I believe that, with the whole genome sequence information for *Halobacterium* species NRC-1 now available (14), the novel protocol described here will be a useful alternative to the existing protocol and its modifications.

REFERENCES


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PCR Primers and Conditions for Non-Marine Ostracods

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Non-marine ostracods, small bi-valved crustaceans, are increasingly used as model organisms for evolutionary studies (3). This is the case for three main reasons. First, ostracods have an excellent fossil record (6) that allows fine-tuned calibration of molecular clocks. Second, the group shows a high frequency of asexual reproduction (4). Third, the ostracod family Darwinulidae is one of the two putative ancient assexual groups in the animal kingdom (7). All of this makes non-marine ostracods particularly interesting to study the “queen of evolutionary problems”, the benefit of sexual reproduction (2,9).

This avenue of evolutionary research requires DNA sequence data, on which phylogenetic reconstructions and estimates of genetic diversities can be based. Although PCR-based techniques are now widely used, few known primers will allow cross-species amplification in ostracods because such primers were mostly derived from distantly related invertebrates, such as *Drosophila*. Specific primers for ostracods are thus far published for two species only (11, 12). Furthermore, these belong to two lineages that have been separate for several hundred million years.

Owing to the small size of non-marine ostracods, the amplification of multi-copy regions facilitates both PCR amplification and subsequent cycle sequencing. Such regions can be found in nuclear and mitochondrial DNA. Both genomes are further known to evolve at different rates (8), and some evolutionary questions such as the origin of asexual clones (11) require nuclear and mitochondrial sequence data. Table 1 therefore includes primers that either amplify part of the nuclear ITS1 region or of the mitochondrial COI or 16S genes. Universal primers are used successfully (5,14, Table 1b), and the obtained DNA sequence data form the base for the design of specific primers. Such specific primers have been found
to significantly improve the success rate of PCR and subsequent automatic sequencing. ITS1 primers (Table 1a) have been designed for *Penthesilenula brasiliensis* of the family Darwinulidae and for *Cytherissa sp.* of the Cytheri-
<table>
<thead>
<tr>
<th>Primer</th>
<th>Locus</th>
<th>GenBank Accession No.</th>
<th>Organism</th>
<th>Sequence (5'→3')</th>
<th>PCR Conditions</th>
<th>Size of PCR Product (bp)</th>
<th>Accession No. of Submitted Sequence</th>
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</thead>
<tbody>
<tr>
<td>Adh</td>
<td>Alcohol Dehydrogenase</td>
<td>AF045113</td>
<td>Drosophila saltans</td>
<td>CCCTATGATGT GACGTCGCC</td>
<td>45°C</td>
<td>279</td>
<td>AJ319728</td>
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<td>Adh</td>
<td>Alcohol Dehydrogenase</td>
<td>AF067280</td>
<td>Drosophila affinis</td>
<td>CATTTGAAACAG TACTGAAACCA</td>
<td>2 mM MgCl₂</td>
<td>(Darwinula stevenson)</td>
<td></td>
</tr>
<tr>
<td>AdhEX</td>
<td></td>
<td>AF059887</td>
<td>Chymomyza (Diptera)</td>
<td>TACTCTGTAAA ATGGCCAGTT</td>
<td>Tag DNA polymerase (Promega)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cad</td>
<td>Calmodulin</td>
<td>X05948- X05951</td>
<td>Drosophila melanogaster</td>
<td>TGCCAAAATTA AACCCTATAT</td>
<td>42°C</td>
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<tr>
<td>Cad</td>
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<td>X05948- X05951</td>
<td>Drosophila melanogaster</td>
<td>AAAAAAATATA CATGTTCTCC</td>
<td>Tag DNA polymerase (Promega)</td>
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<td></td>
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<tr>
<td>Agk</td>
<td>Arginine Kinase</td>
<td>U09809</td>
<td>Limulus polyphemus (Chelicera)</td>
<td>GCTGGCTTCA AAGAACTTGA</td>
<td>45°C</td>
<td>2 mM MgCl₂</td>
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<tr>
<td>Agk</td>
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<td>AL253904</td>
<td>Penaeus monodon (Malacostraca)</td>
<td>TGCTTTCTATC AGGAAATTTAG</td>
<td>Tag DNA polymerase</td>
<td></td>
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<td>CYC1</td>
<td>Cytochrome Oxidase</td>
<td>AF091461-AF091462</td>
<td>Tigriopus californicus (Copepoda)</td>
<td>GGCAAGAAGAT CTTTGCTAGAA</td>
<td>45°C</td>
<td>288</td>
<td>AJ319731</td>
</tr>
<tr>
<td>CYC1</td>
<td>Cytochrome Oxidase</td>
<td>M11382</td>
<td>Manduca sexta (Hexapoda)</td>
<td>TACGGAAAGT GGTGACTTGA</td>
<td>Tag DNA polymerase Progene (Techne)</td>
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<td>Pgd</td>
<td>Phosphoglucomutase</td>
<td>M80598</td>
<td>Drosophila melanogaster</td>
<td>TAATTGGTCAA GGATCGGCG</td>
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<tr>
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<td>S67873</td>
<td>Ceratitis capitata (Diptera)</td>
<td>TCCCTGACCTG TGCCACCTG</td>
<td>2 mM MgCl₂</td>
<td>(Darwinula stevenson)</td>
<td></td>
</tr>
<tr>
<td>TubHA</td>
<td>Tubulin</td>
<td>H41810</td>
<td>Homarus americanus</td>
<td>TTACAATCACAG ACCGCGCAGT GTTGACCTCG CCTGTAAC</td>
<td>45°C</td>
<td>291</td>
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<tr>
<td>TubHA</td>
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<td>Homarus americanus</td>
<td>TTACAATCACAG ACCGCGCAGT GTTGACCTCG CCTGTAAC</td>
<td>HotStar</td>
<td>Progene</td>
<td></td>
</tr>
</tbody>
</table>

*Note: All primers are designed for PCR amplification and sequencing.*
of amplification products with universal ITS1 and ITS4 primers (14) and then provide a nested set of primers.

In a similar way as for ITS, primers have been developed for the mitochondrial COI region of *P. brasiliensis* and *Cyprideis* sp.; the latter species belongs to the same subfamily as *Cytherissa*.

Nested primers for automatic sequencing of the reverse direction have further been designed for two endemic species from Lake Baikal (*Cytherissa elongata* and *Cytherissa donquisotet*). These primers also generate high-quality sequences from other species of the same genus. Based on *Drosophila* sequences from GenBank® (Table 1b), a PCR primer pair for the mitochondrial 16S region has been designed and successfully applied to non-marine ostracods. Again, a nested primer for automatic sequencing, this time for the forward direction, has substantially improved the quality of sequences from the genera *Cytherissa* and *Cyprideis*.

All three multi-copy regions are especially suitable to study genetic variability within or between species, although the observed variability might vary considerably between different lineages of non-marine ostracods. For example, populations of *Darwinula stevensoni* from different continents differed only about 2.2% in COI (12), whereas a maximum of 20.9% was reported between *Eucypris virens* populations within Europe (11).

Single-copy genes from non-marine ostracods are much harder to amplify, probably because of the small amount of template. Nevertheless, their sequences can provide important data, especially for the calibration of molecular clocks or for estimating substitution rates between homologue alleles. Table 2a gives an overview of primers from six different nuclear genes, which have been designed from GenBank sequences and tested on non-marine ostracods. Based on the information available from GenBank, these primers are designed for exon regions that might flank introns, as in the case of Alcohol Dehydrogenase and Calmodulin. Primer design was independently from Reference 10 because these published primers have not been suitable for non-marine ostracods. The primers from Table 2a have been based on sequences from distantly related arthropods, which is why they are equally suitable for other crustaceans such as *Daphnia*, *Artemia*, etc. Because these nuclear genes evolve relatively slowly and code for proteins, they could also offer important, suitable new genetic tools, for example, to reconstruct phylogenies on higher taxonomic levels.

Table 2b summarizes PCR primers that are designed for three nuclear genes of the species *D. stevensoni*. Although these primers only amplify part of each gene, they significantly increase the overall success rate of PCR. They might furthermore not only be suitable for *D. stevensoni* but also for other non-marine ostracod species. Amplifications with primer sets from Table 2a and b, only yield single PCR products; thus, no evidence for pseudogenes or gene duplications has been found.

DNA extractions from single individuals are conducted with a slightly modified protocol of the Chelex® method (13). If not specifically mentioned otherwise, PCRs are carried out in 25-mL volumes with the model 480 DNA thermal cycler (Applied Biosystems, Foster City, CA, USA). Standard
conditions include 0.5 U Taq DNA polymerase (Amersham Pharmacia Biotech, Piscataway, NJ, USA) with the provided buffer, 1.5 mM MgCl₂, 10 mM dNTPs (Promega, Madison, WI, USA), 10 pmol each primer and mineral oil (Sigma, St. Louis, MO, USA). A hot start (denaturation step of 5 min without Taq DNA polymerase before the cycles) is used at all times to increase specificity of the PCR. Modifications of this protocol and the appropriate annealing temperatures are given in Tables 1 and 2. PCR products of multi-copy regions are sequenced directly. PCR products of two successful clones are sequenced directly. PCR products of appropriate annealing temperatures are given in Tables 1 and 2. PCR products of multi-copy regions are sequenced directly. PCR products of two successful clones are sequenced directly. PCR products of appropriate annealing temperatures are given in Tables 1 and 2. PCR products of multi-copy regions are sequenced directly. PCR products of two successful clones are sequenced directly. PCR products of appropriate annealing temperatures are given in Tables 1 and 2.

Sequences are automatically processed following the manufacturer’s protocol. Positive clones are screened by PCR for insert size, using M13 Forward and Reverse primers, standard PCR conditions, and the following program: 3 min at 95°C and 30 cycles of 50 s at 95°C, 50 s at 55°C, and 2 min at 72°C in a Genius Thermal Cycler (Techne, Princeton, NJ, USA) thermal cycler. Mini-preps of positive clones are conducted with the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA) and automatically sequenced in both directions on ABI Prism® 3700 or ABI Prism® 3700 DNA analyzers with M13 primers and the BigDye™ Terminator Kit (Applied Biosystems), following the manufacturer’s protocol. Sequences are automatically processed with Chromas 1.45 and are also checked manually.

The identity of all sequences is verified by BLAST search (1) in GenBank. One sequence of each amplification product has been submitted to GenBank accession nos. AJ319728-AJ319744 (see also Tables 1 and 2).

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