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Isolation of a Cancer-Associated Microchromosome in the Sperm-Dependent Parthenogen *Poecilia formosa*

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Key Words
AFLP · Asexual reproduction · B chromosomes · Gynogenesis · Microdissection · Paternal introgression · Telomeres

Abstract
In the asexual all-female fish species *Poecilia formosa*, the Amazon molly, supernumerary chromosomes have frequently been found in both laboratory-reared and wild-caught individuals. While wild-caught individuals with B chromosomes are phenotypically indifferent from conspecifics, individuals carrying B chromosomes from recent introgression events in the laboratory show phenotypic changes. Former analyses showed that the expression of a pigment cell locus is associated with the presence of these B chromosomes. In addition, they contain a so far unidentified locus that confers a higher susceptibility to tumor formation in the presence of pigmentation pattern. Isolation by microdissection and hybridization to metaphase chromosomes revealed that they contain one or several sequences with similarity to a highly repetitive pericentromeric and subtelomeric sequence in A chromosomes. Isolation of one particular sequence by AFLP showed that the B chromosomes contain at least 1 copy of an A-chromosomal region which is highly conserved in the whole genus *Poecilia*, i.e. more than 5 million years old. We propose it to be a single copy sequence.

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B chromosomes are additional, usually unstable constituents of the genome of many organisms. Their origin is often unclear, and their evolutionary relevance is not well understood. It may range from being deleterious to neutral or even beneficial [for an overview see Jones and Rees, 1982; Camacho et al., 2000; Palestis et al., 2004; Schmid et al., 2010]. Recently, Nanda et al. [2007] have shown that certain B's are stably inherited in the asexual, all-female teleost fish *Poecilia formosa*. In one situation, B chromosomes appear in the form of 1–3 microchromosomes derived from Black molly males that serve as sperm donors for this gynogenetic species under laboratory conditions [Schartl et al., 1995a]. In gynogenesis (= sperm-dependent parthenogenesis), the haploid sperm pronucleus will normally not fuse with the diploid pronucleus of the egg, leading to clonal reproduction. The offspring is, therefore, genetically identical to the mother. In rare cases, however, offspring with black pigmentation pat-

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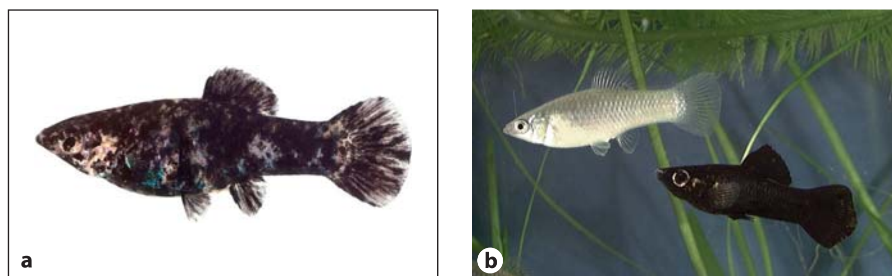

Fig. 1. a Spotted *P. formosa* female (*P.f.* 922) showing the macromelanophore pattern derived from the jet-black sperm donor Black molly. **b** Wild-type *P. formosa* strain I with a Black molly male serving as sperm donor.

tern occurred. The pigment cells turned out to be macromelanophores, a specific pigment cell type present in the skin of Black molly [Schröder, 1964], but not in *P. formosa*, obviously derived from this sperm donor by paternal leakage in form of microchromosomes (allospecific origin) [Schartl et al., 1995a]. Since macromelanophores were never seen in *P. formosa* bred with host males that do not have this cell type and only in the presence of microchromosomes, it is reasonable to assume that a genetic factor either encoding a macromelanophore locus or a *trans*-acting activator for an otherwise not expressed macromelanophore-determining locus *(mdl)* should be located on the microchromosomes. This is an exceptional situation since supernumerary chromosomes are normally considered to be genetically inert [for a review see Camacho, 2005; Schmid et al., 2010].

The microchromosomes of *P. formosa* are small, dot-like chromosomes. Small B chromosomes normally exhibit a tendency to be mitotically unstable and thus may vary in number from cell to cell within the same individual [Camacho, 2005; Schmid et al., 2010]. Accordingly, in the Amazon molly, a mosaic of 1–3 microchromosomes has been found in several organs of adult fish [Schartl et al., 1995a]. For stable inheritance of B chromosomes functional centromeres and telomeres are important. Nanda et al. [2007] showed that all microchromosomes derived from the Black molly have centromeric heterochromatin, but usually only one has a telomere. The authors hypothesize that the latter is stably inherited, while microchromosomes missing the telomeric sequences are prone to be lost, both in the soma and germline. This instability of telomere-less microchromosomes leads to a mosaic of microchromosome abundance within and between individuals. Individuals showing only 1 remaining microchromosome in all cells do not express any pigmentation pattern [Schartl et al., 1995a; Nanda et al., 2007]. Therefore, it is presumed that the pigmentation locus is located on the microchromosomes lacking the telomeres.

Interestingly, a certain percentage of the microchromosome carriers develop pigment cell tumors [Schartl et al., 1997]. It has never been observed that wild-type *Poecilia* or non-spotted *P. formosa* with only 1 microchromosome ever developed such tumors. Black mollies only extremely rarely, and only at very old age, develop pigmented tumors of the skin [M. Schartl, unpubl. data], despite the high content of macromelanophores.

Therefore, the microchromosomes seem to carry a so far unidentified locus that confers a higher susceptibility to tumor formation in the presence of the macromelanophore pigmentation [Schartl et al., 1997]. It is expected that the isolation and molecular characterization of the cancer-associated microchromosomes will provide important insights about the origin and consequences of genes located on these B chromosomes.

Materials and Methods

Fishes

All fish were raised and maintained under standard conditions [Kallman, 1975] in the aquarium of the Biocenter at the University of Würzburg. Fish from the following strains were used:

- Black Amazon I (WLC 533): Animals of this clonal line exhibit a black pigmentation phenotype (fig. 1a) due to the presence of microchromosomes derived from the normal A-chromosome complement of the Black molly by paternal leakage. The founder female was from the wild-type pigmented *P. formosa* strain I (WLC 1357; fig. 1b, left). The introgression event and the origin of this lineage occurred in 1989 and was described by Schartl et al. [1995a]. Several clonal sub-lineages of WLC 533 have been established since (e.g. WLC 637 in 1992, WLC 857 in 1994) [Nanda et al., 2007].
- Black Amazon II (WLC 922-25/IV): Clonal line similar to WLC 533, also derived from an independent introgression event of a Black molly-derived microchromosome into *P. formosa* strain I (WLC 1357).
- Black molly (WLC 1351): Melanistic ornamental strain of unknown genetic origin. These fish are homogeneously dark black colored due to the presence of macromelanophores. Fish are homozygous for the dominant pigmentation loci *Niger (N)* and *Melas (M)* [Schröder, 1964] (fig. 1b, right).

Multilocus DNA Fingerprints

Liver, brain, eyes and muscles of each individual were pooled and stored at −80 °C. DNA was extracted using EDTA buffer and phenol/chloroform according to the method of Blin and Stafford [1976]. *Hin*fI was used for restriction digestion, and the restriction fragments were separated on a 0.8% agarose gel at 1 V/cm. In-gel hybridization was routinely processed in the Department of Human Genetics, Bochum, Germany, essentially as described by Nanda et al. [1988]. The ^{32}P-labeled oligonucleotides $(GATA)_4$, $(GGAT)_4$, $(GA)_8$, and $(CA)_8$ were used as hybridization probes.

Amplified Fragment Length Polymorphism (AFLP)

AFLP analyses were performed as described in Vos et al. [1995]. Shortly, genomic DNA was digested with *Eco*RI and *Mse*I and ligated to linkers. Two subsequent PCR reactions with +1 primers (*Eco*RI: 5′-GACTGCGTACCAATTC *A*-3′, *Mse*I: 5′-GATGAGTCCTGAGTAA *G*-3′) and +3 primers (*Eco*RI: 5′-GACTGCGTACCAATTC *NNN*-3′, *Mse*I: 5′-GATGAGTCCT-GAGTAA *NNN*-3′), respectively, were performed. In +3 primers *NNN* was ACC or AAC (*Eco*RI) and GTG, GAG, GGT, GTC, GCA, GAA, GCC or GAT (*Mse*I). 500 ng of +3 primers were labeled with 100 μC γ^{32}P-ATP. Amplification products were separated for 2 h at 35 W on a denaturing 5% PAA gel in 0.5× TAE buffer. In total, 16 primer combinations were tested. Informative bands were cut out, extracted from the gel, re-amplified (40 cycles, 56 °C), and cloned for sequencing. The fragments were radioactively labeled and re-hybridized under low stringency conditions onto *Eco*RI-digested genomic DNA from microchromosome carriers, wild-type individuals and a Black molly male.

Fibroblast Cell Culture

Due to a very low mitotic index in adult live fish, a fibroblast cell culture was established. A pregnant spotted *P. formosa* female (WLC 922-25/IV) was sacrificed by over-anesthesia in a 0 °C water/ice bath. Thirteen late-stage embryos (stage 19, according to the description given by Tavolga [1949] for the related species *Xiphophorus maculatus*) were prepared under sterile conditions from the mother, washed 1 × 5 min in 100× penicillin/streptomycin (Gibco®) and 4 × 5 min in 4× penicillin/streptomycin in fish PBS (1 mM NaCl, 2 mM KCl, 6 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.4). Subsequently, the embryos were chopped with a sterile scalpel and transferred to 10 ml 1× trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA × 4 Na, Gibco®, diluted in fish PBS) in a Falcon tube. After shaking for 2 h at room temperature, the trypsin reaction was stopped by adding FBS (20% final concentration). Leaving behind remnants of tissue, the cell suspension was centrifuged for 10 min at 450 *g*. The pellet was resuspended in 1.5 ml DMEM, 10% FBS, 10% SC (50% FBS, 10% penicillin/streptomycin, 10% glutamine, 10% pyruvate, 10% non-essential amino acids, 0.5% β-mercaptoethanol) and plated out on 24-well Primaria tissue culture plates (Becton, Dickinson and Co., Franklin Lake, N.J., USA). Incubation was performed at 28 °C, 5% CO_2. Subculturing was carried out until passage 25, and ploidy and presence of microchromosomes (1–3) was regularly checked by flow cytometry and karyotyping, respectively. The cell line was given the designation Pfo2. The cells showed a fibroblast-like phenotype.

Chromosome Preparation

For microdissection, metaphase chromosome spreads were prepared from the cell line Pfo2 not later than passage 6. To obtain metaphase chromosomes, cells were treated with Colcemid® (1.0 μg/ml DMEM; KaryoMAX®, Invitrogen) for 4 h, and metaphases were carefully harvested by trypsin-EDTA treatment. After hypotonic treatment with 40 mM KCl for 30 min at room temperature, cells were fixed in ice-cold Carnoy's fixative (methanol:glacial acetic acid, 3:1) for 10 min. The fixed cell solution was dropped onto wet, ice-cold coverslips, dried at room temperature and stored dry and sterile until use.

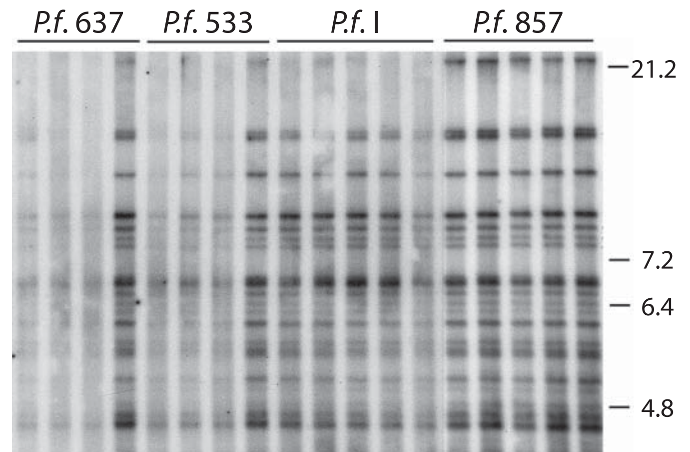

Fig. 2. Multilocus DNA fingerprint of spotted microchromosome-carriers (*P.f.* 637, *P.f.* 533, *P.f.* 857) in comparison to wild-type *P. formosa* (*P.f.* I). Hybridization probe $(GGAT)_4$. No differences can be detected with this method.

Microdissection

Microdissection was carried out as previously described [Henning et al., 2008; Yang et al., 2009]. After proteinase K digestion, DNA of microdissected chromosomes was amplified using degenerate oligonucleotide primers (DOP-PCR) [Telenius et al., 1992]. The obtained products were labeled with biotin, and fluorescent in situ hybridization (FISH) was carried out on the metaphase chromosomes of *P. formosa* and Black molly using a standard protocol [Nanda et al., 1990; Yang et al., 1999; Graphodatsky et al., 2000]. The preparations were analyzed with a Zeiss Axioplan 2 microscope. Images were digitally stored with the help of ISIS FISH imaging system (MetaSystems).

Results

Estimation of the Genomic Contribution of Microchromosomes

In the multilocus DNA fingerprints no differences could be detected between the different microchromosome-carrying strains (*P.f.* 637, *P.f.* 533, *P.f.* 857) and the wild-type strain (*P.f.* I) (fig. 2). This is consistent with earlier data [Schartl et al., 1995a]. Since the resolution of this method is approximately 5%, and the diploid genome size

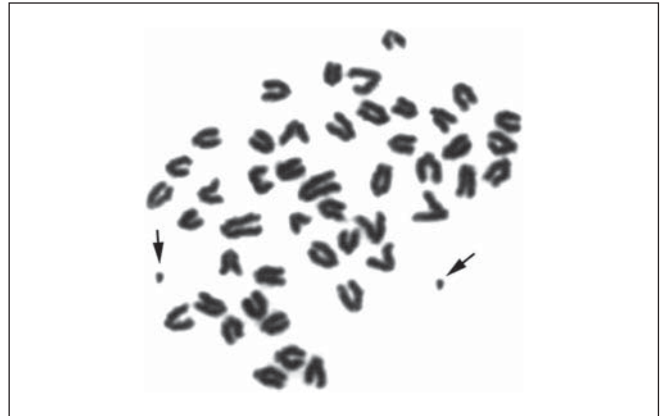

Fig. 3. Metaphase spread of a spotted *P. formosa* female showing 2 microchromosomes (arrows).

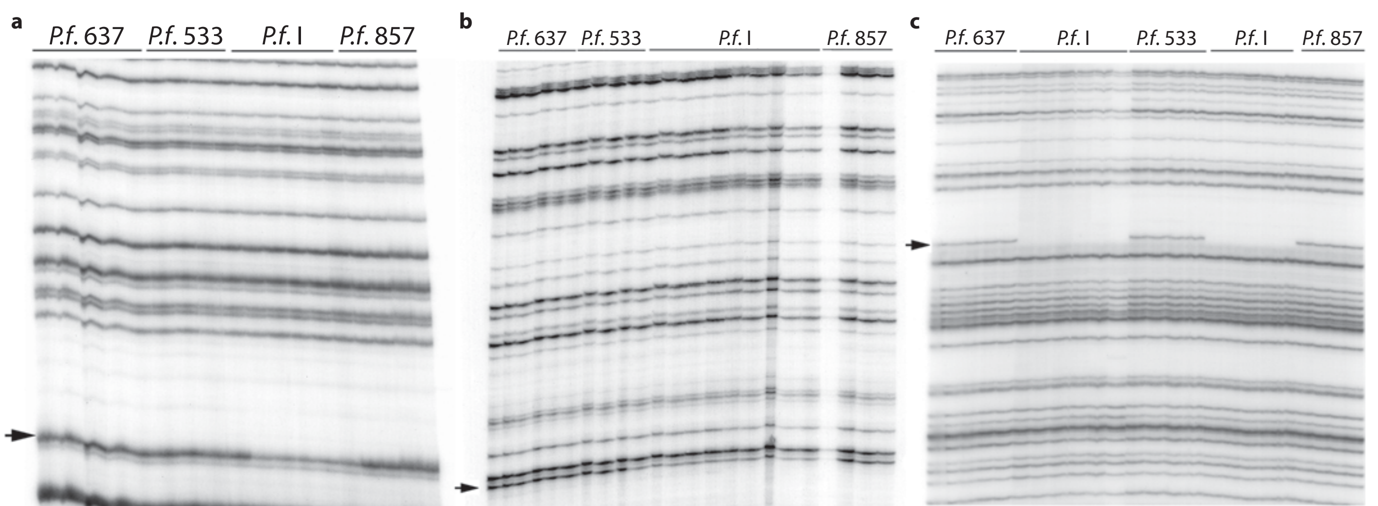

Fig. 4. AFLP analyses of spotted microchromosome carriers (*P.f.* 637, *P.f.* 533, *P.f.* 857) in comparison to wild-type *P. formosa* (*P.f.* I). +3 primer combinations Eco-AAC/Mse-GCC (**a**), Eco-ACC/Mse-GAA (**b**), and Eco-AAC/Mse-GCA (**c**). Arrows indicate microchromosome-specific bands.

of *P. formosa* is approximately 1,900 Mb [Lamatsch et al., 2000], the paternal contribution must be <100 Mb. By visual judgment the microchromosome is approximately 1/10 in size in comparison to an average A chromosome (fig. 3). Hence, it would make up less than ~0.2% of the genome (~4 Mb), and therefore would be far below the limit of detection of the DNA fingerprint method.

Isolation of Microchromosomal Sequences

With the PCR-based AFLP method even small differences between different genomes can be detected. Three out of 16 tested AFLP primer combinations showed differences between microchromosome carriers and non-carriers (fig. 4): The primer combinations Eco-AAC/Mse-GCC and Eco-ACC/Mse-GAA each revealed 1 band that was stronger in the microchromosome carriers than in the non-carriers (fig. 4a, b). This could be due to a fragment which is more abundant in fish with microchromosomes or there might be a co-migrating band of identical size, but unrelated in sequence, that is present in both groups of fishes. With the primer combination Eco-AAC/Mse-GCA, however, 1 band was detected that was exclusively present in animals with microchromosomes (fig. 4c) and always missing in animals without microchromosomes.

Since we could not exclude the extraction of a co-migrating band in the first 2 cases, we only carried on with the band isolated from the Eco-AAC/Mse-GCA primer combination. The band present in the microchromosome

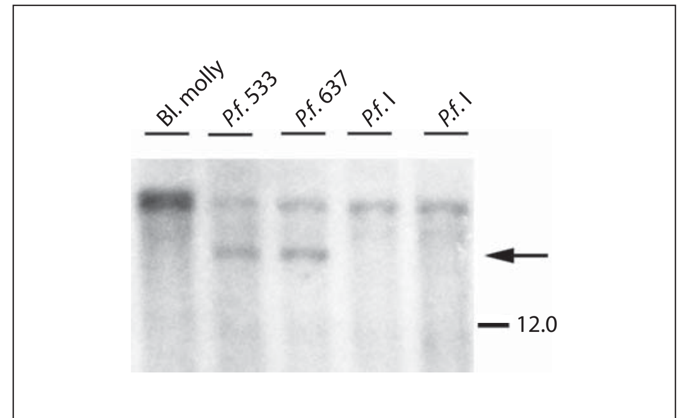

Fig. 5. Southern blot hybridization of the microchromosome-specific sequence (isolated from AFLP) to *Eco*RI-digested genomic DNA of a Black molly male, 2 *P. formosa* females with microchromosomes as well as 2 *P.f.* I females without microchromosomes. Hybridization probe Eco-AAC/Mse-GCA.

Table 1. Microchromosome-specific sequence from *Poecilia formosa* gained by AFLP

AFLP primer combination	Sequence length, bp	AT/GC content, %AT	Sequence
Eco+AAC/Mse+GCA	227	140/87 (61.7)	**AAC**AAATGAGACACAACGTTTTCTGAAGTGAGAGAGCTCAGATGGAGCCA GCTGCAGACTGTCCGGGAAAAAAAAAGAAAGAAAAAAAAATAAGACTTCTTC TTTTTGTGTAGTTGCATATAGAGTACTGCAGCACATTTATTATCAACATA TTTGTTTGGTACCTGTTAGCTGGTTCTCTCTCTCTCTTCCAGATACGTTA GTCACAAATGGGATTTGCAAAACC**TGC**

Bold letters = selective bases of +3 primer.

carriers (1 of each strain) was re-amplified and sequenced (table 1). The 3 sequences were identical and no homologous sequences were found in GenBank using Blastn (blastn E values >0.02) (http://www.ncbi.nlm.nih.gov/blast/). The sequence was desposited at GenBank with accession no. Banklt1467656 Seq1 JN379093.

The re-amplified product from band Eco-AAC/Mse-GCA was 227 bp in size (61.7% AT-content) and was subsequently used as hybridization probe in a Southern blot on *Eco*RI-digested genomic DNA (fig. 5). The autoradiogram showed a hybridization signal of ~14 kb in all individuals investigated and a signal of ~13 kb only in microchromosome carriers. FISH on chromosome spreads did not reveal any signals, probably because the sequences were too short to give a sufficient signal (data not shown).

To find out whether the microchromosomes contain also repetitive sequences (which should give a FISH signal even from short isolated DNA sequences), a microdissection was performed. Twenty-two microchromosomes were isolated by microdissection and unspecifically amplified using DOP-PCR. DNA was labeled with biotin and hybridized to metaphases of microchromosome carriers (fig. 6a). The microchromosomes as well as the centromeres were intensively stained and, more weakly, the telomeres of A chromosomes. As a control, the microchromosome probe was also hybridized to metaphases of the Black molly (fig. 6b). Also in this experiment mainly the centromeric regions were stained, whereas the telomeric regions appeared less intensely labeled.

Discussion

Although approximately 10–15% of all eukaryotic species contain B chromosomes, only a small fraction has been analyzed from a molecular perspective [for a comprehensive overview see Camacho, 2005]. Most B chromosomes consist of constitutive heterochromatin. This compact structure is explained by a high content of repetitive DNA of various types, especially satellite DNA, 18S + 28S ribosomal DNA, and mobile element DNA. Single copy genes seem to be scarce in B chromosomes

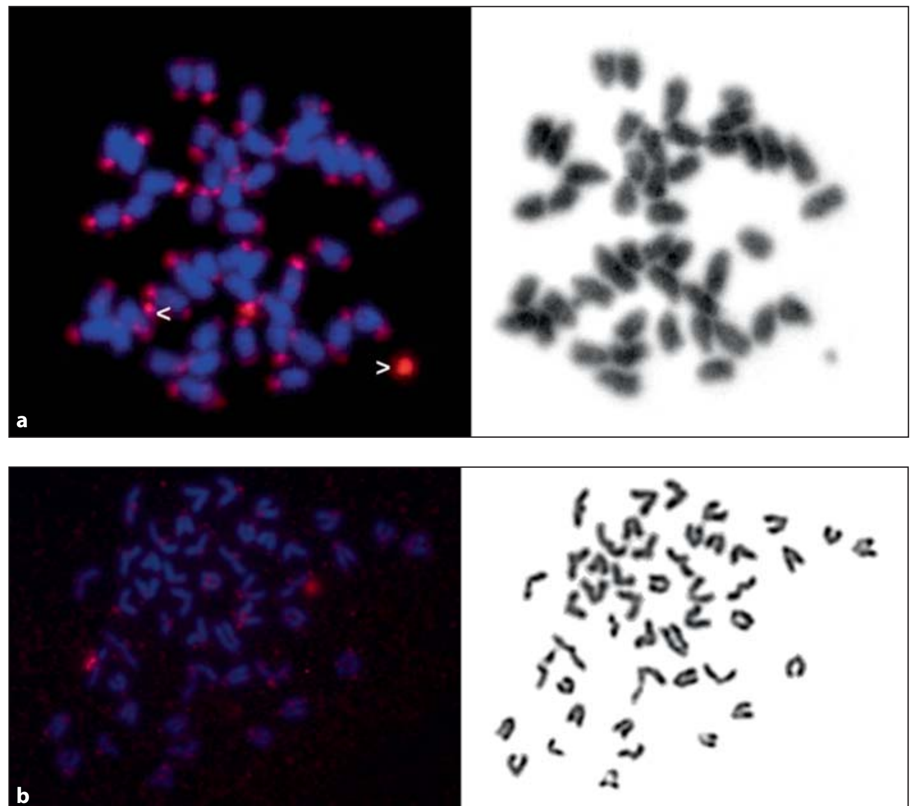

Fig. 6. FISH of DOP-amplified microchromosomes from microdissection onto metaphase chromosome spreads of *Poecilia* (left) and DAPI-inverted images (right). **a** Karyotype of a spotted *P. formosa* female showing 46 A chromosomes and 2 microchromosomes (arrowheads). **b** Karyotype of a Black molly male showing 46 chromosomes.

[Green, 1990; Jones, 1995] and are predicted to be difficult to detect within large stretches of repetitive DNA. Copies of proto-oncogene *C-KIT* and adjacent sequences were found on B chromosomes of foxes and raccoon dogs [Graphodatsky et al., 2005], although these elements were considered as totally heterochromatic before [Yang et al., 1999; Trifonov et al., 2002]. In most cases, evidence for single copy genes comes from an association between B chromosome presence and a certain phenotypic character [Camacho, 2005]. Also in the Amazon molly evidence for single copy genes is due to the association between the presence of microchromosomes and an observable phenotypic change. Importantly, it is one of the few species in which the rise of a supernumerary chromosome was witnessed in the laboratory: the microchromosomes are derived from the sperm-donor Black molly and obviously contain a macromelanophore locus, which is expressed in the foreign genetic background, resulting in a pigmentation pattern of the microchromosome carriers [Schartl et al., 1995a]. Alternatively, the microchromosome may contain a regulatory element that allows the expression of an otherwise silent macromelanophore locus on the A chromosomes of *P. formosa*. There is a growing body of evidence that B chromosomes of most animals are not totally heterochromatic and contain hidden stretches of autosomal segment copies [Trifonov et al., 2010; Duke Becker et al., 2011; in press]. The case of the *P. formosa* microchromosome further confirms this view.

Isolation of B chromosomes (sequences) can be performed with a variety of techniques depending on their size [see Camacho, 2005]. The size of B chromosomes in fishes varies greatly from macrochromosomes (≥ biggest A chromosome), as found in *Alburnus alburnus* [Ziegler et al., 2003; Schmid et al., 2006] and *Astyanax* sp. [Maistro et al., 1992], to medium-sized and small-sized chromosomes (≥ smallest A chromosomes), as described in *Rhamdia* sp. [Lui et al., 2009], as well as microchromosomes (< smallest A chromosome, typically <20 Mb), as in *Prochilodus* [Cavallaro et al., 2000] and *P. formosa* (~4 Mb).

Due to its small size, several techniques were not successful in isolating microchromosome-specific sequences of *P. formosa*, i.e. pulsed-field gel electrophoresis [Lai et al., 1989], flow cytometric chromosome sorting [Ng et al., 2007], laser microbeam microdissection combined with laser pressure catapulting [Schermelleh et al., 1999], and representational difference analysis [Lisitsyn et al.,

1993] (data not shown). We finally succeeded with AFLP and classical microdissection in isolating microchromosome-specific sequences from *P. formosa*.

From the Southern hybridization pattern of the AFLP sequence to *Eco*RI-digested genomic DNA, it can be assumed that the microchromosomes contain 1 copy of an A-chromosomal region, present in *P. formosa* with and without microchromosomes, as well as in Black molly. Since *P. formosa* is a hybrid of *P. mexicana* and *P. latipinna* (2 most distantly related members of the genus *Poecilia*) [Schartl et al., 1995b], this obviously is a highly conserved sequence in the whole genus, i.e. more than 5 million years old. The additional lower band in microchomosome carriers is due to an extra *Eco*RI recognition site. We propose that the low number of bands reflects a single copy sequence since highly repeated sequences would not give single bands in genomic DNA digested with a 6-bp cutter, but rather yield smear signals (in case of fragment heterogeneity) or very intense bands (in case of large tandem repeats).

AFLP was already used in other studies to identify B chromosomal sequences. Ziegler et al. [2003] isolated a B-specific sequence from the cyprinid fish *Alburnus alburnus* showing a high similarity to a *Drosophila* Gypsy/Ty3 retrotransposon. Qi et al. [2002] identified a B-specific sequence by AFLP which contained the telomeric repeat unit AGGGTTT conserved in plant chromosome telomeres. However, all identified sequences contained repetitive DNA, whereas the sequence identified from *P. formosa* has all characteristics that would assign it to the class of single copy sequences. Since the fish lineages are clonal, and the only difference between them is the presence or absence of the microchromosomes, AFLP is ideal for selecting single copy sequences only present in the microchromosomes.

In addition, a classical microdissection was performed in order to obtain a microchromosome-specific DNA library. The FISH pattern on metaphase chromosome spreads showed that the microchromosomes contain one or several sequences with similarity to a highly repetitive pericentromeric and subtelomeric sequence in the A chromosomes of *P. formosa* and Black molly. No major differences could be seen between the hybridization pattern of *P. formosa* and Black molly. This is in accordance with Lamb et al. [2005] who found a striking pattern of enrichment for DNA elements that are normally found at or near centromeres. Single copy sequences, however, would most probably be below the threshold of FISH detection. Besides, we did not conduct any suppression of repetitive elements in our FISH protocol, and thus the overall hybridization picture rather reflects the distribution of repetitive elements present in the library.

Because of their similar morphology, the 2 different types of microchromosomes could not be distinguished in the microdissection procedure: In cells with 3 microchromosomes, 2 should contain the macromelanophore locus, in metaphases with 2 microchromosomes, only 1 will harbor this locus. Therefore, as many microchromosomes as possible were collected per metaphase plate, resulting in a total of 22 microchromosomes. Although quite unlikely, we cannot completely rule out that we have missed the macromelanophore-carrying microchromosome. To identify the macromelanophore locus, a microchromosome-specific library [Enkerli et al., 2000] needs to be constructed and sequenced [Schories et al., in preparation].

Despite many studies on B chromosome systems, the amount of DNA contained in B chromosomes was only determined in a few species: maize (352–430 Mb, 14.7% of C value), the wasp *Nasonia vitripennis* (38 Mb, 5.7% of C value), the grasshoppers *Eyprepocnemis plorans* (500–630 Mb, 5% of C value) and *Locusta migratoria* (147 Mb, 2.5% of C value) [Ruiz-Ruano et al., 2011], and *Alburnus alburnus* (313 Mb, 18.6% of C value) [Schmid et al., 2006] (genome sizes calculated according to Doležel et al. [2003]). *P. formosa*, therefore, does not only contain the smallest B chromosome of the above-mentioned (~4 Mb), but also one of the smallest B chromosomes relative to C content (0.43%).

The combination of the 2 techniques – AFLP and microdissection – provides an important step towards the molecular characterization of one of the smallest B chromosomes (microchromosome) associated with phenotypic change and even tumor formation. Both genetic and environmental risk factors can lead to the malignant transformation of melanocytes and result in the development of tumors. Due to its clonality, *P. formosa* could become a suitable new animal model system for tumor research.

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