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Coral-host specificity of Red Sea *Lithophaga* bivalves: interspecific and intraspecific variation in 12S mitochondrial ribosomal RNA

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Abstract

Comparison of 12S mitochondrial ribosomal DNA sequences was used to approach the question of species specificity between boring bivalves of the genus *Lithophaga* and their coral hosts. A 450-bp long fragment was amplified by PCR from 13 individuals belonging to five subgroups of *Lithophaga* bivalves. These subgroups are defined according to their coral hosts species, and they belong to three currently recognized species: *L. lessepsiana* (1 host), *L. simplex* (2 hosts), and *L. purpurea* (2 hosts). All bivalves were collected from corals growing within an approximately 200-m section of the reef of Eilat, Red Sea. Sequence variation between members of the same species inhabiting different hosts (30–32%) was found to be very similar to the variation exhibited between recognized species. These results, when interpreted together with previously published data concerning variations among *Lithophaga* subgroups, support the notion of a very high degree of species specificity between *Lithophaga* bivalves and their coral hosts in the Red Sea.

Introduction

Lithophaga species are important bioerosion agents (Morton, 1983) that bore into the skeletons of living scleractinian corals (Gohar and Soliman, 1963; Kleemann, 1980; Morton and Scott, 1980). Some of these eroders form associations only with specific coral hosts (Morton, 1990). This specificity was shown to be driven by both selective settlement of *Lithophaga* larvae on specific corals and consequent induction of metamorphosis by the host coral (Mokady et al., 1991, 1992). However, the exact degree of host specificity to a single coral species or genus is still under debate.

Like other bivalves, *Lithophaga* is categorized into species according to distinct morphological characters that are presumably least affected by selective pressures (Kleemann, 1980). Several *Lithophaga* species, such as *L. purpurea* and *L. simplex*, are found boring in more than one coral species (or genus). Subgroups within these species, which inhabit different coral hosts, have been suspected of actually being distinct species (Brickner and Loya, 1990; Mokady et al., 1992; Brickner et al., 1993), thereby demonstrating a very high degree of specificity between the burrowing bivalve and its coral host. However, the resolution necessary to solve such questions is not obtainable by traditional morphological taxonomy. It is therefore desirable to apply other taxonomic methods, such as analyses of molecular data, to resolve the relationships among *Lithophaga* subgroups. Many studies have applied analyses of restriction fragment length polymorphism (RFLP) to resolve taxonomic and phylogenetic questions in bivalves (e.g., Skibinski, 1985; Edwards and Skibinski, 1987; Brown and Paynter, 1991), and some have used DNA sequence analysis (Geller et al., 1993).

The advantages of using ribosomal DNA (rDNA) sequences for constructing phylogenies have been reviewed by Woese (1987) and Hillis and Dixon (1991). These advantages include the substantial differences in the rate of evolution among different regions of rDNA, enabling the inference of phylogenetic history across a very broad spectrum, from studies dealing with very ancient lineages to studies aimed at resolving relationships among closely related species and populations (Hillis and Dixon, 1991). Whereas analysis of sequences of nuclear

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18S rDNA was used to infer phylogenetic divergences older than 500 million years (Field et al., 1988; Stock and Whitt, 1992), 12S and 16S mitochondrial (mt) rDNA were used for resolution of closely related taxa (Geller et al., 1993; Milinkovitch et al., 1993).

We analyzed 12S mt rDNA sequence variations in order to (1) differentiate between subgroups of *Lithophaga* bivalves (including subgroups within the same species) inhabiting different coral hosts, and (2) determine the degree of coral host specificity of these boring bivalves.

Results and Discussion

An approximately 450-bp fragment of the 12S mitochondrial rDNA (small subunit) was amplified by the polymerase chain reaction (PCR) from *Lithophaga* bivalves extracted from five Red Sea coral species (a total of 13 individuals). A 399-bp fragment was unambiguously aligned among all sequenced individuals (Figure 1) and the 12S mt rDNA from *Mytilus edulis* (Hoffmann et al., 1992). Within each of four of the *Lithophaga* subgroups, all sequenced individuals had *identical* sequences: *L. simplex* from *Astreopora myriophthalma* and *Goniastrea pectinata* (referred to in the following as *L. simplex* [A] and *L. simplex* [G]), *L. purpurea* from *Montipora erythraea* (hereafter termed *L. purpurea* [M]), and *L. lessepsiana*. In the fifth population, *L. purpurea* from *Cyphastrea chalcidicum* (2 individuals), 2 distinct haplotypes were revealed (*L. purpurea* [C]1 and [C]2). All six sequences will appear in the EMBL, GenBank, and

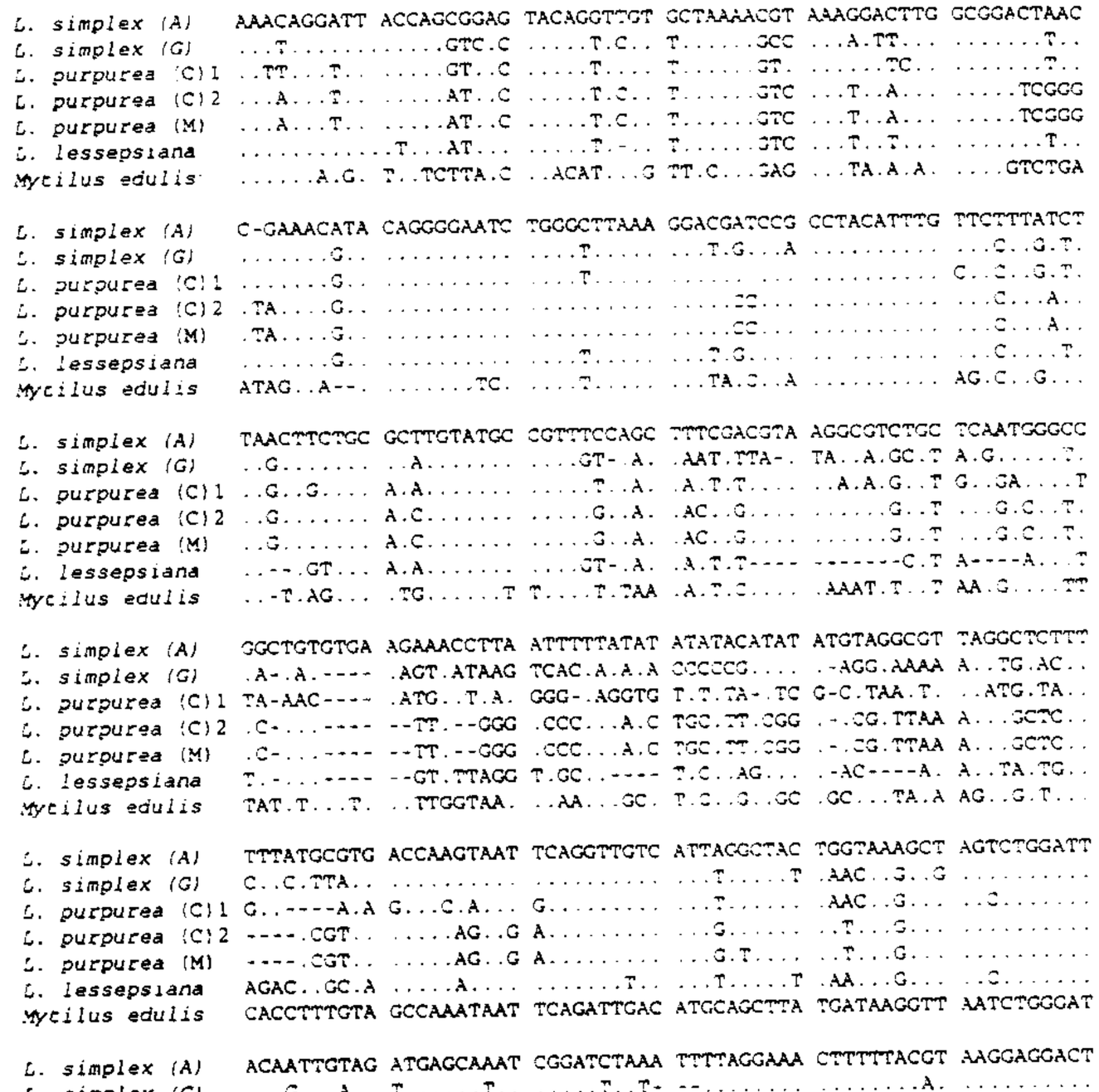

Figure 1. Partial sequence of the 12S mt rDNA from several coral boring *Lithophaga* bivalves, aligned with the homologous sequence from *Mytilus edulis* (Hoffmann, 1992). Names of bivalve species inhabiting more than one coral are followed by the host's initial in parentheses (A = *Astreopora myriophthalma*; G = *Goniastrea pectinata*; C = *Cyphastrea chalcidicum*; M = *Montipora erythraea*). The sequences of *L. simplex* (A), *L. simplex* (G), and *L. purpurea* (M) represent three individuals each. The genotype of *L. lessepsiana* includes two individuals. Each of the two genotypes of *L. purpurea* (C) represents one individual. A dot in a sequence indicates that the nucleotide in this position is the same as in the *L. simplex* (A) sequence.

DDBJ Nucleotide Sequence Databases under accession numbers X75527 through X75532.

Analysis of the whole data set, including all five *Lithophaga* subgroups with *Mytilus* (a bivalve from the same family, Mytilidae) as an outgroup, was performed both including and excluding gaps. One most parsimonious tree was found when gaps were included (Figure 2A), based on a total of 232 variable sites. According to this tree, *L. simplex* (G) clusters more closely with *L. lessepsiana* than with *L. simplex* (A). Thus, the currently defined species *L. simplex*, which inhabits both *A. myriophthalma* and *G. pectinata* corals, may be paraphyletic. *L. purpurea* may also be paraphyletic, because *L. purpurea* (C)1 branches off much lower in the tree than *L. purpurea* (M) and *L. purpurea* (C)2. Exclusion of all gaps resulted in a 347-bp alignment with 180 variable sites, producing three most parsimonious trees (a consensus tree is shown in Figure 2B). One of the three trees was identical to the tree produced from the sequences including the gaps.

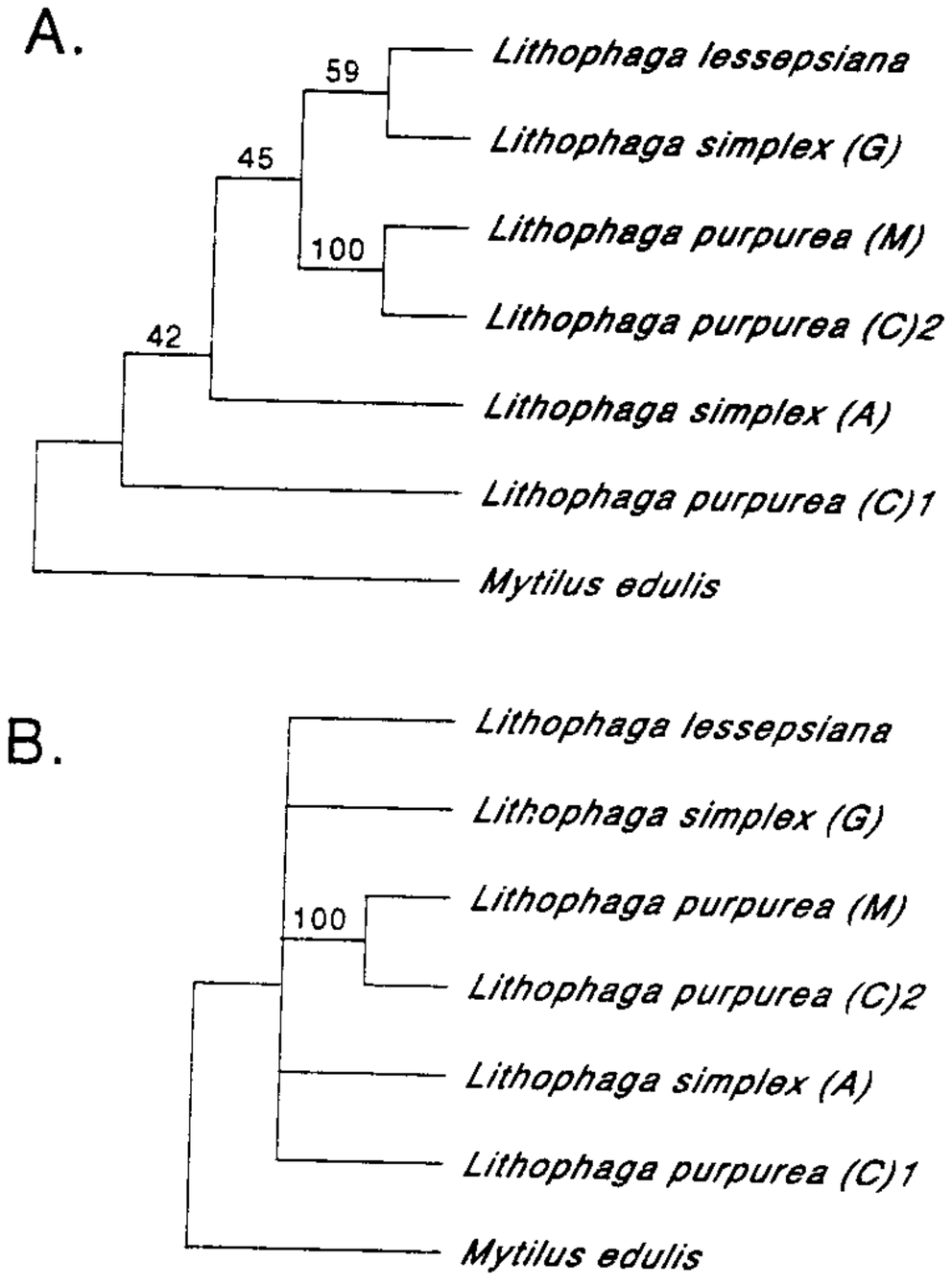

Figure 2. Phylogenetic relationships of live-coral boring *Lithophaga* bivalves, inferred from 12S mt rDNA sequences. (A) The most parsimonious tree found for gapped sequences (399 bp) by DNAPARS (PHYLIP; Felsenstein, 1989). Numbers to the left of a node indicate the percent bootstrap replicates in which the group to the right of that node occurred. (B) A consensus tree of the three most parsimonious trees found for the ungapped sequences (347 bp). Bootstrap replications supporting the nodes are shown for groups that occurred in all three trees.

Differences between coral hosts may act as a driving force for speciation of cryptobionts such as boring bivalves (Wilson, 1979). Brickner et al. (1993) provide ecological, biochemical, and SEM evidence suggesting that *L. purpurea* inhabiting the corals *C. chalcidicum* and *M. erythraea* should be considered two distinct species. The differences they report include presence/absence of 10 μm high denticles on the postlarval shell and slightly larger adult dimensions for *L. purpurea* (M). In the case of *L. simplex*, experimental evidence suggests the same for subgroups inhabiting *A. myriophthalma* and *G. pectinata* (Mokady et al., 1992). No morphological differences between *L. simplex* subgroups have been reported. Both *L. purpurea* and *L. simplex* are morphologically very different from *L. lessepsiana*.

Subsequently, two separate analyses of the 12S mt rDNA sequences were performed to try and resolve the taxonomic relationships between subgroups of a given *Lithophaga* species that inhabit different coral hosts. Each species in question (either *L. purpurea* or *L. simplex*) was subjected to analysis together with *L. lessepsiana*, with *Mytilus edulis* as an outgroup.

Figure 3A shows the one most parsimonious tree found in the analysis concerning *L. simplex*. Two hundred and fourteen variable sites were found in the aligned gapped sequences (400 nucleotides), and bootstrap replications (as well as neighbor-joining analysis) strongly supported a paraphyletic status for *L. simplex*. The same topology was produced when gaps were excluded from the analysis (179 variable sites of 371 nucleotides). Neighbor-joining analysis of the ungapped sequences, however, supports a different topology (Figure 3B). A total of 86 diagnostic nucleotide sites and 11 nucleotide gaps differentiate between *L. simplex* (A) and *L. simplex* (G). These findings agree well with results obtained in experiments concerning differential settlement and metamorphosis of *L. simplex* larvae on different Red Sea corals. Induction of metamorphosis for both larval subgroups, originating from adults extracted from either *A. myriophthalma* or *G. pectinata*, was higher by an order of magnitude for larvae settling on the original host coral, than on the other coral species (Mokady et al., 1992).

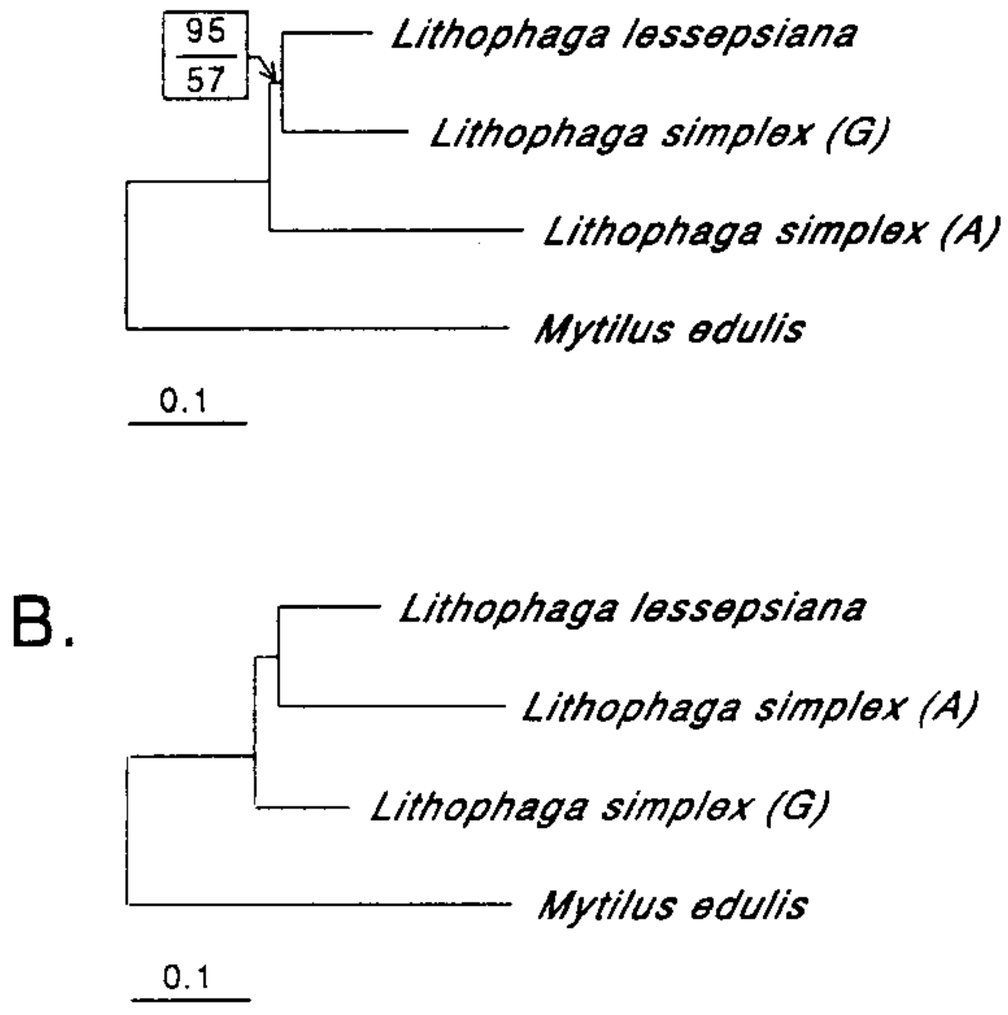

Figure 3. Phylogenetic relationships between the two subgroups of *Lithophaga simplex*, extracted from either the coral *Astreopora myriophthalma* (A) or *Goniastrea pectinata* (G). (A) The most parsimonious tree found for gapped (400 bp) and ungapped (371 bp) sequences by DNAPARS (PHYLIP; Felsenstein, 1989). Numbers to the left of a node indicate the percent bootstrap replicates in which the group to the right of that node occurred. Upper and lower values at each node refer to gapped and ungapped sequences, respectively. The same topology was supported by the neighbor-joining method (NEIGH BOUR81 in PHYLIP; Felsenstein, 1989) for the gapped sequences only. (B) A tree produced by NEIGHBOUR81 for the ungapped sequences. Both trees strongly suggest a paraphyletic status for *Lithophaga simplex*. Lengths of horizontal branches represent the proportion of sequence divergence, according to the scale bar below each figure.

A single most parsimonious tree was also found in the analysis concerning *L. purpurea* (Figure 4). The same tree topology was supported by all analyses, including bootstrap replications and neighbor-joining of sequences with gaps (210 variable sites, 394 nucleotides) or without gaps (165 variable sites, 350 nucleotides). In this case, one of the genotypes of *L. purpurea* (C) clustered with *L. purpurea* (M), whereas the other clustered with *L. lessepsiana*, suggesting considerable heterogeneity within this bivalve species. A total of 87 diagnostic nucleotide sites and 18 nucleotide gaps differentiate between *L. purpurea* (C)1 and the other haplotypes.

The analyses performed after partial exclusion of haplotypes from the database are presented for demonstrative purposes only. Following exclusion, the ingroup consisted of only three and four haplotypes in the analysis concerning *L. simplex* and *L. purpurea*, respectively. The results demonstrate the high extent of sequence variation between members of the same species inhabiting different coral hosts (nearly the same as between recognized species). Tree topologies suggest that both *L. simplex* and *L. purpurea* are each, in fact, paraphyletic. However, because of the small number of haplotypes considered as ingroup and the distance of the outgroup, these results may be regarded as only suggestive for phylogenetic interpretation. In the case of *L. purpurea*, the relatively low bootstrap support further disables phylogenetic conclusions.

Recently, Geller et al. (1993) discussed interspecific and intrapopulation variation in *Mytilus* sp. based on evidence from 16S mt rDNA. The results obtained in the present study are comparable to their findings regarding *M. trossulus*. In both cases, the bivalves showing the differences were sampled within the same geographic area (*M. trossulus* sampled from Tillamook Bay, and *L.*

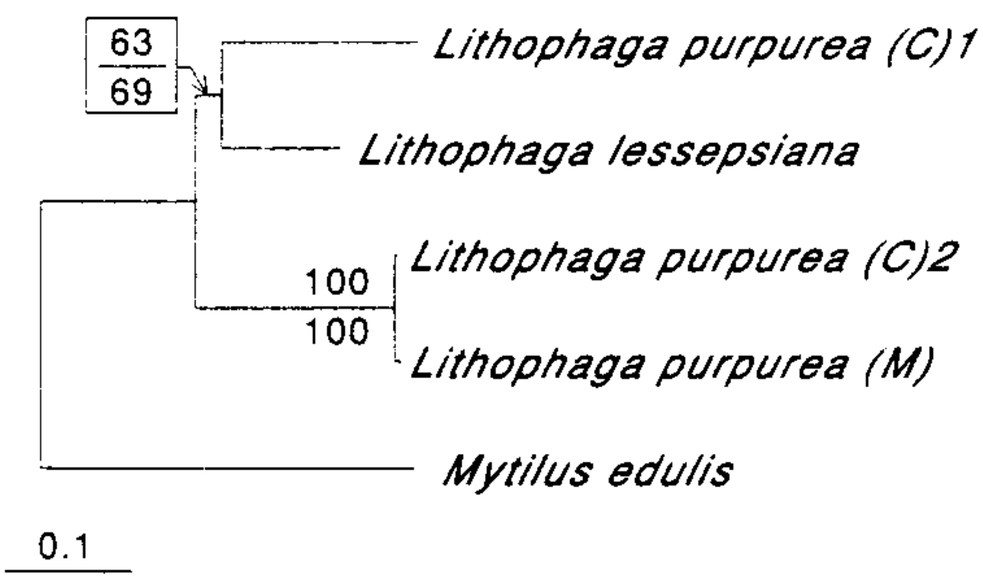

Figure 4. Phylogenetic relationships between the two subgroups of *Lithophaga purpurea*, extracted from either the coral *Cyphastrea chalcidicum* ([C]1 and [C]2) or *Montipora erythraea* (M). One most parsimonious tree was found for gapped (394 bp) and ungapped (350 bp) sequences by DNAPARS (PHYLIP; Felsenstein, 1989). Numbers to the left of a node indicate the percent bootstrap replicates in which the group to the right of that node occurred. Upper and lower values at each node refer to gapped and ungapped sequences, respectively. The same topology was supported by NEIGHBOUR81 (PHY LIP; Felsenstein, 1989). Lengths of horizontal branches represent the proportion of sequence divergence, according to the scale bar below. This tree strongly supports a paraphyletic status for *Lithophaga purpurea*.

simplex or *L. purpurea* from the coral reefs at the northern tip of the Gulf of Eilat). All *Lithophaga* participating in our study were sampled within an approximately 200-m long section of the reef, at approximately the same depth. Because these species reproduce by spawning and their dispersal ranges greatly exceed this distance, it is obvious that geographic variation has no role within the observed differences. Moreover, whereas different *M. trossulus* haplotypes differ in approximately 10% of their sequence (Geller et al., 1993), *L. purpurea* or *L. simplex* inhabiting different coral hosts differ in as much as 30 to 32%. The data regarding specific metamorphic induction in *L. simplex* larvae, by each of the two coral hosts (Mokady et al., 1992), may couple these large differences with adaptation to the host coral.

Finally, these results should be interpreted with the evidence from settlement and metamorphosis experiments (Mokady et al., 1991, 1992), larval development (Mokady et al., 1993), ecological, biochemical, and SEM analyses (Brickner et al., 1993). The emerging picture is that of near-absolute species specificity between boring bivalves of the genus *Lithophaga* and their coral hosts, emphasizing the potential importance of adaptation to a host as a driving force for speciation.

Materials and Methods

Sample collection

Lithophaga bivalves were collected in an approximately 200-m long section of the coral reef of Eilat, Red Sea, Israel. Individual bivalves were collected from subgroups of *Lithophaga* defined according to their host-coral species. Five subgroups, belonging to three currently defined species, were recognized: *L. simplex* boring in the corals *Astreopora myriophthalma* or *Goniastrea pectinata*; *L. purpurea* from the corals *Montipora erythraea* or *Cyphastrea chalcidicum*; and *L. lessepsiana* from the coral *Stylophora pistillata*. Coral colonies showing the figure-eight–shaped apertures typical of burrowing *Lithophaga* bivalves were opened with the aid of a hammer and a chisel. Unharmed bivalves were carefully collected from their burrows. Extreme care was taken to assure minimal damage to the coral colony. The minimal distance between sampled coral colonies of each species was 50 m. Coral species selection was based on existing knowledge regarding the distribution of *Lithophaga* bivalves in Red Sea corals (Table 1) (Loya, unpublished data). The number of bivalves collected from each subgroup and the number of colonies from which they were collected is also indicated in Table 1. Bivalves were kept alive until DNA extraction, which was performed within two to three days.

DNA preparation

Total cellular DNA was prepared by homogenization of the whole soft tissue of individual bivalves in lysis buffer (10 mmol/L TRIS-HCl [pH 8.0], 100 mmol/L NaCl, 20 mmol/L EDTA, 0.5% Lauryl Sarcosine), followed by one hour of digestion in proteinase K (25–50 μg/mL) at 55°C. Nucleic acids were precipitated overnight with 2 volumes of 100% ethanol and 0.1 volume 3 mol/L sodium acetate at −20°C. The pelleted nucleic acids were washed in 100% ethanol, dried, and resuspended in 100 μL H_2O.

DNA amplification

PCR was employed to amplify 12S mt rDNA using primers modified from Kocher et al. (1986) accord-

Table 1. *Lithophaga* bivalves and their coral hosts in the Red Sea.

Lithophaga species and subgroup[a]	No. individuals sampled	Coral host sampled in this study	No. host colonies sampled	Other known hosts[b]
L. simplex (A)	3	*Astreopora myriophthalma*	2	
L. simplex (G)	3	*Goniastrea pectinata*	2	
L. purpurea (M)	3	*Montipora erythraea*	3	*Montipora lobulata* *M. tuberculosa* *Echinopora gemmacea*
L. purpurea (C)	2	*Cyphastrea chalcidicum*	2	*Cyphastrea microphthalma*
L. lessepsiana	2	*Stylophora pistillata*	1	

[a]Subgroups defined according to host-coral species.

[b]These hosts are much less commonly inhabitted by *Lithophaga* bivalves.

ing to the mtDNA sequence of *Mytilus edulis* (Hoffmann et al., 1992): 5′-GAAACCAGGATTA GATACCC, 5′-TTTCCCGCGAGCGACGGGCG. The reaction buffer consisted of 10 mmol/L TRIS-HCl (pH 9.0), 50 mmol/L KCl, 0.1% Triton X-100, and 3.5 mmol/L $MgCl_2$; 55 pmol each primer were added for each reaction, along with 2.5 U *Taq* DNA polymerase (Promega, Madison, WI), 300 μmol/L each dNTP, and 1 μL template DNA solution, in a total volume of 100 μL. The PCR cycle consisted of two minutes denaturation at 92°C, two minutes annealing at 54°C, and three minutes elongation at 72°C. This cycle was repeated 29 times, with a final cycle, in which the elongation time was extended to 10 minutes.

Sequencing

PCR products of the correct size (approximately 450 bp, which in most cases was the only visible band) were cut out of 0.8% agarose gels and purified using JETSORB and the manufacturer's protocol (GENOMED). The PCR product was resuspended in 25 μL H_2O; 100 nmol DNA (typically 6–9 μL purified DNA solution) were separately mixed with 10 pmol each primer in a total volume of 10 μL for bidirectional sequencing. Sequences were determined with an automatic sequencer (Applied Biosystems 373A; Milinkovitch et al., 1993).

Sequence alignment and phylogenetic analyses

Sequences were aligned using CLUSTAL V (Higgins et al., 1992). Aligned sequences were analyzed by the following programs, which are part of the PHYLIP 3.4.1 software package (Felsenstein, 1989). DNAPARS was used to find the most parsimonious trees, supported by bootstrap replications (DNA BOOT, 100 replications). Distance matrices of the sequences were produced by DNADIST81, and analyzed by NEIGHBOUR81 (neighbor-joining).

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