum</i>	Genbank	EU082411	NA	KF484231	EU082371		Jones et al., 2000		
		<i>Octolasmis angulata</i>	Genbank	AB551727	NA	KC138498	NA		Jones et al., 2000		
		<i>Octolasmis cor</i>	Genbank	EU082407	NA	MH753552	EU082366		Jones et al., 2000		
		<i>Octolasmis lowei</i>	Genbank	L26518	NA	NA	NA		Jones et al., 2000		
		<i>Octolasmis unguisiformis</i>	YN190121	LC467957	LC467955	LC467960	LC467958	(1)	Kobayashi and Kato, 2003		
		<i>Octolasmis warwickii</i>	Genbank	AB551728	NA	NA	NA		Jones et al., 2000		
		<i>Poecilasma kaempferi</i>	Genbank	EU082410	NA	NA	EU082369		Jones et al., 2000		
		<i>Poecilasma inaequilaterale</i>	Genbank	AY520654	AY520754	NA	AY520722		Williams and Brown, 1972		
		<i>Temnaspis amygdalum</i>	Genbank	AB551730	NA	NA	NA		Jones et al., 2000		
		<i>Rugilepas pearsei</i>	KUZ 2473	LC467956	LC467954	LC467959	NA	(2)	Grygier, 1991		
		Heteralepadomorpha	Koleolepadidae	<i>Koleolepas avis</i>	Genbank	NA	AB551734	NA	NA		Yusa et al., 2001
				<i>Paralepas dannevigii</i>	Genbank	EU082318	EU082399	NA	EU082358		Brock, 1922
Heteralepadidae	<i>Paralepas palinuri</i>		Genbank	NA	AF057561	NA	NA		Newman, 1960		
	<i>Paralepas xenophorae</i>		Genbank	NA	AB551733	NA	NA		Newman, 1960		

(1), Yanyu, Kagoshima, Japan; (2), Manzamo, Okinawa, Japan.

Transparent Methods

Study site

Our study was conducted on a coral reef off Manzamo in Okinawa Prefecture, Japan. No specific permissions were required for sampling at this location, and neither endangered nor protected species were involved in the field study. Most corals in the inner lagoon were dead. Four *Echinometra* species (*Em. mathaei*, *Em. oblonga*, *Em. ryukyuensis*, and *Em. tsumajiro*) burrowed trenches on the dead corals. The black sea urchin *Stomopneustes variolaris* inhabited the undersurfaces of the corals. Most of the corals in the outer lagoon were alive. *Echinothrix diadema* occurred on the undersurfaces of living corals. On the wave-swept reef edge, the slate pencil urchin *Heterocentrotus mammillatus* inhabited narrow crevices in the reefs. In July 2017, we found the unusual gall-inducing stalked barnacle *Rugilepas pearsei* on the test of *Et. diadema*. In May 2018, we conducted an extensive search for this barnacle on the dominant sea urchin species *Em. tsumajiro*, *S. variolaris*, and *Et. diadema*. We collected 100 individuals of *Em. tsumajiro* and *S. variolaris* and 43 individuals of *Et. diadema*. Additionally, we also searched for the stalked barnacle on 56 individuals of *Et. calamaris* found on a coral reef off Bise, which is located northward from Manzamo in Okinawa Prefecture. All sampled sea urchins were examined for symbionts; only those harboring stalked barnacles were collected and preserved in 99% and 70% ethanol for molecular phylogenetic analysis and observations of morphology and gastric contents, respectively.

Morphological observations

One individual of *Et. diadema*, which bore a gall harboring two stalked barnacles, was preserved directly in 70% ethanol and examined by computed topography at the Japan Multi-Industrial Company Cooperation, Kanagawa. After computed topography scanning, the

stalked barnacles were detached from the host sea urchin. To examine the surface morphology of the gall, the sea urchin was soaked in an undiluted sodium hypochlorite solution for ~2 h until all of the spines and tube feet had detached from the test.

Trophic analysis

To explore the trophic interaction between the barnacle and host sea urchin, four barnacles that had been fixed immediately after collection were dissected, and their gastric contents were observed under an optical microscope. To determine their trophic levels in the food chain, we measured the stable isotope ratios of the barnacle and host. The muscle tissues (30 mg dry weight) of two barnacle individuals and the tube feet of two *Et. diadema* individuals were subjected to stable isotope analysis at the Isotope Research Institute, Tokyo to determine the $^{13}\text{C}/^{14}\text{C}$ and $^{14}\text{N}/^{15}\text{N}$ ratios.

Molecular phylogenetic analysis

We performed direct PCR using the Ampdirect® Plus reagent (SHIMADZU) to amplify the DNA from the ethanol-preserved skin (3–5 mg dry weight) of two stalked barnacle species: *Octolasmis unguisiformis* and *R. pearsei*. The tissue was dried, soaked in 50 μl lysis buffer (20 mM Tris-HCl at pH 8.0, 5 mM EDTA, 400 mM NaCl, 3% SDS, and 200 $\mu\text{mol}/\text{ml}$ proteinase) and incubated at 60°C for 12 h. PCR was used to amplify four genes: 18S rRNA (~1850 bp amplicon) and 28S rRNA (1600 bp amplicon) using previously described primers (Rees et al., 2014), histone 3 (H3; 350 bp amplicon) using the universal primers H3F/H3R (Colgan et al., 1998), and mitochondrial CO1 (700 bp amplicon) using the universal primers LCO1490/HCO2198 (Vrijenhoek, 1994). The purified PCR products (≥ 50 ng/ μl) were subjected to direct sequencing (Macrogen, Kyoto, Japan) using the primer sets described above. The sequences obtained were

deposited in the DDBJ/EMBL/GenBank databases under the accession numbers listed in Table S1. The sequences obtained in this analysis and additional sequences provided by GenBank were aligned using the Muscle package (Edgar, 2004) implemented in Seaview software (Galtier et al., 1996; Gouy et al., 2010) with the default settings. We employed Gblocks v. 0.91b software (Castresana, 2000; Talavera and Castresana, 2007) to eliminate any ambiguously aligned regions of the 18S and 28S sequences. The sequence lengths of the 18S gene before and after Gblocks treatment were 1792 and 1753 bp, respectively, and the respective lengths of the 28S gene were 1720 and 1561 bp. The alignments of the CO1 and H3 genes did not contain any insertions or gaps and were therefore unambiguous. Bayesian and maximum likelihood phylogenetic analyses were performed on the combined data set (18S + 28S + CO1 + H3) using the MrBayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003) and RAxML v. 7.4.2 (Stamatakis, 2006) packages implemented in raxmlGUI v. 1.31 software (Silvestro and Michalak, 2012). We selected the model GTRGAMMA for the RAxML analysis and used Kakusan4 software (Tanabe, 2011) to select the appropriate models for the MrBayes analysis. The models selected for MrBayes analysis were GTR_GAMMA for the 18S and 28S genes, HYK85_GAMMA, GTR_GAMMA and F81_GAMMA for each codon of the CO1 gene, GTR_GAMMA for the first codon of the H3 gene, and J69_Homogeneous for the second and third codons of the H3 gene. Two independent runs of the Metropolis-coupled Markov chain Monte Carlo procedure were performed simultaneously for 5,000,000 generations. Voucher specimens of the stalked barnacles examined for taxonomic assignment were deposited in the Kyoto University Museum.

Data and Software Availability

The gene sequences gained in this study are listed in the Table S2.

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