



A molecular method for the detection of sally lightfoot crab larvae (*Grapsus grapsus*, Brachyura, Grapsidae) in plankton samples

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Abstract

The decapod *Grapsus grapsus* is commonly found on oceanic islands of the Pacific and Atlantic coasts of the Americas. In this study, a simple, quick and reliable method for detecting its larvae in plankton samples is described, which makes it ideal for large-scale studies of larval dispersal patterns in the species.

Key words: dispersal, aratu, sayapa, molecular marker.

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Grapsus grapsus (Linnaeus, 1758) (Brachyura: Grapsidae), commonly known as the “sally lightfoot” crab in English, “aratu” in Portuguese and “sayapa” in Spanish, occurs on the eastern Pacific continental coast and islands from Baja California to northern Chile, as well as in large numbers on the Galapagos Islands. In the Atlantic, although absent along the coast, it occurs on the Caribbean Islands, and very abundantly on the western oceanic islands, viz., Saint Paul’s Rocks, the Rocas Atoll, Fernando de Noronha and Trindade. According to Hartnoll (2009), due to the occurrence of *Grapsus adscensionis* (Osbeck, 1765) on various East Atlantic islands, and along much of the West African coast, the status of the crabs from Trinidad Island is yet to be defined. As morphological differences are very slight (Manning and Chace Jr, 1990), according to Ratti (PhD thesis, Universidade de São Paulo, 2004), there is no justification in considering the two as distinct species (but see Guerao *et al.*, 2001). Although available information on *G. grapsus* is scarce, its reproductive biology was the object of a recent study by Freire *et al.* (2011). Phenotypic plasticity is manifest, both in the pronounced carapace coloration pattern of *G. grapsus*; the red color, an important factor in mating also being associated with aggressive intra-specific behaviour, as well as the larger size of the males at sexual maturity.

The occurrence of *G. grapsus* on several remote oceanic islands poses intriguing questions about the demographic and genetic relationships within the species. Given the involved distances, one would expect pronounced ge-

netic differentiation and little recruitment from alternative locations. For instance, plankton sampling using conventional methods clearly revealed *G. grapsus* larvae retention in the vicinity of Saint Paul’s Rocks (Koettker *et al.*, 2009). Nevertheless, external morphology among populations appears to be very similar, thereby suggesting some level of gene flow between them. Elucidating these questions necessarily requires understanding the larval biology of *G. grapsus*, especially as to timing and the distances that its larvae are capable of travelling. Traditionally, brachyuran larvae are studied by screening plankton samples for the presence of those of interest by stereoscopic microscopy. However, this approach is time-consuming and often inaccurate (Boeger *et al.*, 2007). Methods based on molecular markers have brought important new tools to the study of the biology of planktonic larvae by facilitating their precise and rapid detection (Pie *et al.*, 2006; Boeger *et al.*, 2007; Pardo *et al.*, 2009). Herein, we developed a set of specific primers for identifying *G. grapsus* larvae in plankton samples, whereby, irrespective of the presence of the DNA of various species in a given reaction, the designed primers will only anneal to target-species DNA.

A 803 bp fragment of the mitochondrial control region of *G. grapsus* (GenBank accession number JF444484) was sequenced from specimens collected at the Fernando de Noronha archipelago (Brazil, 51°30’ N, 0°7’ W). Total genomic DNA was extracted from a muscle sample using the EZ-DNA kit (Biosystems, Brazil), according to manufacturer’s instructions. The fragment was amplified using primers dlussaf1 (5’-GTATAACCGCGAATGCTGGCA C-3’) and ileucar2 (5’-CCTTTTAAATCAGGCACTATA -3’) (Oliveira-Neto *et al.*, 2007), with the following PCR protocol: 5 min at 95 °C, 35 cycles of 92 °C for 30 s,

48-51 °C for 30 s and 68 °C for 30 s, followed by final extension at 68 °C for 2 min. Reactions were done in 25 µL reactions with 1.25 units of AmpliTaq DNA polymerase, 1X PCR buffer, 2 mM of MgCl₂, 0.4 mM of dNTPs and 0.5-1 µM of each primer. Unique regions were identified by comparing the *G. grapsus* sequence with the same region in two other brachyuran species: *Ucides cordatus* (Linnaeus, 1763) (Ocypodidae) and *Cardisoma guanhumi* Latreille, 1825 (Gecarcinidae), to date the only closely related species in which sequencing had been undertaken (Pie *et al.*, 2008). The lack of similarity furnished ample proof of propriety for developing species-specific primers. Of the four thus developed, the best set in terms of sensitivity and accuracy was used in further analyses, namely Dlussaf1 (5'-GTATAACCGCGAATGCTGGCAC-3') and Grapsus.R1 (5'-CCCTTCTCTTTTCTTTGGGATG-3'), thereby generating a 401 bp fragment. In addition, a second primer pair was used in the same reaction to amplify a ≈ 540-bp region of the 16S ribosomal gene to serve as positive control (16SL, 5'-CGCCTGTTTATCAAAAACAT-3' and 16SH, 5'-CCGGTCTGAACTCAGATCACGT-3') (Palumbi *et al.*, 1991). Thus, given that 16S primers are universal, PCR amplification of a sample of plankton that lacked *G. grapsus* would only generate the 16S band, and not the specific. The PCR protocol to obtain specific bands consisted of 4 min at 94 °C, 32 cycles of 94 °C for 30 s, 58.2 °C for 30 s and 70 °C for 60 s, followed by final extension at 70 °C for 3 min, all done in 25 µL reactions with 0.625 units of AmpliTaq DNA polymerase, 1X PCR buffer, 1.5 mM of MgCl₂, and 0.5 mM of dNTPs. Primer concentrations for the 16S and control region primers were 0.8 and 4 µM, respectively. PCR products were electrophoresed on 2% agarose gels. Specificity tests were undertaken using four closely related crab species: *Goniopsis cruentata* (Latreille, 1803) (Grapsidae), *Ocypode quadrata* (Fabricius, 1787) (Ocypodidae), *Cardisoma guanhumi* (Grapsoidae, Gecarcinidae), and *Ucides cordatus* (Ocypodidae). In addition, sensitivity tests were carried out with increasingly smaller concentrations of template DNA (300 ng, 30 ng, 3 ng, 0.3, 0.03 ng) to determine detection limits of the method.

The very sensitive method thus developed was capable of detecting DNA amounts as low as 0.03 ng (Figure 1). Notably, an extraction from a single larva of a planktonic species often provides twice as much DNA (*e.g.* Pie *et al.*, 2006). Moreover, in amplifications using the specific primers, the specific band was consistently detected in *G. grapsus* samples, but not so when using DNA from other species (Figure. 2). DNA performance was similar in additional tests (not shown) using known concentrations of environmental plankton samples and increasingly smaller amounts of *G. grapsus*, a sure indication of the utility of this primer for investigating patterns of larval occurrence of *G. grapsus* in the field. Moreover, the combination of extensive field collections and automated procedures for DNA extraction and amplification, is a potential means of obtain-

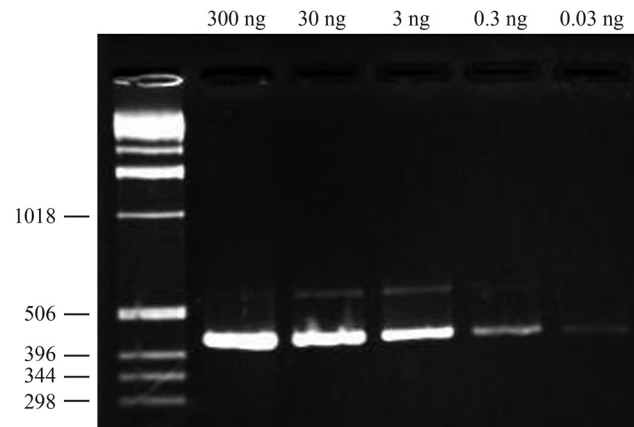


Figure 1 - Sensitivity tests of the molecular protocol developed in the present study to detect larvae of *Grapsus grapsus*, including the amplification of a ≈ 540-bp region in the 16S ribosomal gene that served as a positive control, and the 401 bp fragment corresponding to the specific marker. Each lane received increasingly lower concentrations of template DNA from 300 ng to 0.03 ng. See text for details.

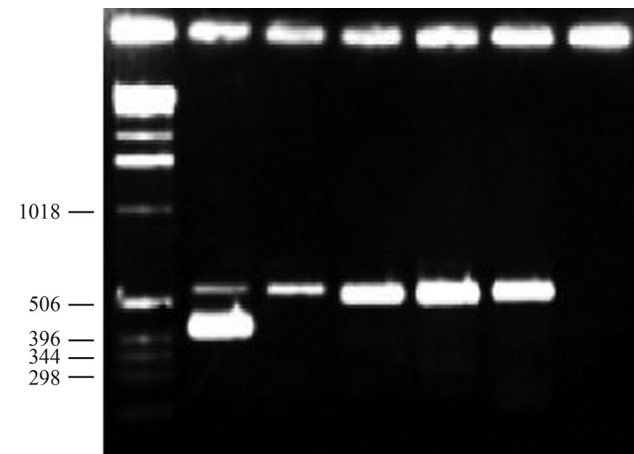


Figure 2 - Specificity tests of the molecular protocol developed in the present study to detect larvae of *Grapsus grapsus*, all of which including both the positive control and specific primer sets. According to the size of the marker, each lane represents the application of the molecular protocol for each species: (1) *Grapsus grapsus* (Grapsidae); (2) *Goniopsis cruentata* (Grapsidae); (3) *Ocypode quadrata* (Ocypodidae); (4) *Cardisoma guanhumi* (Gecarcinidae); (5) *Ucides cordatus* (Ocypodidae); (6) Negative control.

ing unprecedented high-resolution information on spatial and temporal variation in the abundance of *G. grapsus* larvae throughout its range.

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