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Independent and adaptive evolution of phenotypic novelties driven by coral symbiosis in barnacle larvae

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Author contributions. ND performed microscopy, collected data, collected and cultured Facetoteta and Ascothoracida specimens, performed molecular work, analyzed the data, inferred phylogenies, designed photo plates, and wrote the first manuscript drafts; PCT collected and reared Bernditia specimens; JO performed microscopy, cultured Baccalaureus cyprids, secured funding for the Facotetota and Ascothoracida material, and revised the manuscript; JTH cultured the Scalpellum, Peltogaster and Trypetesa material, designed videos and revised the manuscript; GAK provided background information on burrowing barnacles and revised the manuscript; BKKC conceived the study, secured funding and supervised the project. All authors agreed upon the final version of the manuscript.

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Availability of data and materials. All relevant data are presented in the manuscript and the electronic supplementary material. The figures and videos can be downloaded from Figshare: https://doi.org/10.6084/m9.figshare.13198676. The specimens on slides and SEM stubs are stored in the Coastal Ecology Laboratory at Academia Sinica and the Natural History Museum of Denmark (ND, BKKC & JO).

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ABSTRACT

The invasion of novel habitats is recognized as a major promotor of adaptive trait evolution in animals. We tested whether similar ecological niches entail independent and adaptive evolution of key phenotypic structures related to larval host invasion in distantly related taxa. We use disparately related clades of coral barnacles as our model system (Acrothoracica: Bernditia and Thoracica: Pyrgomatidae). We analyze the larval antennular phenotypes and functional morphologies facilitating host invasion. Extensive video recordings show that coral host invasion is carried out exclusively by cypris larvae with spear-shaped antennules. These first exercise a series of complex probing behaviors followed by repeated antennular penetration of the soft host tissues, which subsequently facilitates permanent invasion. Phylogenetic mapping of larval form and function related to niche invasion in 99 species of barnacles (Thecostraca) compellingly shows that the spear-phenotype is uniquely associated with corals and penetrative behaviors. These features evolved independently in the two coral
barnacle clades and from ancestors with fundamentally different antennular phenotypes. The larval host invasion system in coral barnacles likely evolved adaptively across millions of years for overcoming challenges associated with invading and entering demanding coral hosts.

Key words: adaptive host invasion, larval phenotypes, coral barnacle, barnacle phylogeny

INTRODUCTION

The invasion of novel habitats is recognized as a major promotor of adaptive trait evolution and is a key process in all animals that perform any type of movement, whether being passive dispersal of marine invertebrate larvae or migration powered by flight in birds (1-5). When and where to move are critical decisions in the lifecycles of animals residing permanently within other organisms with important consequences for survival and access to partners and food (1, 2, 4).

Scleractinian corals are some of the most extreme habitats permanently colonized by other animals as they possess nematocyst cells and various biochemical defense mechanisms that in concert deter symbiont invasion (6-10). On the one hand, this challenge is exacerbated in animals that permanently invade live corals as they face being overgrown and engulfed by the hosts (7, 11, 12). On the other hand, they gain shelter from predators and may enjoy increased longevity compared to freeliving relatives. A remarkable range of symbiotic invertebrate taxa take residency in or on corals, and these play crucial roles in the maintenance and stability of reef systems (6-9, 11-12). Yet, despite the significance of symbiotic relationships on coral reefs, the underlying mechanisms of how such intimate relationships are functionally established remain poorly understood.

Many coral-dwelling invertebrates can spatially translocate between sites and hosts through time and space (7). Thus, they offer few clues as to how permanent coral symbiosis is functionally achieved. Coral barnacles are extraordinary because their adult stages are permanently affixed deep within their hosts (Fig 1; 7, 11). This immobility critically exposes them to host defense systems. Extensive phenotypic modifications affected by shifts in ecological niches are widespread across metazoan taxa, and recent studies have documented that these may arise from pre-existing morphological plasticity (13). Adult coral associated invertebrates expectedly exhibit host-driven and adaptive traits related to feeding, self-protection, mating and reproduction (Fig 1A-I; 7, 11). A series of recent studies have documented remarkable convergent and adaptive evolution of larval form and function in invertebrates (8, 14-17). However, a significant knowledge gap is whether the features that enable localization, exploration, invasion, and establishment are adaptively selected for by niche requirements in invertebrate larvae.

For any permanently attached invertebrate the decision of a lifetime is whether to irreversibly invade a particular surface or host (18, 19). Barnacles are no exception (20). Here, habitat selection and permanent attachment is exclusively facilitated by a single, short-lived, and non-feeding larval stage, the cyprid (21-27). Succeeding a series of naupliar instars that usually serve for dispersal and feeding, the cyprid explores a habitat by complex, surface-interactive behaviors (23, 24, 27). It first
propels itself toward a surface using powerful swimming legs, and subsequently walks on the
substratum in a bipedal fashion using a pair of prehensile and flexible antennules that carries a battery
of sensory structures (27-29). Upon locating an attractive site, the cyprid finally secures permanent
adhesion by secreting a protein substance that polymerizes into an exceedingly strong cement (27),
which marks the transition to the permanently affixed juvenile and adult stages (22). The functional
and structural biology of barnacle cypris antennules have recently attracted much attention as they are
critical in understanding how and why barnacles are found in such an astounding range of niches,
which comprise the surfaces of whales, sea snakes, sharks, polychaetes, bryozoans, hydroids,
crustaceans, mollusks, rocky shores, deep-sea hydrothermal vents, sea-spiders, sponges, and corals
(Fig 1; 8, 21, 22).

Barnacle larval settlement is thus a decisive event and surfaces may be entirely rejected even
in cosmopolitan species (30-33). Due to its comprehensive behavioral toolbox and innate capabilities
to discriminate between habitats and surfaces, the site-selective cypris larva is celebrated as a model
system in larval evolutionary ecology (20, 21).

Here, we test whether similar ecological niches entail adaptive evolution of key larval
phenotypic traits related to permanent host invasion. Stimulated by the discoveries that sponge and
rocky shore barnacles sport adaptive larval antennular traits (8), we investigate the antennular
biomechanics and structure, mode of host invasion and exploration, and larval development of three
coral-dwelling barnacles: the burrowing barnacles Berndtia utinomii and B. purpurea (Acroro thoracica)
and the epibiotic balanomorph barnacle Darwiniella angularis (Thoracica; Pyrgmatidae). These
species are separated by more than 100my of barnacle evolution and are distantly related. Yet they all
live surrounded by the soft tissues and skeleton of corals (34-44). The two groups evolved from
ancestors living on abiotic surfaces such as rocks or shells, and they are thus primed for testing how
niche shifts impact larval strategies and structure. We extend our experimental and structural data to a
wider ecological and phylogenetic context by assessing how mode of host invasion and larval
phenotypes evolved across 99 species of barnacles (Thecostraca).

MATERIALS AND METHODS

DEFINITIONS AND SYSTEMATICS

To avoid confusion when using vernacular names, we emphasize that coral associated barnacles
consist of at least three separate clades (34-44). The first belongs to the burrowing barnacles
(Acrothoracica), most of which are not coral associated; these refer to the Berndtia species studied
here, which we informally call “burrowing coral barnacles”. The second, small clade is associated
with fire- and hydrocorals (genus Wanella). The third and by far largest clade, comprise the species
belonging to the acorn barnacles (Thoracica: Balanomorpha) and the family Pyrgomatidae. As these
are not strictly acorn nor live entirely endosymbiotically, we here refer to these as “epibiotic

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balanomorphan coral barnacles”. Figure 1 shows a variety of barnacles including burrowing (Fig 1A) and epibiotic balanomorphan coral barnacles (Fig 1F).

Upon inferring to antennular phenotype, we refer to the shape of the third antennular segment and the attachment disc found distally on the antennules of the terminal larval stage, the cyprid, which is found in all barnacle species. We showcase the four antennular phenotypes in Figure 2, and categorize these as hook/spear/bell/shoe-shaped antennules by following the detailed account in Yu et al. (8) and Martin, Olesen & Høeg (21). We define symbiosis as ‘any close association between dissimilar organisms living together irrespective of how harmful or beneficial it may be for either’ following Dreyer & Chan (9). Unless otherwise stated, we define barnacles as ‘Thecostraca’ following Chan et al. (39).

CORAL BARNACLE COLLECTIONS

Live corals of Leptastrea purpurea and Psammocora profundacella bearing burrowing and adult females of Berndtia barnacles were collected by SCUBA diving on coral reefs in the vicinity of the Northeast (NE) coast of Taiwan. Adult specimens on approximately 3 cm² host colonies were transferred live to the lab and kept in 1-liter beakers containing aerated, filtered seawater with a 10L:14D cycle under LED lamps. The seawater was changed daily, and the tanks changed regularly. All tanks were checked daily for the release naupliar larvae (which precede the invasive cyprid larva). Once released, the nauplius larvae were concentrated using a pointed light source and then transferred using glass pipettes to sterilized aerated petri dishes. The larval material of the acorn coral barnacle Darwiniella angularis (Thoracica: Pyrgomatidae; inhabiting Cyphastrea chalcidicum) originate from Liu et al. (37).

Larval culture and imaging

Hatched nauplius larvae of Berndtia from the field-collected females were cultured in autoclaved seawater in petri dishes at ~26°C. As the larvae of Berndtia are lecithotrophic (non-feeding; Video 2) and no food was supplied during the culture. Seawater was changed at daily intervals until the nauplii metamorphosed to the cyprid stage (seen by the emergence of two compound eyes laterally to the nauplius eye; Fig 3A). To assess the larval swimming behaviors and gross structure in culture, both nauplii and cyprids were video recorded and photographed alive in either an Olympus SZX7 stereo microscope (SM) or a Zeiss AX10 light microscope (LM) fitted with differential interference contrast (DIC) optics.

All larvae investigated originated from mixed broods of various females. To gain higher morphological resolution of the larval structure, >50 nauplii (all instars) and >100 cyprids were fixed and prepared for LM and scanning electron microscopy (SEM) following the guidelines in (8, 29).
Digital editing of images was done using Corel PHOTO-PAINT X8 and the photo plates were assembled in Corel DRAW X8. The videos were edited in ACDSee Photo Studio.

Larval experiments

To track how the Berndtia cypris larvae explore and invade their hosts experimentally, we released between six and 50 live cyprids from different broods in 10-litre transparent, rectangular aquarium tank containing host colonies. All tanks were aerated by air pumps and sterile glass pipettes. The filtered seawater was changed daily. The cyprids of all three species are non-feeding. The condition of the cyprids were checked every 10-15 minutes during daytime under a stereomicroscope (SM) after exposure to the hosts. The SM was fitted with a digital Lumix G8 camera. Live videos were recorded at 15-minute intervals for 4-30 minutes from about 06:30am-11:00pm but were on several occasions followed for 24 hours. This was repeated for 32 days for all three species. To document larval and juvenile metamorphosis, observations and videos were made every 30 minutes after larval invasion.

COLLECTION, REARING AND IMAGING OF OTHER BARNACLE SPECIES

To contextualize and compare the functional antennular biomechanics and structure of the three coral barnacle species, we provide novel video, LM and SEM data of 13 species that span the spectrum of barnacle (Thecostraca) larval diversity (Video 1). Collection, rearing and imaging details of these species are described in the Supplementary Text.

ANCESTRAL STATE RECONSTRUCTION AND LARVAL PHENOTYPIC EVOLUTION

The purpose of this study is specifically not to recover the systematic relationships between barnacle lineages but rather to generate a tree used for ancestral state reconstruction (ASR) of larval phenotypes and mode of substratum invasion. Previous comprehensive molecular phylogenetic analyses (39-44) do not include both species of Berndtia and epibiotic balanomorphan coral barnacles. To reliably infer ancestral states of larval phenotypic evolution, it was therefore necessary to generate a new phylogenetic tree. Our tree covers all three Thecostraca (barnacles in the widest sense) subclasses (Facetotecta, Ascothoracica and Cirripedia, the latter containing the stalked and acorn barnacles (Supplementary Tables S1 and S2). The Facetotecta, or y-larvae, is an enigmatic taxon for which only larvae are known, and their adults are thus entirely unknown. To adequately root our trees, it was necessary to expand the marker coverage of the Facetotecta. The collection, imaging, extraction, and amplification details of these experiments are documented in the Supplementary Text and Table S2.
We included 99 species of barnacles (Thecostraca; Fig 6; Table S1) where cypris larval traits and habitats are known. For each species, we downloaded the longest possible nucleotide sequences of selected markers comprising 12S, 16S, 18S and 28S ribosomal DNA, Histone-3, Cytochrome oxidase subunit I (COI) and RNA Polymerase Largest Subunit II (RPII) (2563bp in total). The sequences were aligned with MAFFT v 7.450 (45, 46) in Geneious Prime v. 2020.2.2. We assessed uncertainty in our alignments by using the less stringent options in Gblocks v. 0.91b (47). No ambiguous sites were found in any of the protein coding genes. We concatenated the individual Gblocks alignments in Geneious Prime using the concatenation tool. We used the concatenated alignment to infer maximum likelihood (ML) trees using IQ-TREE multicore version 2.0.3 (48). We first assessed the best-fit evolutionary model for each partition in the alignment using ModelFinder (-m TESTNEWONLY). The best-fit models and alignment details are shown in Supplementary Table 3.

ML trees (Figs 1, 6, S1-S3) were then generated using the best partition scheme and substitution model from the ModelFinder test and by allowing individual partitions to have independent evolutionary rates (edge-proportional partition models; -spp) with a more thorough nearest neighbor interchange search (NNI; -allnni) and the ModelFinder tree as a guide tree (-g). This tree reflected, with a few exceptions, e.g., Pollicipomorpha being nested within Balanomorpha, the recently revised and accepted phylogenetic relationships between higher and lower barnacle lineages (39, 44). We assessed clade support for the maximum likelihood trees using 10000 ultrafast bootstrap replicates (-B 10000; Fig S1).

Using our ML tree as input tree, we used MBASR (Mr. Bayes Ancestral State Reconstruction, 49) with 10000 samples (=1000000 number of generations) in R under a continuous-time Markov Model (50) to infer ancestral states and evolutionary patterns of larval phenotypes and mode of host invasion (Fig 6; Fig S2; Table 1, S1, S4). Our R-code is placed in the Supplementary Text. ML trees were visualized in R using MBASR Heritage (49) and further edited in Corel DRAW X8.

RESULTS

We describe the functional antennular morphology and the entire host invasion process in Berndtia purpurea, B. utinomii and Darwiniella angularis. Unless otherwise stated, general descriptions concern all three species. Whenever we state ‘Berndtia’ we refer to both B. purpurea and B. utinomii.

FUNCTIONAL LARVAL MORPHOLOGY & THE UNDERLYING MECHANISMS OF HOST INVASION IN CORAL BARNACLES

Larval biology & development prior to host invasion

The cyprids of both species of Berndtia and Darwiniella possess bean-shaped cement glands, suggesting permanent invasion with cement (Fig 2B, 3B-D; video 2), and the four-segmented antennules are invariably spear-shaped with numerous sensory structures scattered around segment three and four (Fig 1J, L, 2B, 3B-D, 5, 6, S3). The spear shaped third segment houses the attachment
disc from which exit pores lead to the multicellular cement gland (Fig 1J, S3). This spear-phenotype is morphologically highly distinct from other antennular phenotypes seen in barnacle larvae (Fig 1J, S3). We found minute differences in the attachment disc of these species, with *Darwiniella* having white-pigmented cells of unknown function and with much fewer but wider cuticular villi on the attachment disc (Fig 5).

We observed six naupliar instars (Video 1) in all three coral barnacle species. In *Berndtia* the nauplii lack a labrum, a functional alimentary tract, gut and masticatory spines on the antennal and mandibular appendages and are thus non-feeding (lecithotrophic). In *Darwiniella*, these structures were present, and the nauplii are feeding (planktotrophic). The dispersive larval development (nauplii) lasts seven days under ambient sea-water temperatures (26-28°C) with a span of one day per stage. In *Darwiniella*, development into cyprids lasted 8-10 days at 26°C. We found no differences in either nauplii or cyprids related to the dioecious sexual system in *Berndtia*. The cyprids are invariably transparent and possess lipid droplets serving as food reserve (Fig 2B; videos 2-6, 10, 11). The thorax is equipped with six pairs of bifurcated, flexible swimming legs (Fig 2B, 3B-D, S3; videos 2-6, 10, 11). The thoracopods are used mainly for swimming towards the hosts upon exposure to the experimental habitat arena but are also seen beating during host exploration (Videos 4, 10).

**Larval swimming & host exploration**

The cyprid larvae immediately started exploring the host upon exposure (Video 11). A coral host was never rejected in favor of the aquarium surface. Once “attached” to the host, the cyprid would only cease to explore upon permanent attachment. Hosts were explored by the *Berndtia* cyprids for two days before permanent invasion of the host tissue commenced (Fig 2A-E, 3B-D; videos 2-8), whereas this process lasted less than 12h in *Darwiniella*. After permanent settlement, *Berndtia* cyprids remain superficially visible for up to eight days where after it is engulfed by live host tissue (Fig 2F-O; video 7). The total non-feeding time was 18-19 days, which spans from hatching, over larval development, host exposure and settlement to juvenile emergence from the host tissue (Fig 2F-O). Plankton-feeding of juveniles commenced after 23 days after settlement (Fig 2O). In *Darwiniella*, cyprids remain externally visible only for up to 3-4 days with feeding juveniles emerging after 10 days after settlement.

Forward movement was facilitated by the first two antennal segments (Fig 3C; videos 3-8, 10, 11). The third segment serves the purpose of host attachment and sensory exploration and is the structure physically adhering to the substratum, while the fourth segment also probes the host for sensory purposes; Fig 3C; videos 3-7, 9). The antennal walking was slow (Videos 3-8, 10, 11), and the step frequency is slower than observed in rocky-shore barnacles (Videos 1, 10, 11, 14).

The host exploratory and invasive behaviors are divided into three distinct categories (i.e., i.e., wide searching, close searching, inspection), which are not entirely sequential except that once permanently attachment commenced host exploration ceased. The antennules move independently of each other and flex up to ~100° in the dorsal-ventral plane (Videos 1, 2, 10). The third segment is capable of independent movement from the proximal two segments and can bend ~45° up- and downwards (Videos 1, 2-6, 10, 11). During surface exploration, the hosts expelled a calcareous
structure (putative digestive filaments) and mesenteries several times. The cyprids survived being almost completely covered in this structure (Videos 8, 11; Fig 2F, G, H).

Wide searching behavior

Wide searching (WS) is facilitated by counteractive bi-pedal movements (Fig 3C, D; Videos 3-8; Table 2). The antennules always move in a forward-down/backward-up motion in the sagittal plane; Videos 1, 2-5, 10). One antennule extends either straight antero-ventrally or almost entirely anteriorly, here in a range of ~90° difference between antennular positions (Fig 3C-D; Videos 2, 3, 10). It then attaches with the third segment to the coral epithelial surface or a nearby tentacle either with the tip only or with the entire ventral surface of the segment (Fig 3C-D; Videos 3, 4, 10, 11). Subsequently, the unattached antennule is lifted slightly and moved forward either next to or just behind the formerly attached antennule (Video 3, 10, 11). Before the step is taken, the tip of the third and fourth antennular segment may probe the host epithelium several times (Videos 2-4). The coral is never penetrated here, and the cycle may be repeated for more 100 total steps in Berndtia purpurea.

In Berndtia, WS could last more than 24 hours and is the most exercised one (Fig 3C, D; Videos 3, 10, 11). WS was interrupted several times by close searching and inspection behaviors (video 8) but could be resumed. WS take place on both the epithelium surrounding individual polyps, on their tentacles, on, in and around the polyp mouth open and in the orifice of adult conspecifics (Fig 2C-P, 3C, 4A-E; videos 3-6, 8). The behaviors exhibited during the WS phase range from straight walking, directional change, vertical walking from polyp colony epithelium to the coral host mouth (and vice versa), elevating the carapace ~90°, slight body rotation on the spot and “grabbing” polyp tentacles with the tip of an extended antennule (Fig 3C-D; Videos 3-5, 8).

The cyprid body may elevate ~90° relative to the substratum during exploratory walking (Fig 3C; Videos 10, 11) and can rotate around ~45° on the spot while being attached with one antennule (Videos 3, 4). The Berndtia cyprids lying on the lateral sides in culture (Videos 1, 2, 12) showed that the second segment may bend more ~90° relative to body mid-axis. The time spent walking during the WS phase ranges from 30 seconds to several hours in the case of host tentacle exploration. In Berndtia purpurea, a new antennulary step was taken every 1-75 seconds ranging from 1/10 to 1/2 of the total cyprid length (~52um-259um).

The behaviors exhibited during WS between the polyp epithelium and the tentacles varies slightly, mostly in the sense that the tentacle-exploring cyprids would usually move from tentacle to tentacle by remaining affixed with one antennule and grabbing an adjacent tentacle with the tip or ventral surface of the other and drag itself towards the latter while releasing the grip with the former. The cyprids would thus not just walk in a straight line as observed in the cyprids of free-living species (Videos 1, 13, 14). We never observed backward movements. Darwiniella exhibited an extremely slow walking behavior that could only be detected using fast-forwarding on video recordings. Apparently, wide searching behavior is not obvious in this species.

Close searching

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Both *Berndtia* and *Darwinella* have close searching (CS) behavior. CS is characterized by a clearly decreased step frequency (Videos 4, 5, 8, 10, 11), although the cyprid is mostly still during this behavior while probing the host surface with the non-affixed antennule (Table 2; Videos 5, 10, 11). The antennular movements are divided into two distinct patterns: either non-moving cyprids remain affixed to the host with the tip of the antennules and are wriggling back and forth just above the host or elevated in the 90° stance, or one antennule would attach while the free antennule repeatedly probes the polyp or tentacle epithelium using the antennular tip (i.e., the third segment; Fig 3D; Videos 4, 5, 10, 11).

CS may take place on both the colony epithelium surrounding individual polyps, on and around the mouth opening and the tentacles of the latter, and at the orifice of adult conspecifics. The cyprid may here remain affixed for up to several hours and then resume either CS or wide searching to other areas of the host. Close searching thus includes both walking and temporary “attachment”.

**Inspection**

Inspection (Videos 5, 10, 11) commences when the cyprid actively stops bipedal walking while pushing down the antennule on the host surface, initially penetrating this (Table 2). We only observed full penetration at this stage in *Berndtia purpurea*. We consider the penetration ‘initial’ because it never leads to permanent attachment. This behavior is also consistently observed in non-coral barnacle cyprids (Videos 1, 13, 14; Table 1) yet without exhibiting any down pushing or penetrating behaviors. During inspection, one antennule remains affixed to the epithelium medioventrally to the carapace while the other antennule actively pushes down on the host epithelium or penetrates the host (Fig 3D; Videos 10, 11). The cyprid remains penetrated for up to several minutes but may resume any searching behavior shortly after. Inspection is therefore distinguished clearly from permanent, irreversible settlement by not leading to juvenile metamorphosis. We also interpret the clear pushing down/penetrations and the ceasing of bipedal walking as the main difference between inspection and close searching (Table 2). That the cyprid can distinguish behaviors to this extent suggests that the suite of invasive behaviors is decisively executed.

Inspection leading to settlement/invasion is slightly different from the above movements but only in the penetration cycle being repeated several times and by both antennules bipedally (Fig 3D; Video 6). The antennules are often penetrating fully but a ~45° bending of the third antennular segment in combination with up- and downwards movements in an “excavating” manner was also observed in *Berndtia purpurea* (Video 6). In *Darwinella*, we observed putative biochemical degradation of the host epithelium around the permanently penetrated antennule (Fig 5).

**Permanent invasion & metamorphosis**

The cyprids never settled permanently on the host tentacles but always in the tissue lining the coral skeleton (Fig 1A, 2A, C-P; Videos 3-8). Permanent settlement is ultimately reached when the cyprid ceases to walk, explore, and penetrate (Fig 3D; Videos 6, 11). After several minutes of excavating behaviors, which are similar to the antennular movements observed in inspection leading to
permanent settlement, the cyprid cease moving and positions itself in a ~45° angle relatively to the coral epithelium. Permanent settlement is completed when the cyprid aligns ventrally with the host surface. We could not observe cement secretion, but the presence of a distinct cement gland in all three species suggests that a secretory product is eventually released during settlement.

We did not recover the metamorphosis to the juvenile in *Berndtia utinomii*. The larval-juvenile metamorphosis lasts 18 days (Fig 2N; Video 7) in *Berndtia purpurea*. These cyprids are surrounded by the growing host tissue after one week (Fig 2C-M) and the juvenile emerges from the host epithelium 18 days after permanent settlement. We only observed tentative feeding in one *B. purpurea* juvenile, which started beating the cirri after 23 days. The juvenile body and its cirri are rotated ~180° relative to the substratum since permanent attachment (Fig 2O, P; Video 7). In *B. purpurea*, which is a species with separate sexes (dioecy), we observed putative dwarf male settlement as a cyprid first explored the exterior and subsequently the burrow of an adult female. In *D. angularis*, settlement and initiation of juvenile metamorphosis was faster than in *B. purpurea* and commences within the first 12h of arriving on the host. Ecdysis of the cyprid cuticle is completed after two days with a feeding juvenile appearing after 12 days (Video 11).

**COMPARATIVE FUNCTIONAL MORPHOLOGY OF BARNACLE CYPRIS LARVAE**

Bipedal walking, 100° flexing of the antennules in the dorsal/ventral plane, individual probing, and dorsal/ventral movement of the third segment and individual flexing of the fourth segment, were nearly identical in all cirripede cyprids (Videos 1, 3-14; Table 2). This suggests constraints in the biomechanics and functional morphology of the cyprid antennules during exploration.

Variation almost entirely concerns the structure and usage of the distal most antennular segment, which functionally is the third segment (Fig 1, S2, S3). The first, second and forth antennular segments are structurally similar in shape and sensory armament (Fig 1, S2, S3). These similarities include first segments consisting of two independently moving sclerites, a trapezoid second segment and a rod-shaped fourth segment with up to four subterminal setae and up to five terminal setae; Figs 1, 2, 5, 6, S3; videos 1, 3, 10-14).

While the burrowing, parasitic and scalpellid barnacles all sport shoe-shaped antennules (Videos 1, 13; Figs 1, 6, S2; Tables 1, 2, S2, S4), the rocky intertidal and fire-coral living forms invariably possess bell-shaped antennules (Videos 1, 14; Fig 1, 6, S2; Tables 1, 2, S1, S2). These species explored and walked on the culture vessels in a more complex (e.g., faster, and backwards walking and a 180° turn on the spot) manner than other cyprid morphotypes did (Videos 1, 14). Most scleractinian coral barnacles exhibit spear-shaped antennules that are rarely used for walking bipedally on culture dish surfaces (Video 1-12; Fig 1, 5, 6). The *Bacculaureus ‘a-cyprid’ Ascothoracida*) and the y-cyprid (Facetotecta) has a six-segmented antennule, very different from that seen in cirripede cyprids and distally equipped with a cuticular hook (Video 1; Fig 6, S3). Although the host infection has never been observed for these parasites, live recordings revealed that the antennules are extremely flexible and may extend close to 180° in the dorsal-ventral plane (Video 1). The hook is putatively involved with epithelial piercing/penetration and subsequent injection of a primordial parasite. We did not observe this phenotype in any other species (Fig 6, S2, S3).
ANTENNULAR PHENOTYPIC EVOLUTION IN BARNACLE LARVAE

The barnacle (Thecostraca) cypris larvae invariably exhibit multi-segmented antennules used for habitat invasions (Video 1; Fig 1, 6; Table 1, 2, S1, S4). The Facetotecta (y-larvae) and Ascothoracida possess hooked shaped antennules (Video 1; Fig 6, S2; Table 1, 2, S1). In the ‘true’ barnacles (Cirripedia), the range of antennules morphologies comprises shoe-, spear-, hook- or bell-shapes (Videos 1, 4-14; Fig 1, 6, S2; Table 1, 2, S1, S4).

Video (LM, stereo microscope), structural (LM, SEM) and multilocus phylogenetic data confirm our hypothesis that the larval phenotypes (Fig 1, 6, S2, S3) and invasion systems are driven by host symbiosis in coral barnacles. Antennular penetration using spear-shaped antennules evolved exclusively, independently, and adaptively between burrowing (Berndtia) and epibiotic balanomorphan coral barnacles (Pyrgomatidae), which are very distant relatives (Videos 1-12; Figs 1-6, S2; Table 1, S1). The ancestral traits leading to this spear-phenotype are fundamentally different. The shoe-shaped antennule that gave rise to the spear-shaped antennule in Berndtia cyprids are encircled by a tiny, cuticular ‘skirt’ and is covered by a dilute carpet of microscopic villi (Video 1, 13; Figs 2, 6, S2, S3). The spear phenotype found in pyrgomatids evolved, in turn, from ancestors with bell-shaped antennules (Figs 1, 5, 6, S2, S3; Videos 1, 11, 12), which is basally (posteriorly) surrounded by a series of velar flaps (Figs 6, S3). Apart from the convergence observed between antennular phenotypes in Berndtia and pyrgomatid cyprids, a reversal from both spear-shaped antennules to shoe-shaped antennules occurred in the pyrgomatid barnacle Conopea minyrostrum, which settles superficially, and never penetrates, the hosts (Figs 6, S2). Convergent evolution of functional antennular penetration also arose independently between the ‘akentrogonid’ parasitic barnacles (Rhizocephala) and the two distant coral barnacle clades (Figs 6, S2). Antennular penetration thus arose independently three times in cirripede barnacles, but only twice using spear-shaped antennules since those in ‘akentrogonids’ retain the shoe-shape found in other rhizocephalans (Figs 6, S2).

All other barnacle cyprids sport either hook-, shoe- or bell-shaped antennular invasion devices (Video 1; Figs 1, 6, S2, S3; Table 1, 2, S1, S4). While it is equally likely that the ancestor to all barnacles (Thecostraca) had hooked or shoe-shaped antennules (Fig S2), we recover the latter as the ancestral phenotypic state of the cirripede barnacles as well as the burrowing (subclass Acrothoracica), parasitic (subclass Rhizocephala) and the stalked and acorn barnacles (subclass Thoracica) (Figs 1, 6, S2). We found that shoe-shaped antennules are the phylogenetically and ecologically most versatile in the cirripede cypris larvae (Fig 1, 6, S2), being used for exploration and settlement on a diverse array of substrata including shells, crustaceans, elasmobranch egg cases, hydroids, sea urchin spines, gorgonian corals (externally) and floating man-made objects (Fig 1; Table 1, 2, S1). We confirm the analyses by Yu et al. (8) who used a smaller phylogenetic dataset to infer unravel antennular evolution in sponge barnacles. These evolved shoe-shaped antennules from bell-shaped ancestors at least three times independently, suggesting an adaptive mechanism to this substratum (see 8 and discussion). The ancestor to the acorn barnacles (Balanomorpha) possessed bell-shaped antennules (Fig 6, S2). Most acorn barnacles exhibit this phenotype, including the barnacles that inhabit fire- and hydrocorals, marine mammals, and reptiles (Video 1; Fig 1, 6, S2).

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DISCUSSION

Symbiosis has evolved independently numerous times in marine invertebrates. Here, host invasion is most often accomplished by a larval stage that differs from the adult in both structure and function. Considerable knowledge gaps exist with respect to how larval phenotypes and modes of host invasion evolve in concert with the type and range of the niche. Both burrowing (Acrothoracica) and epibiotic balanomorphan (Thoracica; Pyrgomatidae) coral barnacles are highly specialized suspension feeders embedded within the tissue of coral hosts. We hypothesized that similarities in larval functional morphology and invasion biology evolved independently and adaptively because of the shared and demanding ecological niche. We have shown that in both groups, larvae enter the host soft tissues by first exploring them in a complex array of behaviors and then by active, decisive penetration using uniquely spear-shaped antennules, which differ from the antennular outlines seen in other barnacles. These larval phenotypes and mode of invasion are, across >2000 species of barnacles, exclusively seen in these two monophyletic and phylogenetically disparate clades. Our results demonstrate that the antennular spear phenotype evolved independently from non-symbiotic barnacle ancestors that never perform antennular penetration. Although conclusions on adaptively evolution should always be treated with caution, we find that these remarkable similarities are best explained by adaptive, structural evolution of larval form and function, which are in concert driven by niche requirements, and operating on an inherent plasticity in the antennular morphology and function.

EVOLUTION OF LARVAL FORM AND FUNCTION

Irrespective of niche, functional data suggests considerable biomechanical constraints on cypris larvae as all cirripede barnacles utilize essentially the same behavioral toolbox for substratum exploration (this study, 22-26; Videos 1, 3-14). Examples of this are the almost identical walking biomechanics and general outline of the larvae (Video 1).

The only significant variation in the larvae concerns the structures that physically probe the substratum during the invasion/settlement process. Some barnacles sport modifications of antennular structure to habitat and invasion mode, which we here explain as adaptations (this study; 8, 24, 51-54). Importantly, coral barnacles do not unanimously exhibit spear-shaped antennules used for host penetration. The soft-coral species Conopea minyrostrum, which belongs to the epibiotic balanomorphan barnacles (Pyrgomatidae; Fig 6), possess shoe-shaped antennules that never penetrate. This constitutes a putatively adaptive reversal from a spear-shaped ancestral state within the clade (Fig 6; 8). Similarly, the fire-coral inhabiting barnacle Megabalanus ajax retain bell-shaped antennules (Video 1; Fig 6, S1). Although the settlement has not yet been observed on hosts but only in petri dishes (this study), decisive penetration is unlikely as barnacles with bell-shaped antennules never penetrate but also because it resides epibiotically on the hydrocoral hosts in contrast to other coral-dwelling barnacles that are embedded in the host tissues. As all burrowing barnacles also initially settle epibiotically (51), these examples strongly suggest that entering live corals
independently selected for the evolution of antennular penetration using a unique, novel phenotype (the spear shaped antennule).

Whale barnacles also reside in the integuments of their hosts. Yet, all such species have large, bell-shaped antennules that resemble those seen in closely related species settling on rocky shores, and they never perform tissue penetration (51, 55-58). The bell-shaped antennule is unanimously selected for in acorn barnacle species that inhabit high-energy, intertidal shores and the integument of marine vertebrates (this study; 51, 56-58), yet it remains unknown how or whether this phenotype contributes to improved settlement under increased water velocities. It has been suggested that a larger attachment disc may increase the surface area of the secreted cement thus putatively increasing adhesion, although this also needs experimental validation.

What makes the barnacle cyprid antennule so unique? In many parasitic crustacean taxa (21), cuticular hooks used for host attachment are commonly encountered, and evolved numerous times convergently presumably to facilitate effective host invasion. Crustacean carp lice (Branchiura) attach to their fish hosts by means of simple hooks. Interestingly, some derived forms evolved muscular first maxillary “suction discs” (21). Similarly, mechanical attachment by antennular hooks as seen in the parasitic Ascothoracida and Facetotecta represents a plesiomorphic pattern, which was lost entirely in the ancestor to all cirripede barnacles (Fig 6). Here, a four-segmented antennule with a complex musculature combined with a unique glandular and sensory apparatus clearly allowed barnacle cyprids to settle on virtually any surface found in the marine environment using water-soluble cement (21, 22).

Settlement with cement, not hooks, even persisted in the parasitic barnacles that demonstrate the most extreme evolution of host invasive mechanisms. After wide searching and inspection phases, the so-called ‘akentrogonid’ parasitic barnacles, an advanced clade within the Rhizocephala, cement themselves with one antennule on the crustacean host while the other penetrates its cuticle (24, 51). Thus, penetration mediated by the cypris antennule evolved at least three times in cirripede barnacles, in all cases with the purpose of ensuring endosymbiotic development of ensuing juvenile stages.

Surprisingly, one remarkable sponge barnacle was recently shown to exhibit a large, cuticular hook protruding from the attachment disc on the cypris antennule (8). This is the only known example of such a morphology in any cirripede cyprid. Whether this hook evolved as a true phenotypic novelty for promoting host invasion (8) is unknown nor is it known whether it represents a reversal to the state found in Facetotecta and Ascothoracida, which attach by purely mechanical means.

It is at present unknown whether the phenotype (spear) or the function (penetration) emerged first in coral barnacle cypris larvae. In Berndtia, it is likely that the spear ensued penetration as the shoe phenotype (its ancestral state) can penetrate (24). This is also supported by all burrowing barnacle cyprids having shoe shaped antennules that are exclusively used for epibiotic attachment (51). Bell-shaped antennules are never used for penetration (8, 24), suggesting that the spear may have evolved first in epibiotic, balanomorph coral barnacles. The fossil record of these species is poorly studied when compared to free living species, but a possible scenario is that they ancestrally were entirely epibiotic and equipped with bell-shaped antennules and cement as seen in fire coral barnacles (Video 1; Table 1; Fig 6). With the potential fitness advantage of gaining more protection by ‘digging’ deeper into the hosts, it is not unlikely that this selected for a structural modification of bell- to elongated
spear-shaped antennules. This again entails independent antennular evolution in the two widely separated clades of coral barnacles.

We observed putative enzymatic digestion of the host epithelium in *Darwiniella angularis* (Fig 5). Although we cannot rule out mechanical excavation, Turquier (59) documented substrate perforation by carbonic anhydrase secretion in females of burrowing barnacles. This may dissolve the calcareous substrate they inhabit. Similar digestion of host integument has been suggested in the larvae of parasitic barnacles and adult whale barnacles (24, 51), whence this mechanism may be a widespread means of entering host tissues across symbiotic barnacle taxa.

**PHENOTYPIC NOVELTY FROM PRE-EXISTING MORPHOLOGICAL PLASTICITY**

The spear-shaped antennule in coral barnacle larvae could possibly have evolved independently from ancestors with either shoe- or bell-shaped antennules due to pre-existing phenotypic plasticity in the barnacle ancestor as shown for tadpoles by Levis et al. (13). Using morphological investigations, gene expression profiles and growth patterns, the authors showed that plastic phenotypic evolution sparked evolutionary novelty in the form of a carnivorous tadpole morph that, driven by diet, adaptively changed certain trophic traits to use an unexploited niche. These results are remarkable because they show that phenotypic plasticity may precede and facilitate morphological novelties.

We did not use gene expression or follow the ontogeny of the antennules, but the phenotypic shift between multi-segmented, hooked antennules in Facetotecta and Ascothoracidia (Fig 6) and four-segmented, shoe-shaped antennules in the cirripedes (Fig 6), is both structurally and functionally significant. With this followed segmental fusions, the evolution of a glandular cement and muscular system and re-modelling of existing, or addition of several novel and ultrastructurally different, mechanical, and chemosensory setae (24-29). That similar niche demands, i.e., entering live corals, independently selected for the evolution of the same spear phenotype from fundamentally different structures (shoe- and bell-shaped antennules) across millions of years also highlights standing phenotypic plasticity as a likely explanation for the morphological novelties observed in cypris larvae.

Structural plasticity likely persisted throughout the cirripede barnacle radiation, and the most dramatic changes are seen in acorn barnacles. These inhabit the widest range of substrata with the broadest array of antennular morphotypes (hook, shoe, bell, and spear shapes; 8). The evolutionary phenotypic plasticity is especially pronounced in forms where the adults live embedded within host tissues. This dovetails well with the notion that sponges, corals and whales are some of most challenging niches inhabited by barnacles (8, 51). The structural origins of the spear shapes may be explained by allometric changes to the same structural elements, at least in *Bernditia* that originate from ancestors with shoe-shaped antennules in which case the spear is essentially just an elongated and laterally truncated shoe. Thus, in cirripede cyprids dramatic changes in performance can apparently be achieved by relatively simple structural changes to an inherently plastic antennular morphology. It is obvious that more molecular and biochemical studies are needed to further understand the adaptive significance of phenotypic change to challenging niches like sponges and corals, not least of the critical cement that ultimately ensures larval survival.
Reports on host invasion by coral-dwelling invertebrates are scarce, likely because culturing and live experimentation is challenging. The coral-parasitic copepod *Xarifia obesa* enters the host exclusively in the adult phase and through the mouth and gastrovascular cavity of the host. They never perform any type of host tissue penetration as seen in coral barnacles (60). Scott (61) showed that the larvae of *Lithophaga* bivalves enter the hosts through their mouths, and never perform any penetrating behaviors with their muscular foot (i.e., their host exploratory device). Larval exploration never occurs on the host tentacles, and they explore dead corals longer than live ones, in the latter case only for around 30 seconds (61). In our experiments the larvae never cease to explore the hosts until permanent settlement commenced, and we found that this process lasts up to two days. Furthermore, the cyprids willingly entered and escaped the mouth of a host and explored and walked around the tentacles (Fig 4).

One obvious avenue for further exploration is detailed analysis and quantification of the behavioral repertoire of barnacle larvae. Aldred et al. (24) developed a system to accurately monitor cyprid behaviors in petri dish-like arenas. Due to experimental limitations in colony and partner numbers and sizes, and the number of cyprids released in the tanks, we could not sufficiently measure the step frequencies quantitatively between the different behaviors exhibited by the cyprids. Nevertheless, it seems that rocky intertidal barnacle larvae possess a more complex behavioral toolbox than other barnacle cyprids (Video 1, 22), likely because high-energy, intertidal environments may impose physical challenges not experienced on other substrata. This may also explain why whale and dolphin barnacles are structurally and behaviorally so similar to rocky shore barnacles (51, 56-58). Similarly, the reduced wide search walking speed in coral, hydroid and sponge barnacles (Videos 1, 10, 11; 8) may, in turn, arise from them analyzing live surfaces instead of bare rocks or petri dishes. Interestingly, *Darwiniella* did not perform fast wide searching behaviors in our trials. This may correlate with the cyprids having reduced villus density on the spear shaped attachment disc, but a functional link would require a deeper understanding of how the villi aid adhesion to the substratum (53).

Matsumura and Qian (62) found that intertidal barnacles locate adult conspecifics in the absence of chemical cues, apparently by using visual cues with compound eyes to actively discriminate color patterns in the shells of conspecific adults. Whether color vision also plays a role in the settlement of coral barnacles remains unknown, but the spectacular color patterns seen in adult *Berndtia* and *Darwiniella* barnacles in our study are suggestive. As our experimental aquarium housed fewer adults than are available in the field, the combined effect of actively ‘seeing’ fewer adult females and receiving fewer chemical stimuli by these may also explain the prolonged exploration time we observed in *Berndtia purpurea*.

Barnacles demonstrate widespread adaptive traits in their adult stages. To this we can now add that the shape and configuration of the larval antennular phenotypes are extremely similar in form and function between disparately related coral barnacles that exhibit early endosymbiotic development (i.e., surrounded entirely by host tissue during larval-adult transition; the adults are not considered endosymbionts). No other barnacle cyprid exhibit the spear phenotype, nor do any such larva commit to burying the entire cypris body deep within live host tissue as seen in these species.
Yet, as also argued by Gould and Lewontin (63), it is necessary to assess to what extent antennular phenotypes evolved adaptively.

The two phylogenetically independent origins of spear-shaped antennules with penetrative settlement are, although depending on test and approach, obviously not adequate to yield statistical significance. However, using the criteria for adaptive/functional networks among traits and taxa outlined by Maddison and FitzJohn (64), the phylogenetic, morphological, and functional patterns documented by us lends compelling support for an association between endosymbiotic juvenile development and spear-shaped antennules used for active, decisive penetration. We thus argue that antennular form and function evolved adaptively in the two clades coral barnacles as remarkably similar functional and structural morphologies arose independently from morphologically different ancestral states over millions of years. The fact that barnacles e.g., *Megabalanus ajax* and *Wanella milleporae* (both Thoracica: Balanomorpha; 8) invading other cnidarians, such as fire corals, develop externally on the hosts and never penetrate with spear-shaped antennules further supports the correlation between invasion mode and phenotype in the *Berndtia* and epibiotic balanomorphan coral barnacles studied here. We therefore argue that the most likely explanation for this link is an adaptive, *de novo* shift from abiotic settlement to entering live coral hosts.

References


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Data accessibility. Data available in the manuscript. Electronic supplementary material can be downloaded from the Figshare data repository (https://figshare.com/articles/online_resource/Videos/13198676)

Figure legends.

Fig 1. Host and larval attachment device diversity across the barnacle phylogeny. A-I, Diversity of adult barnacles and their hosts/substrata. J-N Phylogenetic mapping and examples of structure in the cypris larval device (antennules) used for host/substratum invasion. A Berndtia purpurea on its coral host, B Peltogasterella sulcata on its hermit crab host, C Scalpellum scalpellum on sea-urchin spines, white box shows dwarf male larvae attached to a hermaphrodite partner, D Lepas sp. on a ray egg, E Dosima fascicularis attached to a bottle, F The coral barnacle Darwiniella angularis embedded in its coral host, G Balanus improvisus aggregation on a rock, H A balanid barnacle on a hermit crab shell, I Chelonibia testudinaria on the host crab carapace, indicated by the white arrow. J Synthesized phylogeny of barnacles with mapping of primary habitat/lifestyle and shape of the invasion device. Colored circles indicate the shape of cypris antennular attachment/invasion devices. K The barnacle cypris larva with extended antennules used for invading/settling on hosts or substrata. Box highlights the third antennular segment. L the spear-shaped antennule designed for penetration in coral barnacles (yellow circles on tree). M the shoe-shaped third antennular segment seen in cyprids of various symbiotic and abiotic barnacles (orange circles on tree). N the bell-shaped third antennular segment seen in cyprids of acorn barnacles inhabiting both live and abiotic surfaces (blue circles on tree). Roman numbers are segment numbers. The phylogeny is synthesized from the tree produced in the present paper and from several highly congruent trees (39-44). Larval structural data from Supplementary Table 1 and 4. ‘Invasion device’ is synonymous with antennule, attachment- and habitat selective device used in the manuscript.
Fig 2. Host invasion and metamorphosis in the burrowing coral barnacle Berndtia purpurea. 

A Cypris larva arrives on hosts and starts exploring. 
B The cypris larva in light microscopy with magnification of antennules in scanning electron microscopy. 
C Wide searching, cyprids walk and explore host surfaces. 
D Inspection with initial penetration. 
E After 48 hours, permanent invasion with penetration commences. 
F-I Metamorphosis and host embedding. The metamorphosing larvae survive host defense mechanisms. 
J-M Cypris embed fully in the host. 
N, O Juvenile emerges and feeds after 18 and 23 days, respectively. 
P An in-situ adult. 
Stiped circles indicate a cyprid on/in the host. Stiped line mark host epithelium. 
White, curved arrow indicates cyprid movement.
Fig 3. Life history, development, and larval invasive behaviors in burrowing and balanomorphan coral barnacles. A Six nauplius larvae disperse in the plankton. The last nauplius starts metamorphosing to the cyprid stage, which is revealed by the presence of two compound eyes and a nauplius eye. B-C Cyprids arrive on hosts and leave the planktonic phase. All host surfaces are explored by various behaviors (marked in italics). D Overview of settlement behaviors mediating host invasion. E Cypris permanent invasion by antennular penetration. The cyprids enter the host tissues and metamorphose to juveniles and adults. Roman numbers: naupliar stages. Stiped curved arrows: larval movement. Curved arrows: larval relocation. Adult sketch of *Berndtia purpurea*.

Fig 4. Larval entering of the host mouth in the burrowing coral barnacle *Berndtia purpurea*. A-E A cyprid willingly enters and escapes the host mouth alive.

Fig 5. The penetrating cypris larva of the coral acorn barnacle *Darwiniella angularis*. A Cyprid in lateral view exposing spear-shaped antennules, B magnification of the 3rd antennular segment and the attachment disc, C Cyprid in scanning electron microscopy (SEM) settled on host by antennular penetration, D magnification of another cyprid specimen penetrating the host in SEM, E magnification of C showing the third antennular segment penetrated through the host epithelium.
Fig 6. Maximum Likelihood phylogenetic tree and antennular phenotypic evolution of 99 barnacle species (Thecostraca) based on 7 nuclear and mitochondrial markers. Spear-shaped (yellow) antennular phenotypes evolved exclusively in Berndtia and pyrgomatid coral barnacles and coincides with host penetration (C, J). Faciotecta and Ascothoracida (A, B) exhibit hook-shaped invasion devices, which was lost in the ancestor to Cirripedia (Acrothoracica, Rhizocephala and Thoracica). Shoe-shaped antennules (orange circles) is the most versatile phenotype used for both free-living, symbiotic and parasitic settlement in burrowing, parasitic and scalpelloid barnacles (D-G). A bell-shaped antennule evolved exclusively in the epibiotic coral barnacles (H, I, K) and is the ancestral state for all major lineages. Both spear (J) and shoe (L) shaped antennules evolved from the bell. Light microscopy images inserted on major branches obtained from live cypris larvae are representative. SEM inserts around grey circle are cut-off at the intersection between the second and third antennular segments. Upper left insert shows a cirripede cyprid (Megabalanus rosa) antennule for reference. Data from Table S1 and S4. Node colors represent ancestral states inferred with MBASR (Fig S2). Roman numbers are segment numbers. Tree inference details given in Supplementary Tables 1, 2, 3 and 4. Small color light microscopy inserts match those from Fig 1.
Electronic supplementary material.

Supplementary figure legends.

Figure S1. Maximum Likelihood Tree with support values used to analyze trait evolution of barnacle cypris larvae. From IQ-TREE as described in the Text and in Supplementary Tables 1-3.
Node support values derived from 10000 ultrafast bootstrap replicates in IQ-TREE. ML-tree based on 2563bp nucleotides derived from 7 nuclear and mitochondrial markers.

Figure S2. Maximum Likelihood Tree showing Ancestral State Reconstruction of antennular phenotypes and mode of host invasion in barnacle (Thecostraca) cypris larvae. Left: Tree showing that antennular penetration (State 2) evolved three times in Thecostraca; in the burrowing (Acrophyllidae) and balanomorphan (Thoracica) coral barnacles and in the akentrogonid parasitic barnacles (Rhizocephala). In coral barnacles, penetration evolved from ancestors that settled externally with cement (State 1). The ancestors to Thecostraca either attached by hooks (State 3) or by external attachment with cement. The ancestor to all parasitic barnacles attached by external cementation and then piercing by a novel larval stage, the kentrogon. ML-tree based on 2563bp and derived from 7 nuclear and mitochondrial markers. Character coding in Supplementary Table S4. Right: Tree showing that spear-shaped antennules (State 2) evolved independently and convergently between the two coral barnacle clades (Acrophyllidae and Thoracica). Bernditia evolved from ancestors with shoe-shaped antennules (State 1) while the coral barnacles (Pyrgomatidae) in Thoracica evolved from ancestors with bell-shaped antennules (State 4). The ancestor to all barnacles (Thecostraca) was either hook (State 3) or shoe-shaped. ML-tree based on 2563bp and derived from 7 nuclear and mitochondrial markers. Character coding in Supplementary Table S4.


Supplementary tables.

Table S1. Summary of taxonomic coverage, nucleotide markers, NCBI GenBank accession numbers antennular phenotype and mode of settlement in the 99 species of barnacles (Thecostraca) used in the study.

Table S2. Summary of primers and PCR-conditions used for amplifying y-larva (Facetotecta) DNA.

Table S3. Summary of DNA substitution models used to infer maximum likelihood trees in IQ-TREE.
Table S4. Character coding for ancestral state reconstruction on Maximum Likelihood trees inferred using IQ-TREE. Function refers to mode of host/substratum invasion.

Video legends

Video 1. The variation in antennular phenotypes and biomechanics in live barnacle cypris larvae comprising an unknown rocky shore balanomorphan, Balanus amphitrite (rocks, Balanidae), Megabalanus ajax (fire corals, Balanidae), Scalpellum scalpellum (hydroids, Scalpellidae), Peltogaster paguri (crabs, Rhizocephala), Trevithana margarethae (corals, Pyrgomatidae) and Baccalaureus sp. (zoanthids, Ascothoracida).

Video 2. The nauplius larva of Berndtia purpurea.

Video 3. Free cypris larvae of Berndtia purpurea.

Video 4. Wide searching behavior in cypris larvae of Berndtia purpurea.

Video 5. Close searching behaviors in cypris larvae of Berndtia purpurea.

Video 6. Inspection with initial penetration in cypris larvae of Berndtia purpurea.

Video 7. Inspection with initial penetration and permanent settlement in cypris larvae of Berndtia purpurea.

Video 8. Metamorphosis and early juvenile in Berndtia purpurea.

Video 9. Special behaviors exhibited by cyprids of Berndtia purpurea.

Video 10. Larval exploratory behaviors in Berndtia utinomii.

Video 11. Larval exploratory behaviors, settlement, and metamorphosis in Darwiniella angularis.

Video 12. Antennular biomechanics and morphological features of Galkinius cypris larvae.

Video 13. Larval exploratory behaviors and antennular biomechanics in cyprids of two burrowing barnacles.


Table 1. Phenotype, ecological niche and mode of host invasion of the 13 species studied by light microscopy, scanning electron microscopy and live video. Data from this study.

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<td>Thoracica</td>
<td>Darwiniella angularis</td>
<td>Chan, Chen &amp; Liu 2012</td>
<td>Scleractinian corals</td>
<td>Symbiotic Spear</td>
<td>Penetration, cementation</td>
<td>Video 11</td>
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<tr>
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<td>Trevathana margarethae</td>
<td>Bricker, Simon-Blecher &amp; Achituv 2010</td>
<td>Scleractinian corals</td>
<td>Symbiotic Spear</td>
<td>Penetration, cementation</td>
<td>Video 1</td>
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<table>
<thead>
<tr>
<th>Common name group</th>
<th>Coral barnacles</th>
<th>Stalked barnacles</th>
<th>Parasitic barnacles</th>
<th>Symbiotic balanomorph</th>
<th>Intertidal</th>
<th>Y- and A-larvae</th>
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<td>Thoracica Galkinius sp</td>
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<td>Symbiotic</td>
<td>Spear</td>
<td>Penetration, cementation</td>
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<td>Thoracica Megabalanus ajax Darwin 1854</td>
<td>Fire corals</td>
<td>Symbiotic</td>
<td>Bell</td>
<td>Cementation</td>
<td>Video 1</td>
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**Table 2.** Summary of host/substratum niches and larval behavioral and structural characteristics across major barnacle lineages. Data on coral and stalked barnacles, Facetotecta and Ascothoracida from this study. Data on parasitic barnacles from Høeg et al. (1985a, b). Data on symbiotic acorn barnacles from Yu et al. (2020), Nogata and Matsumura (2006) and Dreyer et al. (2020). Data on intertidal barnacles from Aldred et al. (2018) and this study.
<table>
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<th>Taxa</th>
<th>barnacles</th>
<th>barnacles</th>
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<tr>
<td>Berndtia, Pygomatidae</td>
<td>Scallopsidae, Lepadidae</td>
<td>Rhizocephala</td>
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<tr>
<td>Acastinae, Coronuloidea, Megabalanus ajax</td>
<td>Pollicipomorpha, free-living Balanomorpha</td>
<td>Facotetcta and Ascothoracida</td>
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</tbody>
</table>

| Niches | Symbiotic with scleractinian corals | Symbiotic with hydroids, neustonic objects, marine vertebrates | Parasitic in crustaceans | Symbiotic with sponges, marine vertebrates, fire corals | Rocky shores, floating objects, shells, occasionally marine vertebrates | Parasitic in echinoderms and cnidarians, unknown in Facotetcta |

| Wide searching | Slow bipedal walk with 1st and 2nd segment, 3rd segment sets off from surface | As in coral barnacles | As in coral barnacles | As in coral barnacles but faster | As in coral barnacles but faster | No bipedal walking |

| Close searching | Brief, temporary halt and extensive probing with 3rd and 4th segments | As in coral barnacles | As in coral barnacles | As in coral barnacles | As in coral barnacles | Not observed |

| Inspection | Prolonged halt, probing with 3rd segment on narrow area, pushing down host epithelium | Prolonged halt, probing with 3rd segment on narrow area | As in Scallopsidae | As in Scallopsidae | As in Scallopsidae | Not observed |

| Permanent settlement | Repeated penetration with 3rd segment, cementation | External cementation | External cementation or penetration, injection | External cementation | External cementation | Piercing, injection |

| Duration from host arrival to permanent settlement | Up to 48h | Up to 24h | 5-20 min | Less than 12h in sponge barnacles, several days in whale barnacles | Less than 24h, depends on cyprid age | Not observed. Primordial parasite escapes cyprid in 31-72 hours |

| Antennular phenotype | Spear | Shoe | Shoe | Shoe and Bell | Bell | Hook |