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Molecular Phylogenetic and Embryological Evidence That Feeding Larvae Have Been Reacquired in a Marine Gastropod

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Abstract. Evolutionary transitions between different modes of development in marine invertebrates are thought to be biased toward the loss of feeding larvae. Because the morphology of feeding larvae is complex and nonfeeding larvae or encapsulated embryos with benthic development often have simplified morphologies, it is presumed to be easier to lose a larval stage than to reacquire it. Some authors have gone so far as to suggest that feeding larvae, morphologically similar to the ancestral feeding larvae, cannot be reacquired. However, the larval structures of some groups, most notably gastropods, are often retained in the encapsulated embryos of species that hatch as benthic juveniles. Therefore the re-evolution of feeding larvae using the same structures may be possible in these groups. Here we present the first well-substantiated case for the recent re-evolution of feeding larvae within a clade of direct-developers. DNA sequence data show that *Crepipatella fecunda,* a species of calyptraeid gastropod with planktotrophic development, is nested within a clade of species with direct development, and that *Crepipatella dilatata,* a species with direct development, appears to be paraphyletic with respect to *C. fecunda.* Observation of the embryos of *C. dilatata* shows that the features necessary for larval feeding and swimming are retained in the encapsulated veligers, suggesting that heterochronic shifts in hatching time and changes in nurse-egg allotment could have resulted in the re-evolution of feeding larvae in this species.

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Introduction

The mode of development of marine invertebrates (direct or indirect, brooded, encapsulated, or planktonic) has far-reaching evolutionary and ecological consequences that are mediated *via* differences in dispersal ability. Species with planktotrophic (swimming and feeding) development often have higher rates of dispersal than do those with benthic, nonfeeding development (referred to here as "direct development" for simplicity). These differences in dispersal are thought to result in higher rates of gene flow and increased colonization ability (*e.g.,* for gastropods: Hoskin, 1997; Kyle and Boulding, 2000; Wilke and Davis, 2000; Collin, 2001) for planktotrophs, as well as larger geographic ranges (Scheltema, 1989; Emlet, 1995; but see O'Foighil, 1989) and lower speciation and extinction rates when compared to species with direct development (*e.g.,* Hansen, 1978, 1980; Jablonski, 1986; Gili and Martinell, 1994). Since mode of development has such profound evolutionary consequences, and since it is a feature that varies widely among species within most phyla of marine invertebrates, understanding the factors that shape evolution in mode of development is a major goal of marine invertebrate biologists (*e.g.,* Strathmann, 1974, 1978a, b, 1985, 1990; Raff, 1987; Havenhand, 1995; Smith, 1997; Duda and Palumbi, 1999; McEdward, 2000; Jeffery *et al.,* 2003; Collin, 2004).

The evolution of mode of development is presumed to be biased toward the loss of feeding larvae (Strathmann, 1974, 1978a; Hart, 1996). The idea is that species lacking planktonic feeding stages do not experience the selective pressure to retain the complex larval structures for feeding and

swimming in the plankton. This should eventually result in the loss or reduction of such structures, and the likely degeneration of the genetic machinery that supports their development. Indeed, loss of such structures is commonly observed in nonfeeding larvae or embryos of echinoderms and molluscs (*e.g.,* Strathmann, 1978a,b; Hadfield and Iaea, 1989; Emlet, 1994; Wray, 1996). Once lost, the difficulty of re-evolving a set of structures that function effectively for feeding and swimming makes it unlikely that feeding larvae will be reacquired from an ancestor with direct development.

If planktotrophic larvae have been reacquired *de novo*, the morphological structures used for feeding and swimming are expected to differ noticeably from those structures in the ancestral larval type (Strathmann, 1978a,b). The hypothesized ancient acquisition of feeding larvae in lingulid brachiopods through co-option of juvenile structures for function in a feeding larva is an example of this scenario (Strathmann, 1978a). In addition, some groups, most notably polychaete families (Rouse, 2000), have evolved multiple ways of feeding in the plankton that are associated with distinct larval morphologies. The cases identified by Strathmann (1978a) as possible independent acquisitions of feeding larvae were all ancient. Although more recent reacquisitions of feeding larvae are possible, there are no well-documented cases of such an event. Applications of phylogenetic methods to the comparative study of invertebrate development has further supported the idea that losses of feeding larvae are common, while little evidence of the possible re-evolution of feeding larvae has been recovered (Wray, 1996; Hart *et al.,* 1997; Smith, 1997; Duda and Palumbi, 1999; Jeffery *et al.,* 2003; Jeffery and Emlet, 2003).

Most of our ideas about the loss and reacquisition of feeding larvae are based on the extensive study of echinoid echinoderms. Groups such as gastropods, in which larval structures are not so extremely modified in direct-developing species, may be more permissive for the reacquisition of feeding larvae (Strathmann, 1978a). Many direct-developing gastropod species do indeed lose any identifiable larval structures. For example, the direct-developing brooded calyptraeids *Crepidula adunca* and *Crepidula philippiana* lose almost all vestiges of the larval velum and operculum (Gallardo, 1977b; Collin, 2000a). Although the loss of larval structures is also well documented for muricids (*e.g.,* Fioroni, 1966), it is also common for species without a free-living larval stage to retain larval features during development. For example, Moran (1999) demonstrated that the embryonic velum in *Littorina* species with encapsulated development is not simplified or reduced compared to that of species with planktotrophic larvae. Likewise, Pernet (2003) showed that nonfeeding sabellid polychaete larvae retained the ability to capture particles by using the opposed-band ciliary system. The reduction and loss of larval structures appears to be correlated with a proxy for the time since the loss of feeding larvae (Collin, 2004). This suggests that after the evolution of direct development there is a window when larval structures are retained and during which the re-evolution of feeding larvae would be possible (Collin, 2004).

A recent phylogenetic analysis of calyptraeid gastropods did not support the irreversibility of the loss of feeding larvae (Collin, 2004). Maximum likelihood reconstruction of ancestral states did not demonstrate a statistically significant difference between the rate of gains *versus* losses of feeding larvae in calyptraeid gastropods (Collin, 2004). Furthermore, the rate of gains was significantly different from 0. Likewise, parsimony reconstructions suggested that feeding larvae could have been regained if the probability of loss was twice that of gaining feeding larvae (Collin, 2004). This study identified three areas in the phylogeny where a planktotrophic species appeared to be nested within a clade of direct-developing species and were, therefore, likely candidates for the discovery of secondarily evolved feeding larvae. Here we examine one of these clades, the genus *Crepipatella,* in detail and provide more evidence that *C. fecunda* is a species in which planktotrophic development may have been recently reacquired.

Materials and Methods

Biology and natural history of Crepipatella

Calyptraeid gastropods comprise a family of sedentary, filter-feeding, protandrous marine limpets (Fig. 1 A, B). They are tolerant of widely varying ecological conditions but generally occur in intertidal or shallow subtidal habitats. Species in this family have been the focus of many studies of life histories, reproduction, and development. The genus *Crepipatella,* first described by Lesson in 1830, was synonymized with *Crepidula* until phylogenetic analysis of anatomical and DNA sequence data showed that *Crepipatella* groups more closely with other calyptraeid genera, and most closely with *Crucibulum* and *Bostrycapulus,* than with *Crepidula* (Collin, 2003a, b). About 15 *Crepipatella* species have been described since 1800, but recent studies indicate that there are only six distinct species. Three of these co-occur along the coast of Chile. These species are morphologically indistinguishable as adults, but they can be distinguished on the basis of development. Two of these species, *C. dilatata* and *C. fecunda,* are among the most well-studied species of gastropods from the southern hemisphere (Gallardo, 1976, 1977a, 1979; Gallardo and Garrido, 1987; Chaparro and Paschke, 1990; Chaparro *et al.,* 2002, 2005; Veliz *et al.,* 2003).

Gastropod larval morphology

Like larvae of most groups of marine invertebrates, gastropod larvae use bands of compound cilia arranged on a

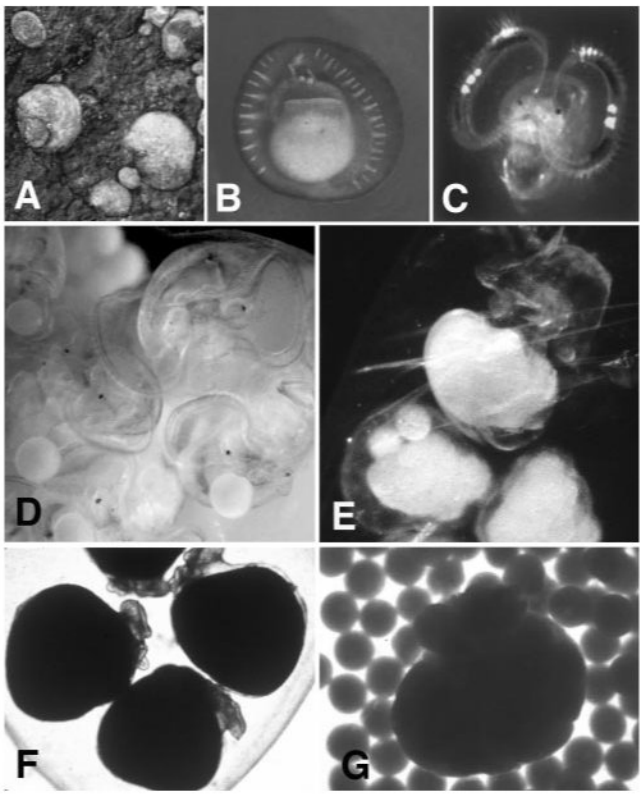

Figure 1. Adults, embryos, and larvae of *Crepipatella* species. (A) Adult *C. dilatata* in the intertidal near Coquimbo, Chile. (B) Adult *C. lingulata* attached to the glass of an aquarium in Friday Harbor, Washington. (C) Planktotrophic larva of *C. lingulata.* (D) Encapsulated development of *C. dilatata.* The uncleaved nurse eggs are about 190 μm, and the extended velum is clearly visible on several of the embryos. (E) Encapsulated embryo (above) and nurse eggs (below) of *Crepipatella* sp. This early in development, the embryos are not much larger than the nurse eggs, but the clearly developed mouth, embryonic kidneys, head-vesicle, and beginnings of the velum are all clearly visible, whereas the nurse eggs are irregular ciliated balls of yolk. (F) Embryos of *C. capensis* after consumption of the nurse eggs. The foot and tentacles are visible, but the embryonic morphology is dominated by the large yolk-filled visceral mass. (G) An embryo of *C. capensis* that has not yet consumed all of the nurse eggs. The irregular outlines of the nurse eggs are just visible at the edges of the visceral mass.

supporting structure to both propel them through the water and, at the same time, capture particles and transport them to the mouth. Gastropod larvae swim and feed with the velum, a pair of lobate flaps that extend from the head and are edged by two bands of compound cilia. These ciliary bands flank a ciliated food groove along which food is transported to the mouth. In direct-developing marine gastropods these structures are often reduced. The velum can either be small or reduced to a raised ciliated ridge, and the cilia are shortened and are often rearranged into a uniform ciliary field covering the head. Other modifications of direct-developing embryos include (1) enlargement or elaboration of the "embryonic kidneys," a pair of round cells located laterally behind the velum that are used for uptake of intracapsular albumin; (2) enlargement of the head vesicle, a ciliated, fluid-filled extension of the head with unknown function; (3) loss of the operculum in species that lack an operculum as adults; and (4) reduction of the coiling and sculpture of the shell (Collin, 2004).

Field collections and observations

Individuals of six species of *Crepipatella* were collected, with their brooded embryos, in the intertidal and shallow subtidal zones. For all animals that were preserved in 95% ethanol for subsequent DNA sequencing, mode of development was scored by examining living embryos with a stereomicroscope. Multiple stages of development were observed and described for all species, and special note was taken of the features that are known to often be modified in direct-developing species (see above). In most cases many additional animals were collected for other ongoing studies by the principal investigators (*e.g.,* Chaparro *et al.,* 2002; Véliz *et al.,* 2003; Collin, 2004, 2006). Locality information for the live-collected specimens that were examined or sequenced for this study are listed in Table 1. Vouchers are deposited at the Field Museum of Natural History, Chicago, USA; The Natural History Museum, London, England; and the Academy of Natural Sciences, Philadelphia, USA (Table 1). We sampled all apparently valid species of the genus *Crepipatella: C. lingulata* (Gould 1846), *C. dorsata* (Broderip 1834), *C. capensis* (Quoy & Gaimard 1832–1833), *C. fecunda* (Gallardo 1979), *C. dilatata* (Lamarck 1822), and a currently undescribed species we refer to as *Crepipatella* sp. The only described species that is not likely to be a synonym of the species included here is *Crepipatella fluctuosa* Taki 1938 from Japan, which is reported from shells only.

DNA sequencing and analysis

A 564-base-pair fragment of the mitochondrial cytochrome oxidase I (COI) gene was sequenced for individuals from each locality (Table 1), and sequences have been deposited in GenBank (AF546051–AF546054; AF550491; DQ811116–DQ811133). DNA was extracted, amplified, and sequenced following the methods of Collin (2003b). DNA was extracted from ethanol-preserved tissue with Puregene (Gentra Systems) or DNeasy (Quiagen) extraction kits, amplified using Ready-To-Go PCR beads (Pharmacia Biotech), and primers and PCR profile of Folmer *et al.* (1994). PCR products were sequenced in both directions with dRhodamine (Perkin Elmer) or Big Dyes cycle sequencing dye terminator kits using the amplification primers and an ABI 377 automated sequencer.

Phylogenetic analyses were conducted using PAUP* 4b02 (Swofford, 1998). An equal-weighted parsimony analysis was performed, using a heuristic search with TBR branch swapping and 100 random additions. Bootstrap sup-

Table 1

Material collected, location, and haplotype designations

Species	Location	Development	Vouchers	Number Sequenced	Haplotypes (number of individuals; GenBank #)
Crepipatella capensis Quoy and Gaimard, 1832–33	Muizenberg, Cape Province, South Africa 34° 4′ S, 18° 20′ E	Direct with nurse eggs	FMNH 282278	3	Cap1 (1; DQ811130) Cap2 (1; DQ811131) Cap3 (1; AF546053)
Crepipatella lingulata Gould, 1846	Shady Cove, Friday Harbor, Washington, USA 48° 20′ N, 123 01′ W	Planktotrophic	FMNH 282293 FMNH 285019	1	Ling2 (1; AF546054)
	Santa Barbara, California, USA 34° 20′ N, 120° 01′ W	Planktotrophic	BM20010466	3	Ling1 (2; DQ811132) Ling3 (1; DQ811133)
Crepipatella dorsata Broderip 1834	Islas de las Perlas, Panama 8° 30′ N 79° 02′ W	Unknown	ANSP 413607	1	1; DQ811116
Crepipatella sp.	Totoralillo, southern, Chile 30° 4′S, 71° 22′W	Direct with nurse eggs	FMN282280	2	Csp1 (1; AF550491) Csp2 (1; DQ811129)
	Bahía Heradura, Chile 29° 58′S 71° 21′W	Direct with nurse eggs	ANSP 413606	3	Csp2 (3; DQ811129)
Crepipatella fecunda Gallardo, 1979	Santa Maria, Peru 12° 25′S, 76° 46′ W	Planktotrophic	ANSP 413601	3	Fec5 (3; AF46051)
	Ancon, Peru 11° 46′S, 77° 10 23′W	Planktotrophic	ANSP 413600	2	Fec5 (1; AF546051) Fec6 (1; DQ811126)
	Bahía de Coquimbo, southern Chile 29° 59′ S, 71° 19′ W	Planktotrophic	FMNH 299425	2	Fec5 (1; AF546051) Fec7 (1; DQ811128) Fec8 (1; DQ811123)
	Bahía de Metrí, Chile 41° 31′S, 72° 42′ W	Planktotrophic	ANSP 413599	2	Fec4 (1; DQ811125) Fec5 (1; AF546051)
	Bahía de Yaldad, Chiloé, Chile 43°08′S, 73°44′W	Planktotrophic	ANSP 413598	10	Fec1 (1; DQ811120), Fec2 (1; DQ811121), Fec3 (2; DQ811122) Fec5 (5; AF546051)
Crepipatella dilatata Lamarck, 1822	Corral Bay, San Carlos, Chile 39° 51′ S, 73° 27′ W	Direct with nurse eggs	BM 20010461	3	Dil2 (1; DQ811119) Dil3 (1; DQ811118) Dil4 (1; AF546052)
	Fjords near Metrí, Chile 41° 32′ S, 72° 42′ W	Direct with nurse eggs	ANSP 413603	2	Dil5 (2; DQ811124)
	Puerto Madryn, Argentina 42° 46′S, 65° 02′W	Direct with nurse eggs	BM20010459, BM20010460	3	Dil1 (1; DQ811117) Dil2 (2; DQ811119)
	Playa Orengo, Argentina 40° 53′S, 64° 29′W	Direct with nurse eggs	ANSP 413604	2	Dil2 (2; DQ811119)
	Rio Quempillen, Chiloé, Chile 41° 52′S, 73°44′W	Direct with nurse eggs	ANSP 413602	9	Dil5 (9; DQ811124)
	Bahía de Coquimbo, southern Chile 29° 59′ S, 71° 19′W	Direct with nurse eggs	ANSP 413605	3	Dil3 (2; DQ811118) Dil6 (1; DQ811127)

port for each clade was assessed on the basis of 1000 bootstrap replicates with TBR branch swapping and five random additions. Likelihood analyses were also conducted with PAUP, using TBR branch swapping and the model of evolution identified as the most appropriate by ModelTest (see below). *Crucibulum auricula* and *Crucibulum* cf. *quiriquinae* were used to root these phylogenies.

DNA sequence data were also analyzed by a Bayesian approach using MrBayes 2.01 (Huelsenbeck and Ronquist, 2001). The appropriate model and starting parameters for Bayesian analysis were chosen for each of the data sets by using the likelihood ratio test implemented in ModelTest 3.06 (Posada and Crandall, 1998, 2001) with the default settings and an α-level of 0.01. The Bayesian analysis, using one cold and three incrementally heated chains, started from a random tree with a uniform prior for branch lengths and for the gamma shape parameter. Invariant sites were retained in the sequences, and their frequency was estimated using the "invgamma" setting. The Metropolis-coupled Markov chain Monte Carlo (MCMCMC) analysis was run five times for 4,000,000 generations, and the number of trees to be discarded as representing a "burn-in" period was

determined graphically. Majority-rule consensus trees for every 50th tree after the "burn-in" period were created using PAUP*, and consensus phylograms were created in MrBayes.

Likelihood reconstructions of character evolution were performed using Discrete (Pagel, 1997, 1999a,b). The maximum likelihood rates of character change were used to map the evolution of development onto the Bayesian topology using the "calculate fossil likelihood" command. Those nodes where the likelihoods of the two states differed by more than 2 log units were considered to provide significant support for one state at that node in preference to the other state (Pagel, 1997, 1999a,b). For comparison, ancestral states were also reconstructed using equal-weighted parsimony.

Results

Observations of development

The gastropod genus *Crepipatella* (Calyptraeidae) contains two species that have typical planktotrophic development (*Crepipatella lingulata* and *Crepipatella fecunda;* Fig. 1) and three species that have direct development with nurse eggs (*Crepipatella capensis, Crepipatella* sp., and *Crepipatella dilatata*). Mode of development was documented for samples from each location where animals were collected. Development did not vary among populations of any of the species.

The development of the planktotrophs has been described in detail previously (Collin, 2000a; Chaparro *et al.,* 2002, 2005). Neither the development nor the larval morphology of either species differs in any substantive way from that described for the well-known larvae of *Crepidula fornicata* (Conklin, 1897; Werner, 1955; Fig. 1C). We observed no modifications to the development of the velum, head vesicle, embryonic kidneys, or operculum that were similar to any features of the embryos of the direct-developing calyptraeid species. There were no intra-specific differences in larval morphology between *C. lingulata* from Washington State and California, or between *C. fecunda* from different populations in Chile.

All three direct-developing species produced small eggs that developed into large, crawling juveniles by ingesting nurse eggs (other eggs deposited in the same egg capsule). The development of *C. dilatata* has been described in some detail previously (Gallardo, 1976, 1977a, 1979; Chaparro *et al.,* 2002). To summarize, a small number of the 220–240-μm eggs in each capsule develop into an intracapsular veliger with a coiled shell, a single embryonic kidney on each side, an operculum, and a distinct velum with a food groove (Fig. 1D). In fact, embryos of *C. dilatata* retain the ability to capture particles and transport them to the mouth via the food groove when removed from the capsule (Chaparro *et al.,* 2002). However, they cannot swim (Chaparro *et al.,* 2002). At this stage, the embryos of *C. dilatata* consume the uncleaved nurse eggs that were deposited in the capsule with them. The embryos lose all larval features before they hatch as crawling juveniles.

The development of *Crepipatella* sp. is similar to that of *C. dilatata.* The embryos develop into intracapsular veligers complete with a coiled shell, an operculum, and a velum with a distinct food groove. Like *C. dilatata,* the embryos do not have any obvious modifications for encapsulated development. However, unlike *C. dilatata,* the nurse eggs cleave in the same way as the embryos, and the two can only be distinguished from each other after gastrulation when the development of the nurse eggs becomes abnormal. Unlike the situation in *C. dilatata* where the nurse eggs are consumed whole, the embryos of *Crepipatella* sp. appear to somehow suck the yolk from the nurse eggs. Early in development the nurse eggs are yolk-filled ciliated balls, while later the yolk is restricted to the center of the ball (Fig. 1E). The nurse eggs are all finally consumed, and the larval features are lost before the embryos hatch as crawling juveniles.

The embryos of *C. capensis* also have direct development with nurse eggs. However, they lack a distinct intracapsular veliger stage (Fig. 1F, G). The embryos begin to consume the uncleaved nurse eggs much earlier in development, somewhat after gastrulation. The shell develops around the yolk-filled embryo and therefore does not develop a distinct coil like that of the other species of *Crepipatella.* The velum is a vestigial ridge with some ciliation but lacks a well-organized food groove. There is no operculum, and the head vesicle is large, although the larval kidneys remain simple.

Crepipatella dorsata is a very rare, subtidal species, about which little is known. None of the four individuals we collected were brooding egg capsules, and they failed to produce eggs when maintained in the laboratory for several months. Therefore, the development of this species remains unknown.

Phylogenetic relationships

Sequences of 564 base pairs of COI representing 33 haplotypes were obtained from 55 individuals (Table 1). Phylogenetic analyses of these sequences using Bayesian, parsimony, and maximum likelihood methods all resulted in congruent tree topologies (Fig. 2). In all cases the genus *Crepipatella* was rooted on the branch between *C. lingulata* + *C. dorsata* and the rest of the clade. *Crepipatella capensis* and *Crepipatella* sp. were the successive sisters to a clade comprising *C. fecunda* and *C. dilatata.* All species except the *fecunda-dilatata* group appeared as distinct and well-supported monophyletic clades (bootstrap $> 70\%$, posterior probability $> 95\%$; Fig. 2), which were separated by 10%–18% Kimura two-parameter distances.

Together the haplotypes of *C. fecunda* and *C. dilatata*

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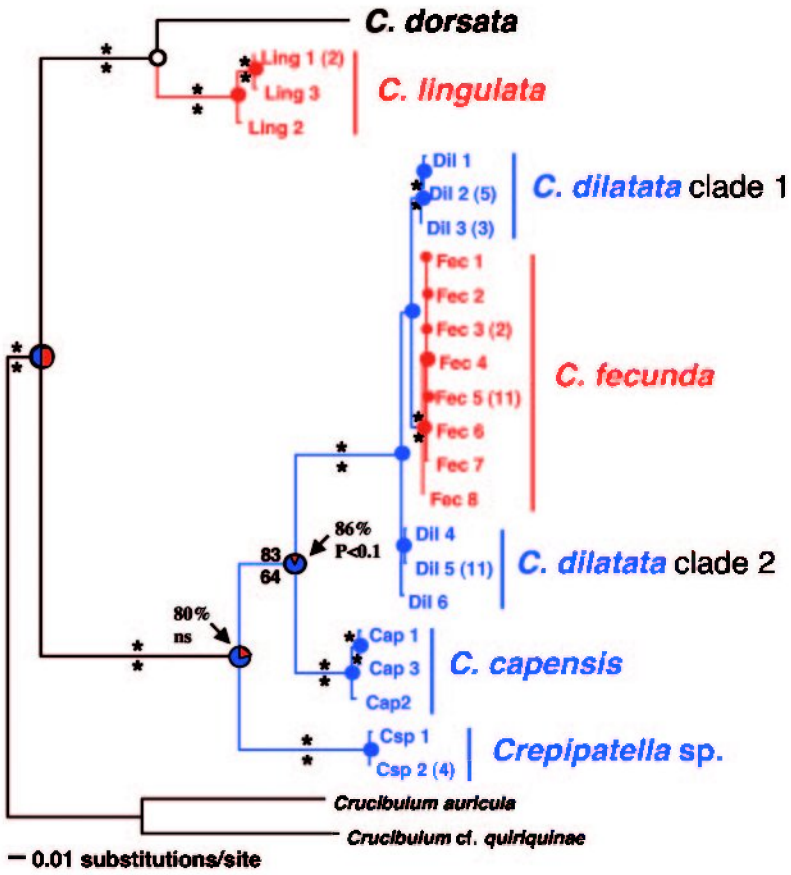

Figure 2. The maximum likelihood topology of the COI haplotypes of *Crepipatella.* Redundant sequences are not shown, but the number of individuals with each haplotype is indicated after each species name. Asterisks above the branches indicate Bayesian support >95%, and those below the branches indicate support of >70% from a nonparametric bootstrap of the maximum likelihood analysis. In Bayesian, parsimony, and likelihood analyses the best trees all showed *C. fecunda* haplotypes nested within *C. dilatata.* Colored branches represent the parsimony state reconstruction of mode of development, and colored circles at the nodes indicate the maximum likelihood reconstruction of the ancestral condition reconstructed by Discrete (Pagel, 1999a, b). Solid blue circles represent nodes where the support for one state over the other was statistically significant and where that state was reconstructed more than 95% of the time. Blue = direct development; red = planktotrophy; black = unknown.

formed a well-supported (bootstrap > 70%, posterior probability > 95%) monophyletic clade, but the haplotypes from the two species were not reciprocally monophyletic. The haplotypes of *C. fecunda* formed a monophyletic clade in all analyses, and this clade was consistently placed within a paraphyletic *C. dilatata* by all three phylogenetic methods. *Crepipatella dilatata* comprised two haplotype clades, one made up predominantly of haplotypes from southern Chile and the other mostly of haplotypes from Argentina. This indication of population structure is what would be expected from a direct-developing species. However, both clades also contained haplotypes from northern Chile. The average Kimura two-parameter distance between sequences from *C. fecunda* and *C. dilatata* was 1.66% (1.26%–1.99%).

Both parsimony and maximum likelihood trait reconstructions support the conclusion that the planktotrophy of *C. fecunda* has re-evolved from direct development. Parsimony reconstructions of mode of development on the phylogeny (Fig. 2) give the ancestral condition at the base of the tree as ambiguous, because the first branching leads to one clade with planktotrophic development and one with direct development, and because the condition in the outgroups is variable (Collin, 2004). Within *Crepipatella,* the ancestral condition for *C. fecunda* is reconstructed as planktotrophy, whereas the four nodes between *C. fecunda* and the base of the tree, including the base of the *fecunda-dilatata* clade, are all reconstructed as direct development. Maximum likelihood reconstructions, of the traits using Discrete recovered a similar pattern (Fig. 2). The ancestral condition for *C. fecunda* is reconstructed as significantly more likely to be planktotrophy than direct development (likelihood ratio, $P < 0.05$), whereas the base of the *fecunda-dilatata* clade is

significantly more likely to be direct development (likelihood ratio, $P < 0.05$). The mode of development for the node below the base of the *feeunda-dilatata* clade is marginally significant for direct development ($P < 0.10$; Fig. 2).

Discussion

In the evaluation of the two alternate hypotheses—secondary planktotrophy in *Crepipatella feeunda versus* ancestral planktotrophy in *C. feeunda*—two lines of evidence are consistent with the idea that planktotrophy has been reacquired: the reconstruction of mode of development on the molecular phylogeny of the genus, and comparative observations of the embryology and larval morphology. The reconstruction of mode of development on the phylogeny shows that the planktotrophic *C. feeunda* has four successive sister clades with direct development (two clades of *C. dilatata, C. eapensis,* and the *Crepipatella* sp.). The nesting of *C. feeunda* deeply within a paraphyletic clade of *C. dilatata* suggests that *C. dilatata* is the extant paraphyletic ancestor of *C. fecunda.* Thus, the direct-developer is reconstructed as the extant ancestor of the planktotrophic *C. fecunda,* giving support to the idea that feeding larvae have been reacquired. Finally, nodes ancestral to the most recent common ancestor of *C. feeunda* are reconstructed by Discrete as statistically significantly more likely to have direct development. If further sampling leads to a re-rooting of this clade such that *C. feeunda* is sister to *C. dilatata* or that there is an unresolved polytomy at the base of the *fecunda-dilatata* clade, the argument would be weakened somewhat, but *C. feeunda* would still be nested within three successive sister clades of direct-developers. Such a topology would still support the reacquisition of feeding larvae under parsimony analysis and is likely to be supported by maximum likelihood, although such a change could also result in an ambiguous reconstruction. Either way, the alternate hypothesis—that feeding larvae are the retained ancestral state—would not receive support from the topology.

The reacquisition of planktotrophy in *C. fecunda* is also consistent with the dynamics of evolution of development across the entire family. If planktotrophy is assumed for the ancestral state at the base of the *Crepipatella* clade, the hypothesis of reacquisition of planktotrophy requires a single loss of feeding larvae and one subsequent gain in *C. feeunda.* The alternate hypothesis—that planktotrophy is the ancestral condition retained in *C. feeunda*—would require that direct development with nurse eggs evolved three times independently (assuming that direct development evolved only once in *C. dilatata*), and that feeding larvae never re-evolve. Global maximum likelihood analysis of mode of development for 72 species of calyptraeids found no statistically significant differences in rates of loss and gain of feeding larvae across the family (Collin, 2004). This study also found that the loss of nurse eggs was almost 5 times more likely than the gain of nurse eggs. Therefore, the global analysis suggests that (1) there is no strong bias toward the loss of feeding larvae and (2) nurse eggs are more likely to be lost than gained. These results are more consistent with the pattern of character-state changes generated by the secondary gain of feeding larvae in *C. fecunda* than by the retention of ancestral planktotrophy. If the ancestral state for *Crepipatella* is reconstructed as direct development or if *C. dorsata* (development currently unknown) has direct development, the case for the reacquisition of feeding larvae becomes stronger.

Observations of embryonic and larval morphologies, an independent line of evidence, are also consistent with the idea that feeding larvae could have re-evolved in *C. fecunda.* The closest direct-developing sister of *C. fecunda* and one of the other sisters both retain complete larval morphologies during intracapsular development. The closest sister, *C. dilatata,* also retains the ability to capture particles from suspension (using the opposed-band ciliary mechanism), and its inability to swim is apparently due only to its large size (Chaparro *et al.,* 2002). If this kind of development with an intracapsular veliger were ancestral at the base of the *C. fecunda* clade, feeding larvae could have been gained by the simple reduction of nurse egg production, as suggested by Chaparro *et al.* (2002). This is not as unexpected as the reacquisition of larvae *de novo.*

How eommon is the reaequisition of feeding larvae?

As described in the introduction of this paper, there is ample evidence from echinoids and asteroids to support the scenario that losses of feeding larvae are relatively common and that feeding larvae have not been reacquired (*e.g.,* Emlet, 1994; Wray, 1996). In contrast, vast variation in mode of development among many families or genera of gastropods, polychaetes (Kupriyanova, 2003; Pernet, 2003), and ophiuroids, and phylogenetic analyses of such groups show that transitions in mode of development have occurred frequently and recently (*e.g.*, Hart *et al.,* 1997; Duda and Palumbi, 1999; Jeffery *et al.,* 2003; Collin, 2004; Ellingson and Krug, 2006). In fact, changes can appear so frequently that our ability to reconstruct the likely pattern of evolution is limited (Collin, 2004). The same groups that display such high levels of variation in mode of development are also the groups in which the retention of structures specialized for planktotrophy is common among direct-developers. This makes it likely that such clades include other examples of secondary larval feeding or swimming. Although independent acquisitions of feeding larvae were predicted by Strathmann (1978a) to have occurred at the levels of order or family in these groups, it is somewhat surprising that well-supported cases of the re-evolution of larvae among closely related species have not come to light before now. There is

a single documented case in which a species with swimming larvae, the starfish *Pteraster tessulatus,* has evolved from a species with completely brooded development (McEdward, 1995). However, these ciliated, yolky swimming larvae do not express any of the morphological features typical of feeding larvae and cannot feed. We are not aware of any similar cases that report the recent re-evolution of feeding larvae.

Are C. fecunda *and* C. dilatata *distinct species?*

The COI sequence data show that *C. fecunda* is as differentiated from the two clades of *C. dilatata* as they are from each other. In addition, these low levels of divergence are similar to intra-specific divergences among other species of calyptraeids (Collin, 2000b, 2001, 2005). These small divergences and the fact that the mitochondrial haplotypes have not yet coalesced suggest that *C. fecunda* and *C. dilatata* diverged very recently relative to other calyptraeid species. A similar conclusion was reached by Véliz *et al.* (2003) on the basis of allozyme variation.

Despite these very similar COI sequences, it is unlikely that *C. fecunda* and *C. dilatata* are conspecific. The variation in development between these otherwise morphologically indistinguishable species is not due to poecilogony (intraspecific variation in mode of development), as evidenced by the fact that many years of research in southern Chile (Gallardo, 1976, 1977a, 1979; Gallardo and Garrido, 1987; Chaparro and Paschke, 1990; Chaparro *et al.,* 2002, 2005) have failed to produce any evidence of intra-specific variation in development among either *C. dilatata* or *C. fecunda.* Observations of embryos and larvae of all populations included here, and therefore species identifications, were standardized by a single worker (R.C.). This cross-validation of developmental characteristics in northern and southern Chile, Peru, and Argentina, as well as many years of research on these species by all of the authors, ensures that development was consistently characterized. Furthermore, poecilogony is unknown for any species of caenogastropod (Hoagland and Robertson, 1988; Bouchet, 1989) and thus is unlikey to occur in these species.

The status of *C. dilatata* and *C. fecunda* as distinct species is supported by several lines of evidence in addition to the consistent differences in development. The geographic ranges of both species, although largely sympatic, do not overlap completely. *Crepipatella fecunda* extends as far north as Lima, Peru, where *C. dilatata* is undocumented (although, *C. fecunda* has been misidentified as *C. dilatata* in this region), and *C. dilatata* occurs commonly along the southern Argentine coast where *C. fecunda* is unknown. Allozyme data for the three sympatrically occurring species from Northern Chile show that there are fixed differences between *C. fecunda* and *C. dilatata* in ACP, EST-2, and PEPB-1 (Véliz *et al.,* 2003) and frequency differences or private alleles in three other enzymes. In addition, cytogenetic data also support the distinct status of the two species. Contreras (1999) found that *Crepipatella* sp. has half the chromosome number of *C. fecunda* and *C. dilatata,* although the large number of chromosomes in these last species (2N = 120–160) mades it difficult to obtain a precise count. Despite having a similar number of chromosomes as *C. fecunda, C. dilatata* has significantly more DNA (almost 4 times more) than the other two species (Contreras, 1999; F. Winkler, unpubl. data).

Conclusion

Here we show that a clade of direct-developing marine snails, which retains the morphological features necessary for planktotrophy within the egg capsule, appears to have recently given rise to a species with planktotropic development. As larval characteristics are often retained by direct-developing gastropods, the recent re-evolution of feeding larvae may be more common than previously expected among some groups.

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