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A preliminary linkage map using spotted melanic laboratory strains of the livebearing fish *Phalloceros caudimaculatus* var. *reticulata* (Cyprinodontiformes: Poeciliidae)

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Introduction

The uniform light-grey pigmentation pattern constitutes the wildtype and most frequent phenotypic class in *Phalloceros caudimaculatus* (Hensel, 1868) a small livebearing Neotropical freshwater fish (figure 1a). An alternative chromatic variation due to the presence of cell-type specific macromelanophores, was described in *P. caudimaculatus* var. *reticulata* (Hensel, 1868). The pigmentation originating in macromelanophores is encoded by dominant genes, as it was described in other poeciliids (Schartl *et al*. 1995; Weis and Schartl 1998). DNA sequences playing a role in melanic patterns have been described in fish such as *Xiphophorus maculatus* (Weis and Schartl 1998; Schartl *et al*. 1999), *Poecilia sphenops* (Schröeder 1976) and *Poecilia formosa* (Schartl *et al*. 1995); in all cases, melanic patterns resulted from dominant allele activity, whereas the absence of macromelanophores was due to recessive ones.

In *P. caudimaculatus* this dominant gene(s) could arise from low frequency spontaneous mutation events as this variation has been only found in two females from different collecting sites in natural populations from Uruguay (figure 1b) (Azpelicueta and García 2001). A founder mutant female, presenting a melanic spotted pattern was used in breeding experiments with wildtype males from the same natural populations and new phenotypic classes were generated: T1 (wildtype pigmented); T2 (slightly spotted fish; not present in adult fish); T3 (heavily spotted fish), and T4 (highly spotted fish whose spots blend into condensed black patches). Also, comparative cytogenetic studies in these classes showed a significant association between the presence of one or two bi-armed chromosomes sharing heterochromatic blocks in the spotted phenotypic classes (Gutiérrez and García 2007).

To search for the melanic mutation causative genes, the implementation of genetic linkage map through the identification of tightly linked markers spread in a genome, allows the localization and further isolation of a gene of interest. Genetic maps constructed by random amplified polymorphic DNA (RAPD) method (Welsh and McClelland 1990; Williams *et al*. 1990) which requires no previous DNA sequence information, have been reported for many fish organisms such as *Poecilia reticulata* (Khoo *et al*. 2003) and *Astyanax mexicanus* (Borowsky and Wilkens 2002), among others.

In the present study we first attempt to construct the preliminary genetic linkage map of *P. caudimaculatus* var. *reticulatus* in laboratory strains by RAPD markers and second, to identify melanic mutation-associated markers.

Materials and methods

Phalloceros caudimaculatus strains, genetic crosses and mapping population

The samples used in the present study belong to strains obtained from the founder mutant female found in natural populations from Uruguay and wildtype males from the same population. Three strains of *P. caudimaculatus* (T1, T3 and T4) were used in laboratory crosses. The experimental population used for mapping resulted from full-sibs, half-sibs and parental–offspring breed crosses involving two kinds of crosses: parental and offspring in arbitrary crosses (AC) were anonymous fishes derived from the founder female lineage displaying uncertain level of inbreeding, whereas offspring in genetic crosses (GC) resulted from rigorous mating designs of different parents as follows: T1×T1,

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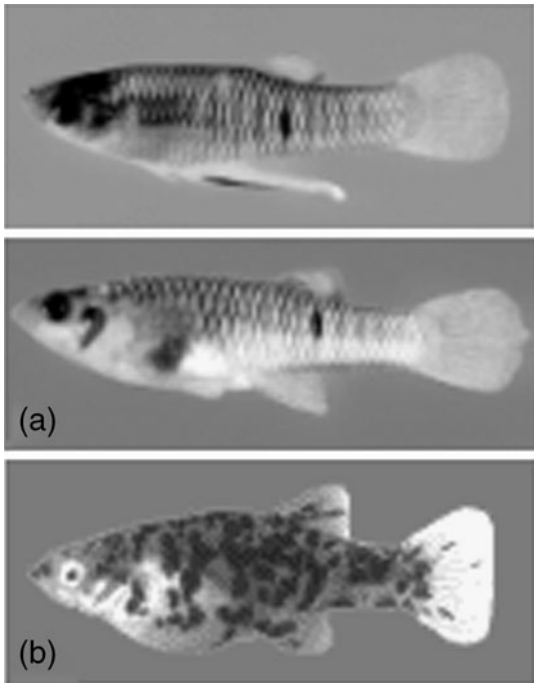

Figure 1. *Phalloceros caudimaculatus* pigmentation phenotypes. (a) wildtype pigmentation in males (above) and females (below) (http://www.fishbase.net). (b) *P. caudimaculatus* var. *reticulata* female from Bañados del Este, Uruguay.

T3×T3, T4×T4 and ♀T1×♂T3 (figure 2). Twenty-one F_1 individuals (10 showing wildtype phenotype and 11 with the melanic phenotype) from AC and 27 F_2 individuals (seven sharing wildtype phenotype and 20 with the melanic phenotype) from GC, were used. Each cross experiment was replicated twice. Data and samples of ♀T3× ♂T1 backcross were obtained from previous experimental crosses due to the offspring absence from the backcross implemented in the present study; an offspring proportion result of 1:1 wildtype/melanic was obtained in this backcross (G. García, personal observation.). Fish maintenance and offspring classification was performed as described by (Gutiérrez and García 2007). Tissues and voucher specimens are deposited in Sección Genética Evolutiva, Facultad de Ciencias, Universidad de la República, Uruguay.

Extraction, amplification of genomic DNA and visualization of amplified fragments

DNA was extracted from muscle, fin and total body (in conserved embryos) (Medrano *et al*. 1990; Westerfield 2000). For RAPD-PCR reactions, a total of 16, 10-mer primers (Operon Technologies, Huntsville, USA) (table 1) were assayed under the following conditions: total reaction volumes of 10 μL were used with final concentrations of 1.5 mM $MgCl_2$, 1.25 μM primer, 0.2 mM each dNTP, 0.5 U *Taq* DNA polymerase (Biotools, Madrid, Spain), 1 μL of 10× reaction buffer, and 1 μL of DNA sample. Amplifications were performed for 35 cycles using a PTC-100 (MJ Research, Waltham, USA) thermocycler with the following experimental parameters: an initial denaturation of 5 min at 95°C, followed by 35 cycles of 1 min at 95°C, annealing for 1 min at 36°C and elongation of 1 min at 72°C. An additional elongation period of 5 min at 72°C followed the last cycle. Amplified RAPD products were electrophoresed in 6% acrylamide gels at 140 V in 1× TBE buffer until the dye front reached the end of the gel and stained with silver nitrate (Sanguinetti *et al*. 1994). Band sizes were estimated against 1-kb DNA ladder of Promega (Madison, USA) and scored between ranges from 200 to 1080 bp for detection of presence or absence of polymorphic markers. Names of polymorphic RAPD loci were as given in table 1, followed by the molecular size (in base pairs) to the right of a decimal.

Experimental RAPD design and band analysis

At first, we screened the total amount of 16 primers against AC of *P. caudimaculatus* laboratory stains by bulked segregant analysis (BSA) (Michelmore *et al*. 1991) using separate pools of melanic and wildtype DNA ($n = 18$) (table 1). Highly reproducible and resolvable RAPD fragments were selected by comparing the oligonucleotide-specific amplification profile of the founder female which was included

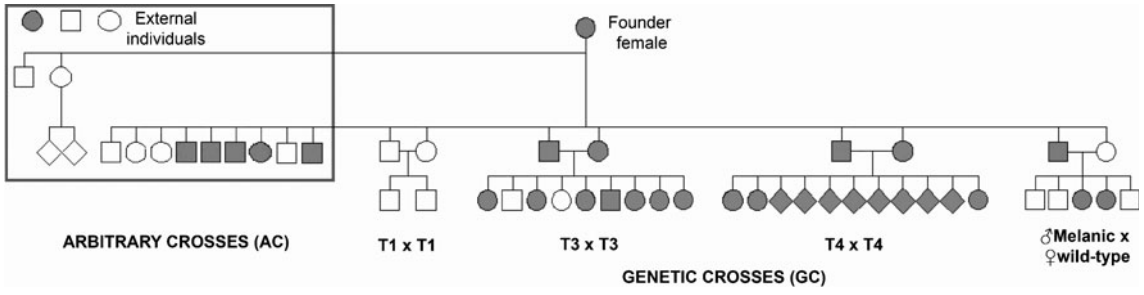

Figure 2. *Phalloceros caudimaculatus* laboratory pedigree. Melanic individuals are shown in filled figures while wildtype individuals are shown in empty figures. Square figures, males; circle figures, females; rhombus, unknown sex and phenotype (embryo individuals).

Table 1. Summary of RAPD primers and size range of markers used for linkage mapping in *P. caudimaculatus*.

RAPD primer	Nucleotide sequence (5′– 3′)	Marker size range, bp	Sample subset
OPA01	CAGGCCCTTC	201–987	AC, GC
OPA02	TGCCGAGCTG	No amplification	–
OPA03	AGTCAGCCAC	No amplification	–
OPA07	GAAACGGGTG	196–956	AC, GC
OPA08	GTGACGTAGG	220–558	AC
OPA09	GGGTAACGCC	175–977	AC, GC
OPA11	CAATCGCCGT	159–900	AC
OPA12	TCGGCGATAG	228–916	AC
OPA13	CAGCACCCAC	170–757	AC, GC
OPA16	AGCCAGCGAA	225–999	AC
OPA17	GACCGCTTGT	292–937	AC
OPA19	CAAACGTCGG	No amplification	–
OPA20	GTTGCGATCC	220–887	AC
OPE03	CCAGATGCAC	150–932	AC
OPE04	GTGACATGCC	201–1000	AC, GC
OPE06	AAGACCCCTC	201–927	AC

AC, arbitrary crosses; GC, genetic crosses.

as positive control in each RAPD experiment to ensure the reliability in markers assessment.

Five of the 16 primers assayed (OPA01, 07, 09, 13 and OPE04) which yielded phenotypic associated markers and clear banding patterns were selected for mapping. Reproducible loci amplified in AC and GC individuals by these oligonucleotides were selected to construct a corrected to reproducibility band matrix that was used to perform linkage analysis.

Analysis of RAPD loci linked to the melanic associated causative loci in *P. caudimaculatus* were implemented in separate pools of DNA from melanic and wildtype individuals from AC ($n = 18$) and were screened by BSA. Phenotypic associated RAPD markers obtained from AC were then rescreened in GC to evaluate their segregation in the laboratory crosses.

Linkage analysis

A mapping population of 46 individuals derived from the founder mutant female and belonging to AC and GC, was used for map construction. Datasets for linkage analysis were coded with Mapmanager QTX 3.0 software package (Manly *et al*. 2001) and oriented to reflect the ancestry of each parental line. For all loci scored, the approach included only loci for which one parent was scored as band-present and band-absent in the other parent.

Three alternative maps were constructed by using different sets of data: excluding and including markers and using different stringency between markers association (data not shown). In the final map (figure 3), a total of 189 loci were incorporated derived from the corrected for reproducibility matrix. The preliminary *P. caudimaculatus* genetic map was constructed using the arbitrary crosses function implemented by the Mapmanager QTX 3.0 in which populations derived by crossing any parents—at least one of which is heterozygous at some loci—are used. The minimum logarithm of the likelihood of odds (LOD) score for determining linkage was taken as 3.0, while a significant level to provide evidence of linkage of $P = 0.001$ was used. Map units used were Kosambi centimorgans (cM_K) (Kosambi 1944).

Estimation of genetic and physical lengths

The genetic length of *P. caudimaculatus* genome was estimated according to Ohtsuka *et al*. (1999). To estimate the length of 1 cM_K in kilobase pairs of DNA, the size of *P. caudimaculatus* physical map has to be compared with the length of its genetic map. The haploid genome of *P. caudimaculatus* was calculated according to Khoo *et al*. (2003). This yielded an average of 0.7×10^9 bp and allowed the length of 1 cM_K of *P. caudimaculatus* DNA to be calculated as (0.7×10^9)/genetic length of genome) in bp

Results

Primer screening and RAPD analysis

A total of 827 loci were amplified from AC experiments by using 13 of the 16 primers assayed with a 89.5% of reproducibility in the *P. caudimaculatus* genome. Five phenotype-associated RAPD markers were found by the amplification of four of these 13 primers by BSA: three of them associated to the melanic phenotype (OPA01.498, OPA07.448 and OPA07.799) and two others associated to the wildtype phenotype (OPA09.887 and OPA04.1686). RAPD amplification with OPA01, 07, 09 and OPA04 in GC experiments revealed that phenotype associated RAPD markers found in AC are not completely associated to pigmentation in line crosses (GC).

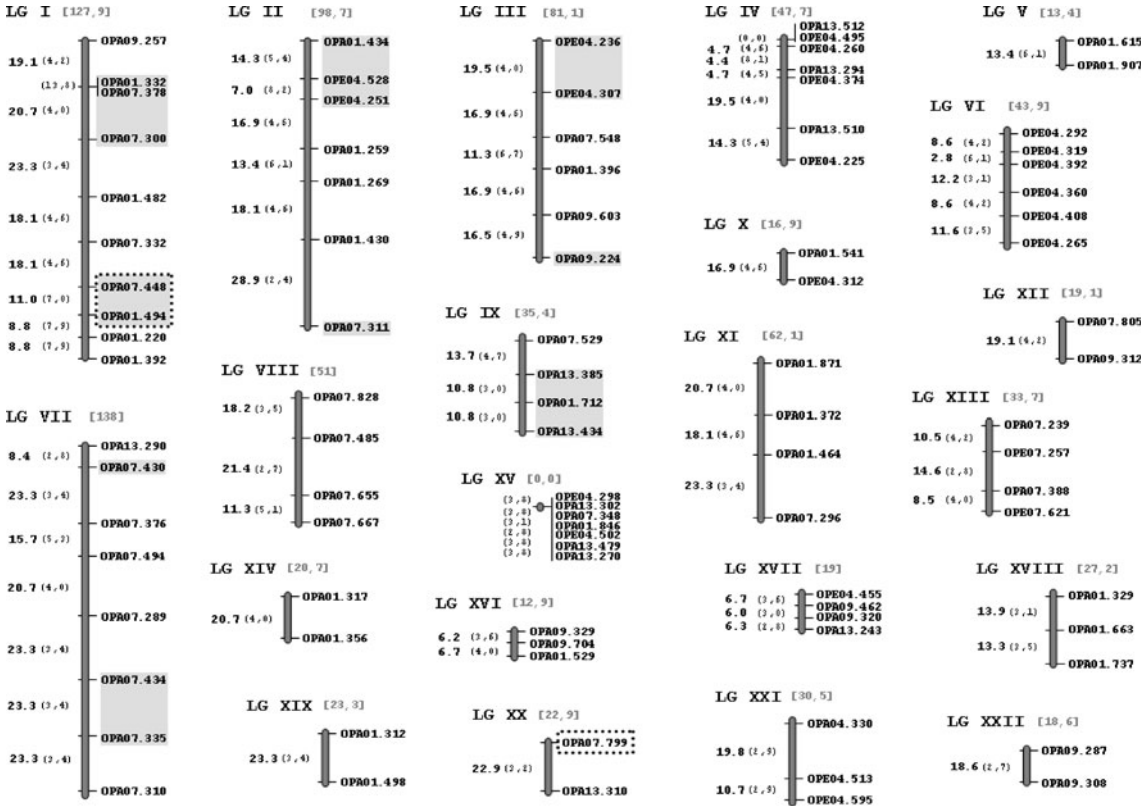

Figure 3. Preliminary genetic linkage map of *P. caudimaculatus* based on RAPD markers on the laboratory breed strains ($n = 46$). Assignment of linkage groups, positioning of markers and application of mapping function were carried out with MapManager QTX. All markers were linked at LOD > 3.0, while those that did not were excluded. RAPD markers are identified according to the following: OPA01.238, where the number of the right is the size in base pairs and the remaining characters are the primer designation. Map intervals in cM_K and LOD score within parentheses are on the left of each LG. Total distance of the mapped markers in each LG is shown within brackets. Blocks of syntenic markers are shown in shaded areas. RAPD phenotype associated markers are boxed.

Linkage mapping

Among the 189 RAPD loci used to perform linkage analysis, 95 (50.3%) could not be linked to any linkage group (LG) (LOD < 3) and hence were excluded. The remaining linked loci, (94 loci; 49.7%) were distributed over 22 LGs (LGI-XXII) (figure 3). The LG length obtained ranged from 0.0 cM_K (LG-XV) to 138 cM_K (LG-VII). Three of the 22 groups had three loci (LG-XVI, XVIII and XXI), while other seven had only two loci (LG-V, X, XII, XIV, XIX, XX and XXII). Total recombination distance over the 22 LGs was 944 cM_K, this gave an average marker interval of 11.1 cM_K between markers calculated according to Nichols *et al*. (2003).

Genetic mapping of RAPD phenotype associated markers revealed that the three melanic phenotype associated markers mapped in LG-I (OPA01.498 and OPA07.448, 11 cM_K apart; LOD= 7.0) and in LG-XX (OPA7.799, 22.9 cM_K from the nearest locus; LOD= 3.2). Regarding the wildtype phenotype associated markers, OPA09.887 did not map in any LG remaining unlinked, while OPA04.1686 locus was excluded from genetic map analysis as it displayed molecular size higher than 1080 bp.

Comparative analysis of loci distribution within the three different alternative maps allowed us to determine highly linked marker blocks (defined as highly associated loci mapping in the same LG independently of the analysis condition performed) distributed on LG-I, II, III, VII and IX (figure 3).

Estimation of genetic and physical lengths

P. caudimaculatus genome length was calculated as 1477 cM_K based on AC. Given that the haploid genome of *P. caudimaculatus* is predicted to have about 0.7×10^9 bp, the average physical equivalent of 1 cM_K would be 474 kb.

Discussion

Linkage analysis based on RAPD markers was carried out in *P. caudimaculatus* laboratory crosses. To the best of our knowledge, this work represents the first preliminary genetic map constructed for this species.

Several important factors, particularly expedited RAPD markers production such as the use of polyacryalmide gels coupled with silver staining and optimization of the

PCR programme. The implementation of these methodologies has resulted in the resolution and detection of highly reproducible numerous fragments for linkage analysis in *P. caudimaculatus* genome.

Five primers were selected for mapping as they could produce a large number of highly polymorphic, reproducible and resolvable RAPD fragments. Twenty-two LGs were constructed by linkage analysis, two less than the 24 pairs of chromosomes previously reported for these laboratory strains (Gutiérrez and García 2007). This lack of correspondence between LG and chromosomes suggests that genome coverage was insufficient perhaps due to a failure in loci amplification by the primers tested or nonrandom distribution of RAPD in *P. caudimaculatus* genome. Also, some of the 22 LGs may themselves map to the same chromosomes. On the other hand, a high proportion of unlinked loci suggest frequent recombination events occurring among loci, as it was proposed in the guppy genome (Khoo *et al*. 2003). More RAPD or other type (i.e., microsatellites SNP) linked loci might be necessary to obtain 24 LGs for further analyses.

Three melanic phenotype associated RAPD markers were found in AC, however they were not associated with pigmentation in line crosses. These results could be plausible if we consider that the aforementioned markers are not tightly linked in relation to melanic causative genes. One of the three markers, the OPA07.799, localize on LG-XX away from the other two melanic phenotype associated RAPD markers on LG-I. This fact might be due to a large gap among loci. Adding new reproducible RAPD loci could cover this possible gap between LG-I and XX, joining OPA07.799 to LG-I. This hypothesis is consistent with less strict analysis in alternative maps constructed where these three RAPD markers maps in the same LG (data not shown). Alternatively, more than one locus involved in pigmentation and mapping in different LGs could be implicated.

Comparative analysis of the three different alternative maps in the present study yielded in identification of five blocks of syntenic markers. Interestingly, OPA01.494 and OPA07.448 loci mapped in a syntenic block on LG-I; these results could provide indirect evidence for possible chromosome rearrangements occurrence preventing crossing over events in syntenic block regions, excluding independent markers segregation along consecutive generations. Further, constitutive heterochromatic blocks associated with putative rearranged chromosomal regions of biarmed elements exclusively detected in the melanic laboratory individuals (Gutiérrez and García 2007), supports this hypothesis.

In the present study, the mapping population derived from the founder mutant female pedigree yielded to an estimate of the total genetic length of *P. caudimaculatus* genome at 1477 cM_K, with a physical length equivalent estimated in 474 kb/cM_K. Although sample size was not enough for a precise estimation, this result represents a proper approximation of the total map coverage with respect to the 1776 cM_K and the 1354.5-cM length calculated for *Oryzias latipes* (Ohtsuka *et al*. 1999; Naruse *et al*. 2000). Conversely, for the genetic length arrived in *P. caudimaculatus* is in disagreement with data from species of the same order as *Xiphophorus maculatus* (2600 cM and 2178 cM_K) (Morizot *et al*. 2001; Walter *et al*. 2004) or *Poecilia reticulata* (4410.7 and 4055.6 cM_K) (Khoo *et al*. 2003). A reason for this difference could be due to the large number of unlinked loci and five extra LGs considered in *P. reticulata*. Also, *P. caudimaculatus* genetic length exposed here is an underestimate of the real total length because it was calculated to give a minimum length map. The addition of new markers could allow enrolling of unlinked loci to obtain 24 LGs corresponding to haploid number of the specie as well as increment the number of markers per LG.

The availability of genetic linkage maps with moderate to high marker densities makes feasible the identification, isolation and cloning of molecular markers linked to phenotypic traits such as colour patterns. Development of these will allow further molecular assays in *P. caudimaculatus* to achieve the physical mapping of melanic markers and to evaluate their location in reference to the biarmed chromosomes associated with melanic strains (Gutiérrez and García 2007).

Further genetic maps from the reciprocal crosses ♀wildtype × ♂melanic and ♀melanic × ♂wildtype, will anchor the present preliminary map and provide relevant data about possible sex associated differences. This will require to improve management of imbred strains with a high quantity of offspring per cross.

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