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## Sexual reproduction in freshwater fairy shrimp, *Dendrocephalus brasiliensis* (Pesta, 1921) inferred by Amplified Fragment Length Polymorphism markers

Reprodução sexual do microcrustáceo de água doce, *Dendrocephalus brasiliensis* (Pesta, 1921), deduzida a partir de marcadores de Polimorfismo de Comprimento de Fragmento Amplificado

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### ABSTRACT

Fluctuations in world harvests of *Artemia* sp cysts have increased prices sharply in the past years. Several organisms have been tested as alternative sources for a total or partial replacement of *Artemia* sp, among which the microcrustacean (*Anostraca*) *Dendrocephalus brasiliensis* stands out. The objective of this study was to investigate the reproductive strategy of *D. brasiliensis* by means of AFLP markers. The distinction between sexual and parthenogenetic reproduction was conducted using 7 EcoRI and MseI primer combinations in a full-sib family composed of 13 progenies. Forty polymorphic markers were obtained, of these, 35% were from paternal origin and 65% were maternally inherited. Results showed mendelian segregation in all markers through the chi-square at  $P \leq 0.05$ . Sex changes were observed after 72 hours of grouping individuals of the same sex in a container, suggesting a possible sex density-dependent behavior. Paternal inheritance revealed that this species adopts sexual reproduction under experimental conditions.

**Key words:** Microcrustacea, AFLP, Paternal inheritance, Sexual reproduction.

### RESUMO

Há vários anos que a produção de cistos de *Artemia* sp. apresenta flutuações, o que aumenta consideravelmente o seu valor. Vários organismos têm sido testados para a substituição total ou parcial da *Artemia*, dentre os quais se destaca o microcrustáceo (*Anostraca*) *Dendrocephalus brasiliensis*. O objetivo do presente trabalho foi investigar a estratégia reprodutiva de *D. brasiliensis* por meio de marcadores de AFLP. A distinção entre uma reprodução sexuada e partenogenética foi feita a partir da genotipagem de sete combinações de primers de AFLP em uma família composta de 13 progênes. Quarenta marcadores polimórficos foram obtidos, dos quais 35% foram de origem paterna e 65%, materna. Os resultados mostraram a segregação mendeliana de todos os marcadores através do teste de qui-quadrado a  $P \leq 0.05$ . Mudanças de sexo

foram observadas após 72 horas do agrupamento de indivíduos de mesmo sexo em um recipiente, sugerindo uma possível razão sexual populacional densidade-dependente. A herança paterna mostrou que a reprodução sexual é adotada por essa espécie sob condições experimentais.

**Palavras-chave:** microcrustáceo, AFLP, herança paterna, reprodução sexual.

### INTRODUCTION

Aquaculture production plays a substantial role in global supplies of fish and fishery products, with a total production of 68.3 million tonnes in 2008 (FAO, 2010). In Brazil, aquaculture production has expanded considerably over the past decade, maintaining an annual growth rate of around 20%, with a total production of 271,695 tonnes in 2006 (IBAMA, 2008). Though, the larval nutrition of aquatic animals has proven to be a major obstacle to aquaculture development, for species such as salmon, the presence of a large yolk sac ensures the development of a digestive system able to accept formulated feeds. However, most viable species for marine aquaculture have a small yolk sac at hatching and a primitive digestive system, which limits survival (SORGELOOS et al., 1991).

With the development of commercial hatcheries in the 1990s, the use of *Artemia* sp. as a live diet for the larvae of many species has increased worldwide (LAVENS & SORGELOOS, 2000).

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However, the availability and high cost of *Artemia* cysts due to periodic fluctuations in supply have contributed to a lack of predictability with regard to production costs (D'ABRAMO et al., 2006). The cladoceran *Moina* sp. (ALAM et al., 1991; MARTÍN et al., 2006) and rotifer *Brachionus plicatilis* (LOVETT & FELDER, 1988) have been tested as replacements for *Artemia* sp. in the post-larval production of the species *Macrobrachium rosenbergii* and *Litopenaeus schmitti*. Another potential substitute is the microcrustacean (Anostraca) *Dendrocephalus brasiliensis* as a living or inert food in hatcheries for freshwater carnivorous fingerlings as well as the post-larvae of *Litopenaeus vannamei* (YFLAAR & OLIVERA, 2003).

*D. brasiliensis* displays sexual dimorphism. Females are identified by the brood pouch near the tail and flat, round-ended antennae, while males have a vertical appendix and spineless eyes (COHEN, 1995; RABET & THIÉRY, 1996). However, little is known regarding reproduction of the species beyond an indication of sexual reproduction suggested by COHEN (1995).

Molecular markers have proven to be of considerable usefulness in the investigation of genetic inheritance patterns. The PCR-based fingerprinting technique of Amplified Fragment Length Polymorphism (AFLP) is based on the detection of genomic restriction fragments without prior sequence information using a limited set of generic primers, resulting in large numbers of markers per reaction (VOS et al., 1995). AFLP have been used in *Artemia* for describing genetic diversity (TRIANAPHYLLIDIS et al., 1997; SUN et al., 1999). In the present study, the occurrence of sexual reproduction in *D. brasiliensis* was investigated in a full-sib family by AFLP markers.

## MATERIALS AND METHODS

*D. brasiliensis* cysts were obtained from the Paulo Afonso Fish Aquaculture Station (09°22'38"S and 38°13'58"W) in northeast Brazil. The cysts were maintained in a 1-L hatching container at 25°C for 24h. Sixteen pairs (A to P) were isolated soon after hatching in order to ensure at least one male and female pair. The specimens were transferred to a 1-L nylon screen container and maintained for a period of 30 days at approximately 27°C in a fertilised pond measuring 50m<sup>2</sup> and a mean depth of 0.80m. As the specimens displayed sexual dimorphism, they were placed in new 1-L nylon screen containers for effective mating and breeding under the same culture

conditions. A single pair generated progenies and tissue from the entire family was collected for DNA extraction.

As only one pair generated progenies, a second experiment was carried out indoors with 20 additional pairs of nauplii (A' to T') maintained in 30-mL glass recipients containing distilled water with a daily renewal rate of 10% and fed on the microalgae *Chlorella* sp. at a concentration of 30,000cells mL<sup>-1</sup> day<sup>-1</sup>. The water was maintained at 28°C with a 12h light dark<sup>-1</sup> photoperiod under constant aeration for a period of 35 days.

Total DNA was extracted from the tissue of the breeders and progenies. Tissues were digested in 100mM NaCl, 20mM Tris-HCl (pH 8.0), 100mM EDTA, with a final concentration of 0.05% SDS and 100µg mL<sup>-1</sup> of Proteinase K. After protein digestion, the aqueous phase was extracted once with phenol, phenol/chloroform/isoamyl alcohol and chloroform. DNA was precipitated using ethanol; pellets were washed in 70% ethanol, dried and re-suspended in TE (0.01M Tris-HCl pH 7.4, 2.5mM EDTA pH 8.0). AFLP analysis of the unique family was performed following the protocol described by VOS et al. (1995), with modifications. Genomic DNA (250ng) was digested with 5U of *EcoRI* and *MseI* prior to ligation with restriction site-specific adaptors in 50µL of final volume at 37°C for 12 hours, followed by inactivation at 70°C for 15 minutes. Next, 10µL of a solution containing 1µg of *EcoRI* and *MseI* adapters, 1X T4 DNA ligase buffer and 1U of T4 DNA ligase was added. This reaction was incubated at 20°C for three hours and stored at -20°C. Pre-amplification was carried out using adaptor-specific primers with a single selective base at the 3' termination. Pre-amplification was performed in 20µL reaction volumes with 2µL of digested and adapted ligated DNA and 25ng of each complementary primer. Amplification conditions were implemented as described by VOS et al. (1995) and the AFLP primer combinations available in the AFLP Analysis System I Kit Gibco (USA) were used. Pre-amplification products were diluted in 100µL of ultra-pure water and used as the template for selective amplification. Selective amplification employed two sets of primers, each containing three selective nucleotides at the 3' termination. For each 10µL of selective reaction, 1µL of diluted pre-amplification product and 25ng of *EcoRI*-ANN and *MseI*-CNN primers were used. Reactions were amplified as described by VOS et al. (1995). Selective PCR products were denatured and separated on polyacrylamide gel and visualised by silver staining. For the identification of sexual

reproductive strategy, 28 AFLP primer combinations were investigated (E – *EcoRI*; M – *MseI* and three selective nucleotides): E-ACC / M-CAG; E-ACC / M-CAT; E-ACC / M-CTA; E-ACC / M-CTC; E-AAG / M-CAG; E-AAG / M-CAT; E-AAG / M-CTA; E-AAG / M-CTC; E-ACA / M-CAG; E-ACA / M-CAT; E-ACA / M-CTA; E-ACA / M-CTC; E-ACT / M-CAG; E-ACT / M-CAT; E-ACT / M-CTA; E-ACT / M-CTC; E-AAC / M-CAG; E-AAC / M-CAT; E-AAC / M-CTA; E-AAC / M-CTC; E-ACG / M-CAG; E-ACG / M-CAT; E-ACG / M-CTA; E-ACG / M-CTC; E-AGG / M-CAG; E-AGG / M-CAT; E-AGG / M-CTA; E-AGG / M-CTC. All loci were assessed as dominant markers. The distinction between sexual and parthenogenetic reproduction was based on the observation of markers of exclusively paternal origin in the full-sib progenies, since parthenogenetic reproduction produces diploid offspring that are genetically identical to the mother. Maternal and paternal inheritances were counted across progenies by segregating markers, which were tested for deviations from expected 1:1 Mendelian segregation ratios using chi-square ( $\chi^2$ ) analysis ( $P < 0.05$ ).

## RESULTS AND DISCUSSION

Out of 16 pairs of nauplii in the first experiment, only one family with 13 progenies emerged. This result is associated to the high level of mortality and non-development of cysts by females. In the second experiment, the small number of females and couples as well as the absence of cysts after 29 days of culture prevented the construction of new families.

Although there are no studies on the environmental factors that influence reproduction in *D. brasiliensis*, the supply of *Chlorella* sp. may have provided a suboptimal food condition for reproduction, thereby inducing stress in the second experiment. On the other hand, a large number of pairs consisting of the same sex were observed. Thus, the decision was made to group their members into four male groups composed of four individuals each and three female groups composed of four individuals for daily observation under a microscope. Sex changes were observed over a 72-hour period. Among the four groups of males, two generated a ratio of 1♂:3♀, while all males in the remaining groups turned into females (Figure 1). Similarly, the all-female groups either produced proportions of 1♀:3♂ or the sexual change of all members into males. In both cases, there were no cysts after the sex change. These results

suggest a possible density-dependent control in this population. Further experiments using different densities should be conducted in order to determine the potential density-dependent effect on the sex ratio.

Initially, 28 *EcoRI* and *MseI* primer combinations were tested in breeders and seven were selected to investigate the Mendelian inheritance pattern in the offspring (Table 1). The chi-square test revealed that these combinations (underlined) demonstrated Mendelian segregation (1:1) in a total of 40 polymorphic loci (Table 1), among which 14 fragments were of paternal origin (35%) and 26 were of maternal origin (65%), with molecular weight ranging from 95 to 310 bp ( $P < 0.05$ ).

The distinction between sexual and parthenogenetic reproduction was based on the observation of AFLP-fragments in the full-sib progenies that were of exclusively paternal origin. In Figure 2 (A and B), the presence of marker n°1 for the combination E-ACC / M-CAT and markers numbers 1 and 2 for E-ACC / M-CTA indicates paternal inheritance in the progenies, which confirms the sexual reproduction. Paternal inheritance was also observed in the progenies for the primer combinations E-AAG / M-CAT, E-AAG / M-CTA, E-AAG / M-CTC, E-AAC / M-CTA and E-AAC / M-CTC.

According to CHARNOV (1982), the sex allocation theory predicts equal provision distribution for male and female functions in response to different conditions. This theory also suggests that sex change is favoured when the reproductive value of an individual varies with age or size, and the relationship

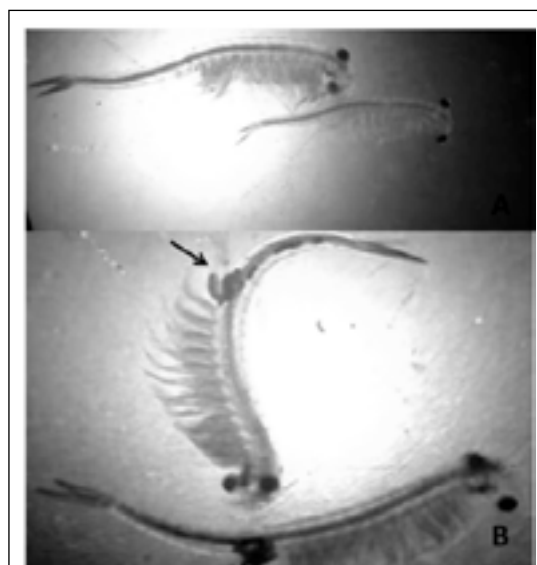


Figure 1 - Sex change of males (A) into females (B). The presence of brood pouch is indicated by arrow.

Table 1 - Number of polymorphic markers scored from AFLP analysis.

Primers <i>Eco</i> RI (E)	-----Number of markers-----			
	-----Primers <i>Mse</i> I (M) -----			
	CAG	CAT	CTA	CTC
ACC	12 <sup>a</sup>	18	15	0
AAG	15	18	14	16
ACA	0	0	11	10
ACT	14	9	11	7
AAC	5	17	19	14
ACG	0	0	0	0
AGG	3	2	0	0
	-----Number of polymorphic markers-----			
E / M	CAT	CTA	CTC	Total
ACC	8 <sup>b</sup>	4	-	12
AAG	5	8	5	18
AAC	-	9	1	10
Total	13	21	6	40

Primer combinations are denoted by the *Eco*RI - AXX and *Mse*I - CXX selective nucleotides, respectively;

<sup>a</sup>Number of markers scored per primer combination;

Underlined – AFLP primer combinations selected to investigate the Mendelian inheritance pattern in the full-sibs;

<sup>b</sup>Total number of polymorphic markers scored.

is different for males and females (ALLSOP & WEST, 2004), being common in species with separate sexes (BAEZA, 2007).

Rotifers have a life history of parthenogenetic reproduction combined with episodes of sexual reproduction (GILBERT, 1977).

During the parthenogenetic phase, which is the more common reproductive mode, females produce diploid eggs through non-meiotic parthenogenesis that produce daughters that are genetically identical to the mothers. After numerous parthenogenetic generations, sex is induced by environmental factors

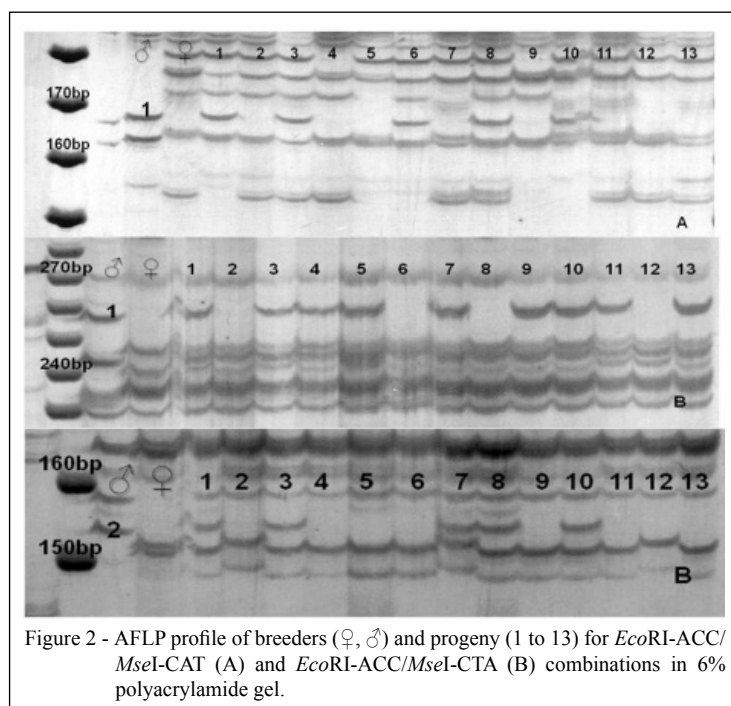


Figure 2 - AFLP profile of breeders (♀, ♂) and progeny (1 to 13) for *Eco*RI-ACC/*Mse*I-CAT (A) and *Eco*RI-ACC/*Mse*I-CTA (B) combinations in 6% polyacrylamide gel.

that can predict habitat deterioration (SERRA et al., 2004). In the case of *Brachionus plicatilis*, sexual reproduction is induced by a density-dependent chemical cue (STELZER & SNELL, 2003). The genus *Artemia* comprises a complex of sexual species defined by criteria of reproductive isolation and a large number of parthenogenetic species (VAN STAPPEN, 1996). Sexual species have been described in the Americas, Africa, Europe and Asia, suggesting that this is the most common form of reproduction, while parthenogenetic species are found only in Europe, Asia and Africa (VAN STAPPEN, 1996). The coexistence of parthenogenetic and sexed *Artemia* species in the same habitat has been reported for the Mediterranean (VAN STAPPEN, 1996).

Overall, sexual species have a large number of progenies and rapid sexual maturation, which is fundamental to population growth and colonisation in new environments where nutrients are limited (VAN STAPPEN, 1996). Moreover, sexual reproduction offers the advantage of producing recombinant types that can make the population better able to adapt to changes in the environment (CROW, 1994).

AFLP proved to be an effective technique in terms of providing a large number of polymorphic fragments without prior knowledge of DNA sequencing of *D. brasiliensis*. The presence of fragments from paternal origin in the progenies of *D. brasiliensis* confirmed sexual reproduction in this freshwater fairy shrimp. Sexual reproduction offers advantages of recombinant types and rapid sexual maturation, which are important characteristics for the large-scale production of the species. In addition, the observed sex change suggests a sexual plasticity as a response to population sex density, which is advantageous in obtaining individuals of both sexes.

## BIOETHICS AND BIOSSECURITY COMMITTEE

The Animal Ethics Commission of UFRPE ensures that the experiment on the reproductive strategy of branchoneta, *Dendrocephalus brasiliensis* (Pesta, 1921), is not an issue of ethical analysis, since this species is not classified as sentient animal, and therefore it is not regulated by Law 11.974/2008.

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