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Stable Inheritance of Host Species-Derived Microchromosomes in the Gynogenetic Fish *Poecilia formosa*

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ABSTRACT

B chromosomes are additional, usually unstable constituents of the genome of many organisms. Their origin, however, is often unclear and their evolutionary relevance is not well understood. They may range from being deleterious to neutral or even beneficial. We have followed the genetic fate of B chromosomes in the asexual, all-female fish *Poecilia formosa* over eight generations. In this species, B chromosomes come in the form of one to three tiny microchromosomes derived from males of the host species that serve as sperm donors for this gynogenetic species. All microchromosomes have centromeric heterochromatin but usually only one has a telomere. Such microchromosomes are stably inherited, while the telomereless are prone to be lost in both the soma and germline. In some cases the stable microchromosome carries a functional gene lending support to the hypothesis that the B chromosomes in *P. formosa* could increase the genetic diversity of the clonal lineage in this ameiotic organism and to some degree counteract the genomic decay that is supposed to be connected with the lack of recombination.

B chromosomes are supernumerary chromosomes, which do not follow Mendelian rules of inheritance. To date, they have been found in >2000 species of plants, animals, and fungi (Jones and Rees 1982; Camacho *et al.* 2000; Palestis *et al.* 2004). B chromosomes are considered either to arise from a duplicated or fragmented A chromosome within the same genome or to be acquired during a hybridization event from foreign DNA that evolves into the supernumerary chromosome (Jones and Rees 1982; Green 1990; Camacho *et al.* 2000). Within a given species or population, individuals are polymorphic for the presence of B chromosomes, because the chromosomes usually lack a homologous partner to pair with during meiosis and are therefore distributed unequally to the gametes. There can be one or several B chromosomes in one individual. In addition B chromosomes can also be lost during an individual's development because of unequal distribution during cell divisions. Such organisms then may lack B chromosomes in certain organs, tissues, or cells (Palestis *et al.* 2004).

The maintenance and evolution of B chromosomes have been explained in several ways. Traditionally, they have been classified as selfish genetic elements that decrease the fitness of the "host" genome (Shaw and Hewitt 1990; Camacho *et al.* 2000). Thus they generate what has been called a "genetic conflict" between the A and B chromosomes. By virtue of their accumulation mechanisms, they are maintained within populations (Östergren 1945; Thomson 1984; Jones 1985; Nur *et al.* 1988). In the heterotic model (White 1973), it is assumed that B chromosomes are maintained because they increase the fitness of the host when they occur at low frequency. This hypothesis does not require an accumulation mechanism. An "evolutionary arms race" model (Camacho *et al.* 1997) assumes a nonstable, dynamic situation. B chromosomes are considered parasitic and spread through the population because of an accumulation mechanism. But, as they increase their frequency, they are neutralized by the host genome and begin to disappear slowly, unless a new variant of the B, which can counteract the elimination mechanism, replaces the neutralized B.

Only in very few cases do the B chromosomes appear to have a beneficial effect on the host species (Bougourd and Jones 1997), while most are considered to be harmful (Palestis *et al.* 2004). They can, however, escape extinction in outcrossing species because they can continually "infect" new lineages if they drive. In inbred or asexual species, natural selection acts among competing lines of descendants or clones, respectively. Lines or clones without B chromosomes are expected to outcompete those with B chromosomes, if B chromosomes decrease fitness. In the asexual all-female

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fish species *Poecilia formosa*, the Amazon molly, supernumerary chromosomes have frequently been found in both laboratory-reared and wild-caught individuals from the Río Purificación/Río Soto la Marina river system, Mexico (Lamatsch *et al.* 2004). The high frequency of B chromosomes in wild populations supports the idea that the B chromosomes of *P. formosa* are not harmful, but rather may be beneficial (Schartl *et al.* 1995).

The genetic fate of B chromosomes is usually not well documented, and their origin is mostly unknown. For the B chromosomes of *P. formosa* it is clear that they are of hybrid (allospecific) origin. *P. formosa* reproduces by gynogenesis meaning that the parthenogenetic development of the diploid, ameiotic oocytes is triggered by sperm of males from closely related species. In general, the paternal DNA is excluded from the inseminated oocyte; however, in rare cases parts of the sperm genome persist as tiny B chromosomes (microchromosomes) in the karyotype of the developing embryo (Schartl *et al.* 1995). This process of introgression of paternal genes into the asexual lineage is considered a process of bringing in fresh genetic material into the asexual lineage. Organisms that cannot perform recombination should suffer from genetic decay because deleterious mutations cannot be purged and are slow to evolve (Muller 1932; Kondrashov 1988). Thus the B chromosomes in *P. formosa* might be ascribed a beneficial effect. However, a precondition for this is that the microchromosomes can become stable components of the genome of the asexual fish. If not, their evolutionary impact would only be very transient and not of considerable importance. We tested this precondition by following the inheritance of microchromosomes from *P. formosa* in B chromosome containing clones from the wild and from recent introgression events in the laboratory. B chromosomes were found to be inherited stably over many generations in the analyzed clones, and no fish without B chromosomes were recorded.

MATERIALS AND METHODS

Animals: 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 spotted pigmentation phenotype because of the presence of a microchromosome derived from a black molly (see description below). The founder female was from wild-type pigmented *P. formosa* strain I (WLC1357). The introgression event and origin of this line have been described in Schartl *et al.* (1995). Several clonal sublines of WLC 533 were established.

Black Amazon II (WLC 922-25/IV): The clonal line is similar to WLC533, also derived from an independent introgression event of a black molly derived microchromosome into *P. formosa* strain I (WLC1357).

Black Amazon III (WLC 41): The clonal line is of the same origin as WLC 533 from a third independent introgression event in *P. formosa* strain I mated to black molly males.

Black molly (WLC 1351): The melanistic ornamental strain is of unknown genetic origin. From body shape and mitochondrial DNA sequence, it is probably derived from the *P. mexicana/P. sphenops* complex (B. Wilde and M. Schartl, unpublished data). These fish are homogeneously dark-black colored due to the presence of macromelanophores in the skin of the body and fins. Fish are homozygous for the dominant pigmentation loci *Niger* (*N*) and *Melas* (*M*) (Schröder 1964).

P. formosa 573 (WLC 573): The wild-type pigmented strain is derived from nonspotted offspring of black Amazons line I.

P. formosa III/9 (WLC 1612): The wild-type pigmented strain is derived from one female with a single microchromosome of a collection from the Río Purificación near Barretal, Tamaulipas, Mexico.

P. formosa III/4 (WLC 1588): The wild-type pigmented strain is derived from one female with a single microchromosome of a collection from a canal east of Ciudad Mante, Tamaulipas, Mexico.

Chromosome analysis and telomere staining: Mitotic chromosomes were prepared directly from pooled organs (spleen, cephalic kidney, and gills) following the standard procedure described elsewhere (Nanda *et al.* 1995). Giemsa-stained slides were screened under light microscope to check the number of diploid chromosomes as well as the presence of microchromosomes. To visualize the centromeric heterochromatin, metaphase chromosomes were subjected to C-banding following the procedure of Sumner (1972), except that the treatment with alkali was done for 2 min.

To detect the presence of telomere specific $(TTAGGG)_n$ repeats at the end of chromosomes, fluorescence *in situ* hybridization (FISH) was performed with a telomeric peptide nucleic acid (PNA) oligonucleotide $(CCCTAA)_3$ labeled with FITC (Applied Biosystems, Foster City, CA). After pretreatment with pepsin and formaldehyde, slides were denatured at 80° for 3 min under a coverslip in presence of the hybridization mixture containing the labeled probe. Hybridization was performed for 2 hr at room temperature, after which slides were briefly washed in 70% formamide (10 min) and further washed in PBS for 5 min. The slides were dehydrated in ethanol series. Afterwards, slides were mounted in an antifade reagent containing DAPI (4′6-diamidino-2-phenylindole) as counterstain. Digital images of metaphases showing hybridization signals were acquired using a Zeiss epifluorescence microscope coupled with CCD camera and Applied Spectral Imaging software (Neckerhausen, Germany).

Statistical analysis: Differences in the number of wild-type, spotted, and black offspring between the lines (I, II, and III) were calculated using a multidimensional chi-square test. To test for a correlation of brood number and the proportion of wild-type individuals, a Spearman rank correlation was performed using the program SPSS. Over- or undertransmission of the microchromosome was calculated using chi-square goodness-of-fit tests assuming that a microchromosome had a 50% chance of being lost in each progeny by somatic instability.

RESULTS

Origin of microchromosome carrying lines: In Amazon mollies, introgression events of paternal DNA are easily recognized in the laboratory if they involve the pigmentation loci of the black molly, which is routinely used as a host species. Usually triploid animals are evenly spotted (Schultz and Kallman 1968; Nanda

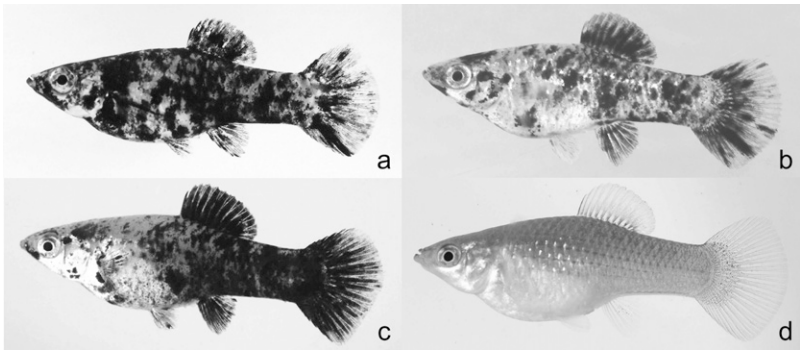

FIGURE 1.—Black Amazons. (a) Line I; (b) line II; (c) line III; and (d) wild-type pigmented *P. formosa* from line III/4.

et al. 1995), while microchromosome carriers have irregularly shaped large black blotches (SCHARTL *et al.* 1995). Since 1993 we recorded in our broods in 10 strains from different localities a total of 64 animals with a spotted phenotype. Offspring was obtained from 29 fish, all of them showing the "microchromosome" phenotype. In 23 cases all offspring were wild type pigmented. Three fish produced wild-type pigmented and spotted offspring, however, the pigmentation phenotype was lost in the next generation. Another three fish transmitted the pigmentation phenotype over all generations to date. They gave rise to the black Amazon lines I (WLC 533), II (WLC 922-25/IV), and III (WLC 41) (Figure 1). In lines I and II a spotted female always gives rise to nonspotted, spotted, and, in very rare cases, almost completely black daughters (Table 1).

Consistent with an earlier study (SCHARTL *et al.* 1995), nonspotted fish had one microchromosome, spotted fish had two, and black fish had three microchromosomes. The proportion of the phenotypes is variable, sometimes nonspotted fish outnumber the spotted fish if mass breeding is carried out. Recording succeeding broods of single females revealed that in line I, the number of nonspotted offspring increased with brood number from ~45% to 80% (Table 2). In line II, the wild-type pigmented fish were much less frequent (between 10 and 15%), and no increase with brood number was obvious. Line I has been bred in the laboratory since 1989 and line II since 1995 (equivalent to ~35 and 20 generations, respectively).

Inheritance of spotted phenotypes: A detailed analysis of microchromosome transmission mode was performed using spotted females of black Amazon lines I and II and their broods (Table 2). Spotted and black offspring were grouped as one since both phenotypes did inherit a melanic B chromosome. A chi-square goodness-of-fit test was performed under the hypothesis that the melanic B present in the mother would have a 50% chance of being lost in each progeny by somatic instability. Significant deviations from this assumption were interpreted as either undertransmission (transmission rate significantly $<50\%$) or overtransmission (transmission significantly $>50\%$). The analysis was performed for each female (broods had to be pooled because of otherwise too low sample sizes) and for each litter (females were pooled for this analysis because of otherwise too low sample sizes).

In line I, 60% of the females (15 of 25) showed significant deviations from a 50% transmission rate. Of these 15 females, four individuals showed undertransmission while 11 females showed overtransmission of the microchromosome. The analyses of the litters showed overtransmission of the microchromosome in litters one to three, random transmission in litters four and five, and undertransmission in litters six to nine.

In line II, 73% of the females (11 of 15) showed significant deviations from a 50% transmission rate. All 11 females showed overtransmission of the microchromosome. In all five litters recorded for line II significant overtransmission was observed.

A significant correlation between the litter number and the proportion of wild-type pigmented individuals was observed in line I ($r_s = 0.905$; $P = 0.002$; $n = 8$) but not in line II ($r_s = 0.100$; $P = 0.87$; $n = 5$). This strong correlation observed between age of the female (as a proxy for number of litters) and loss of the pigmentation locus carrying microchromosome (as measured as the proportion of wild-type individuals) explains 82% of the variability found in the data.

A considerable degree of somatic instability was noted when, in fish of lines I and II, different metaphases of one and the same fish were compared. In each metaphase spread, the microchromosomes appeared as a separate entity, which was not attached to any chromosomes. While the number of A chromosomes was always $n = 46$, either one or two microchromosomes, or even three (only in totally black animals), were present. However, the loss of all microchromosomes was never observed. The proportion of one or two microchromosome-containing metaphases varied between individuals (Table 3).

Line III, established in 2003, differs from the two other lines because after eight generations it produced only a single nonspotted fish out of a total of 319 offspring (Table 1). Karyotype analysis of line III revealed that the spotted fish have only one microchromosome,

TABLE 1

Pigmentation phenotypes of the offspring of individual females in three black Amazon lines of *P. formosa*

	Pedigree								
	I (533)			II (25/IV)			III (41)		
	Wild type	Spotted	Black	Wild type	Spotted	Black	Wild type	Spotted	Black
	30	17	0	5	33	0	0	50	0
	75	1	0	4	17	0	0	91	1
	21	7	0	7	50	1	0	31	0
	130	42	0	5	14	0	0	24	0
	13	3	0	4	24	0	0	26	0
	3	1	0	0	4	0	0	19	0
	4	7	0	2	15	0	0	15	0
	54	81	1	1	4	0	1	29	0
	22	40	1	0	8	0	0	14	0
	27	23	0	3	15	0	0	19	0
	26	18	0	1	8	0			
	62	100	1	0	9	0			
	4	14	0	3	4	0			
	33	68	0	1	12	0			
	55	0	0	3	54	0			
	12	43	0	0	23	0			
	17	42	1	0	8	0			
	4	16	0	1	38	0			
	64	19	0	4	123	0			
Total	654	542	4	44	436	1	1	318	1

The difference between all lines considering the number of wild-type pigmented, spotted, and black offspring was highly significant. Each line gives the number of offspring in the three phenotype classes of a single female. $\chi^2 = 513.14$, d.f. $= 4$, $P < 0.00001$.

which is different from the spotted fish of lines I and II. The single unspotted fish unfortunately died before its karyotype could be analyzed.

The somatic instability observed in lines I and II was absent in fish of black Amazon line III. Like in the wild-type pigmented fish from wild populations ($n = 28$) with one microchromosome (see below), all metaphases consistently had one microchromosome.

Inheritance of microchromosomes: To analyze the inheritance of microchromosomes, females that carried a single microchromosome were mated to black mollies, and the karyotype of the offspring over several consecutive generations was prepared (Figure 2). All animals from a total of nine generations in both lines established from microchromosome-carrying females collected from natural habitats had the microchromosome (Figure 3). In pedigree III/4 in generations G_3 and G_4, spotted fish occurred. They had an additional microchromosome (Figure 2) obviously paternally derived from the black molly that was used for breeding. In pedigree III/9, one female in G_4 and one in G_6 also showed an additional microchromosome in the karyotype, but both fish were wild type pigmented. This additional microchromosome also should have originated from the black molly father, but it evidently did not carry a functional pigmentation locus. The newly recruited microchromosome appeared to be significantly smaller than the original microchromosome in this line (Figure 3). However, the additional new microchromosomes were lost because offspring from these fish consistently had only one microchromosome.

Furthermore, fish were studied from a line of non-spotted black Amazons (WLC573) that were separated from spotted siblings and bred for at least four generations as a closed colony. A single female was isolated from this stock and bred for an additional six generations. All fish had retained a single microchromosome (Figure 2).

Centromeres and telomeres: A prerequisite for the stable inheritance of a chromosome is the presence of a centromere. To analyze whether the microchromosomes have this structure, C-banding was performed on metaphase spreads of black Amazons line I, which shows the most obvious loss of the second microchromosome. Both microchromosomes showed a clear C-band positive staining at one end that might indicate the presence of centromere-associated heterochromatin (Figure 4). This analysis also revealed that the entire microchromosome is not heterochromatic.

To find a possible mechanism for why a single microchromosome is stably inherited while a second and third microchromosome can get lost, telomere staining was performed (Figure 5). All 46 chromosomes in both wild- and laboratory-derived fish display characteristic terminal labeling. Fish of the black Amazon line III, where all fish are spotted and carry a single microchromosome,

TABLE 2

Pigmentation phenotypes in consecutive broods of black Amazons lines I and II

Fem no.	wt/sp Litter one	Litter two	Litter three	Litter four	Litter five	Litter six	Litter seven	Litter eight	Litter nine	All	Total	χ^2	k(sp)	$k - 0.5$	Result
							Line I								
1	1/8	5/2	2/2	3/1	7/4	12/3	18/3	7/3	9/2	64/28	92	14.1	0.304	−0.196	Under
2	2/5	1/6	1/5							4/16	20	7.2	0.800	0.300	Over
3	0/3	0/3	5/3							5/9	14	1.1	0.643	0.143	
4	2/3	1/7	1/3	1/5						5/18	23	7.3	0.783	0.283	Over
5	1/1	0/3	0/8	1/4						2/16	18	10.9	0.889	0.389	Over
6	0/5	3/4								3/9	12	3.0	0.750	0.250	
7	0/2	0/11	1/10	1/6	3/2	1/0	6/1			12/32	44	9.1	0.727	0.227	Over
8	0/2	0/9	3/20	13/11	13/9					29/51	80	6.1	0.638	0.138	Over
9	0/1	3/14								3/15	18	8.0	0.833	0.333	Over
10	0/5	0/9	2/8	3/6	1/1					6/29	35	15.1	0.829	0.329	Over
11	0/4	0/9	1/0	1/1						2/14	16	9.0	0.875	0.375	Over
12	3/5	1/1								4/6	10	0.4	0.600	0.100	
13	4/5	2/0	4/2	1/2	12/3	8/0				31/12	43	8.4	0.279	−0.221	Under
14	0/3	3/1	1/2							4/6	10	0.4	0.600	0.100	
15	3/4	2/2								5/6	11	0.1	0.545	0.045	
16	0/3	0/4	4/6	4/5						8/18	26	3.9	0.692	0.192	Over
17	4/9	4/9	9/0	4/0	5/0					26/18	44	1.5	0.409	−0.091	
18	5/0	3/4	5/3	8/8	6/8					27/23	50	0.3	0.460	−0.040	
19	0/3	1/3	1/6	2/6	0/4					4/22	26	12.5	0.846	0.346	Over
20	1/3	5/4	6/2	6/4						18/13	31	0.0	0.419	−0.081	
21	0/2	0/9	3/9	2/9	4/5	3/3	7/4			19/41	60	0.7	0.683	0.183	Over
22	8/16	10/18	8/3	3/1						29/38	67	1.2	0.567	0.067	
23	0/2	3/2	1/1							4/5	9	0.1	0.556	0.056	
24	1/0	3/1	2/1	6/1						12/3	15	5.4	0.200	−0.300	Under
25	6/4	5/0	9/1	1/3						21/8	29	5.8	0.276	−0.224	Under
All	41/98	55/135	69/95	60/73	51/36	24/6	31/8	7/3	9/2						
Total	139	190	164	133	87	30	39	10	11						
χ^2	23.4	33.7	4.1	1.3	2.6	10.8	13.6	1.6	4.5						
k(sp)	0.705	0.711	0.579	0.549	0.414	0.200	0.205	0.300	0.182						
$k - 0.5$	0.205	0.211	0.079	0.049	−0.086	−0.300	−0.295	−0.200	−0.318						
Result	Over	Over	Over	Random	Random	Under	Under	Random[a]	Under						

(*continued*)

TABLE 2

(Continued)

Fem no.	wt/sp Litter one	Litter two	Litter three	Litter four	Litter five	All	Total	χ^2	k(sp)	$k - 0.5$	Result
					Line II						
1	2/11	0/4				2/15	17	9.9	0.882	0.382	Over
2	0/5	0/3				0/8	8	3.0[b]	1.000	0.500	
3	0/1	0/6	1/4	1/3	1/1	3/15	18	8.0	0.833	0.333	
4	0/5	1/5				1/10	11	7.4	0.909	0.409	Over
5	0/1	0/7	0/1			0/9	9	4.4[b]	1.000	0.500	Over
6	2/6	2/11				4/17	21	8.0	0.810	0.310	Over
7	0/8	1/7	4/11	0/11	2/14	7/51	58	33.4	0.879	0.379	Over
8	0/1	0/5	3/3	2/5		5/14	19	4.3	0.737	0.237	Over
9	0/5	1/8	1/3	2/3	0/5	4/24	28	14.3	0.857	0.357	Over
10	2/6	1/5	2/22			5/33	38	20.6	0.868	0.368	Over
11	1/1	0/2				1/3	4	No statistical test because of low sample size			
12	0/6	0/6	2/16	1/13	0/13	3/54	57	45.6	0.947	0.447	Over
13	0/5	0/7	0/8	0/3		0/23	23	13.6[b]	1.000	0.500	Over
14	0/3	0/5				0/8	8	3.0[b]	1.000	0.500	
15	1/5	0/10	0/7	0/14	0/2	1/38	39	35.1	0.974	0.474	Over
All	8/69	6/91	13/75	6/52	3/35						
Total	77	97	88	58	38						
χ^2	48.3	74.5	43.7	36.5	27.0						
k(sp)	0.896	0.938	0.852	0.897	0.921						
$k - 0.5$	0.396	0.438	0.352	0.397	0.421						
Result	Over	Over	Over	Over	Over						

wt, wild type pigmented; sp, spotted; over, overtransmission; under, undertransmission; underlined, statistically significant (significant deviation from a 50% transmission rate).

[a] Not significant because of low sample numbers.

[b] Fisher test was used.

TABLE 3

Microchromosome heterogeneity in somatic cells

Pedigree/specimen	Pigmentation pattern	Cells with two micros	Cells with one micro
III/4 (WLC 931)	Spotted	4	22
III/4 (WLC 1043)	Spotted	6	33
III/9 (WLC 919)	Wild type	28	4
III/9 (WLC 1147)	Wild type	23	8
Black Amazon II (WLC 922–25/IV)	Spotted	39	8
Black Amazon I (WLC 533)	Spotted	9	2

showed regular telomere staining. Also in wild fish and laboratory lines derived from such fish, the single microchromosome clearly has a visible telomere. In metaphase spreads of spotted fish from the black Amazon line I and II, one microchromosome was labeled with the telomere probe, while the second one was unlabeled.

Hybridization with the telomere-specific probe labeled the terminal ends of all black molly chromosomes (Figure 5c). This excludes the existence of any specific chromosome end with undetectable $(TTAGGG)_n$ repeats as the origin of the material giving rise to the microchromosomes of line I and II is without a telomere.

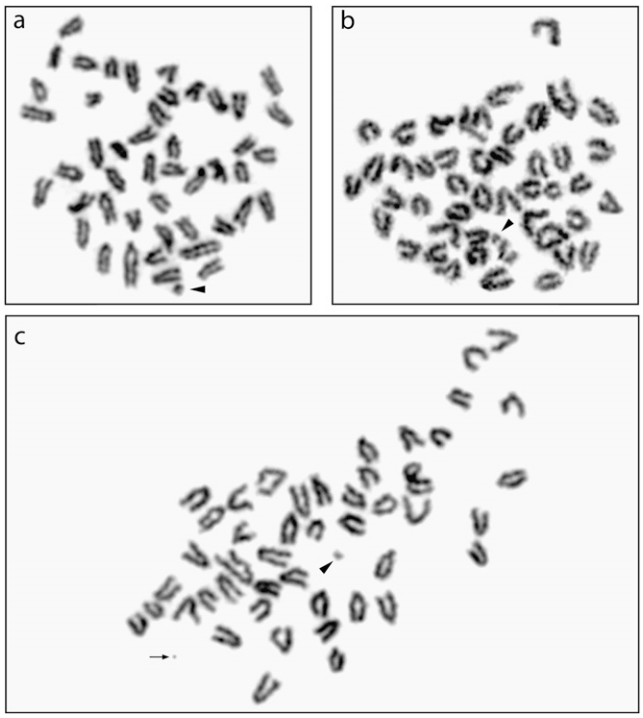

Figure 2.—Metaphase spreads of *P. formosa*. (a) Spotted fish of black Amazon line I; (b) wild type pigmented with one microchromosome (specimen 1199); and (c) spotted animal with two different sized microchromosomes (specimen 1043). Arrowhead represents the stable microchromosome, and the arrow represents "new" microchromosome. b and c are from the breeding experiment to follow the inheritance of microchromosomes.

DISCUSSION

Our analysis of microchromosome-carrying clones of *P. formosa* revealed that B chromosomes were inherited stably over many generations and no fish without B chromosomes were recorded.

Microchromosomes seem to be left over from the enzymatic process that normally clears the diploid ameiotic *P. formosa* eggs of the sperm nucleus after fertilization has occurred. The fact that the microchromosomes are regularly inherited in *P. formosa* pedigrees suggests that foreign DNA once incorporated into the asexual lineage can become a stable constituent of the genome and show germline transmission. The fact that a single microchromosome is found in all offspring despite lacking a homologous partner is obviously due to the absence of meiotic division in the asexual fish that produces diploid eggs.

The somatic instability of B chromosomes is obviously something that is due to mitotic events. It will affect the microchromosomes during the mitotic divisions of the primordial germs and the oogonia as well.

Telomere staining revealed a possible explanation for the observed stability of the single microchromosome situation and the genetic instability of the second and third microchromosome. Cells with one, two, or even three microchromosomes exist in the same individual, but only one microchromosome is ever stained with the telomere probe. This suggests that the microchromosomes lacking telomere staining have been eliminated in those cells of the soma carrying only the stable microchromosome.

The absence of functional telomeres is indicated by the inability to stain certain microchromosomes with the $(CCCTAA)_3$ PNA probe. This may be due to the lack of a threshold level of telomere repeats (short repeats). It is also likely that telomere repeats may be completely absent in the microchromosomes, because microchromosomes are chromosome fragments derived from anywhere along the chromosome and are remnants from the degradation process of the sperm nucleus DNA.

For stable inheritance of a chromosome, functional centromeres and telomeres are important. C-banding indicated the occurrence of heterochromatin, which is usually connected to centromeric regions of chromosomes. Although this technique does not prove the presence of a centromere, the mere presence of microchromosomes in most cells of an individual indicates that it has passed through many divisions, for which a functional centromere is indispensable. However, the absence of functional telomeres correlates with instability and can explain the elimination of certain microchromosomes. In human cancer cells, it has been found that mitotic instability of chromosomes is correlated with dysfunctional telomeres (Gisselsson and Hoglund 2005). In this context, it is the shortest telomeres that mostly constitute telomere dysfunction (Hemann *et al.*

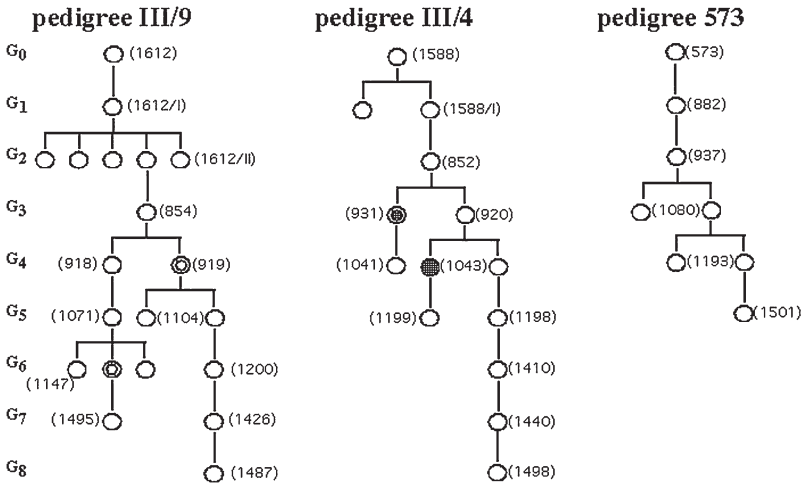

FIGURE 3.—Pedigrees of animals analyzed for inheritance of microchromosomes. Open circle, wild type pigmented; filled circle, spotted; double circle, fish with additional microchromosome. G_0–G_8, generation number. Numbers in parentheses are specimen codes from the chromosomes analysis.

2001). Studies in telomerase-deficient mice lead to the conclusion that functional telomeres are involved in mediating metaphase chromosome alignment and maintaining functional spindles (LIU *et al.* 2002).

Dysfunctional telomeres may also explain the frequent occurrence of unspotted fish in the offspring of spotted black Amazon lines. During mitosis of the germ cells, the microchromosome without functional telomeres may have a tendency to get lost. This would predict that the pigmentation locus in these lines is on the chromosomes without telomeres.

When the offspring of single females were analyzed for inheritance of the spotted pigmentation phenotype, there was a remarkable difference in transmission of microchromosomes of lines I and II, both of which carry microchromosomes with and without a pigmentation locus. Line I showed a tendency to undertransmit the spotted pigment pattern, while line II showed a high tendency of overtransmission. This could be interpreted as a different behavior of melanic B chromosomes. For example, the melanic B chromosome in the younger line II transmits much better than in the older line I. Consistent with CAMACHO *et al.* (1997), it is possible that some resistance has evolved in the old line due to a change in the genotype of the host A chromosomes, where the melanic B chromosome transmits poorly. However, even in line I, a significant number of females showed overtransmission. Line I is a genetic clone with respect to the A chromosomal genome due to the gynogenetic mode of reproduction, hence a different genetic background cannot explain the different genetic behavior of the melanic B chromosome.

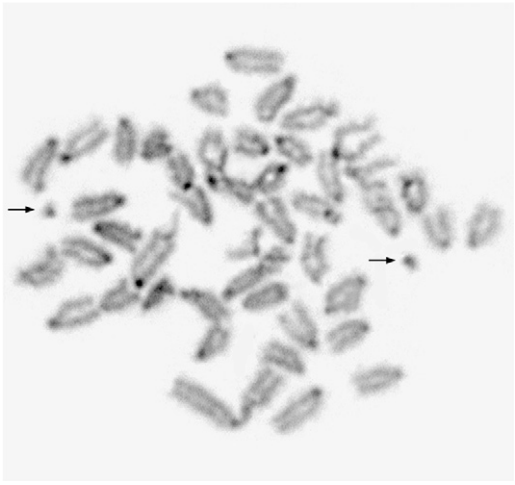

FIGURE 4.—C-banding of metaphase chromosomes of a spotted black Amazon line I (WLC 533) with two microchromosomes. Arrows indicate the dark staining of heterochromatin on both microchromosomes.

An alternative explanation is that the melanic B chromosomes of the separate lines show different inheritance because they are actually dissimilar in nature. This dissimilarity occurs because the microchromosomes originated from different introgressions. The black molly has two nonallelic pigmentation loci from which the melanic B chromosomes can be derived, and genetic material accompanying the pigmentation locus may be different as well.

A statistically well-supported hypothesis is that the inheritance of the melanic B chromosome decreases in consecutive broods of the same female. An increase of genotypic instability with maternal age has been observed in other species with a prolonged meiotic prophase (DJAHANBAKHCH *et al.* 2007). In the ameiotic Amazon molly, a different mechanism might operate. Fish generate primary oocytes throughout adult life. Therefore, oogonia of older females have had more mitotic divisions and thus a higher chance that a mitotically instable microchromosome will be lost. Consequently, the proportion of spotted offspring, which have the pigmentation gene on an instable microchromosome, will decrease. An age effect of transmission of B chromosomes was reported in the grasshopper *Myrmeleotettix maculatus* (SHAW and HEWITT 1984), but not in the grasshopper, *Eyprepocnemis plorans* (BAKKALI *et al.* 2002).

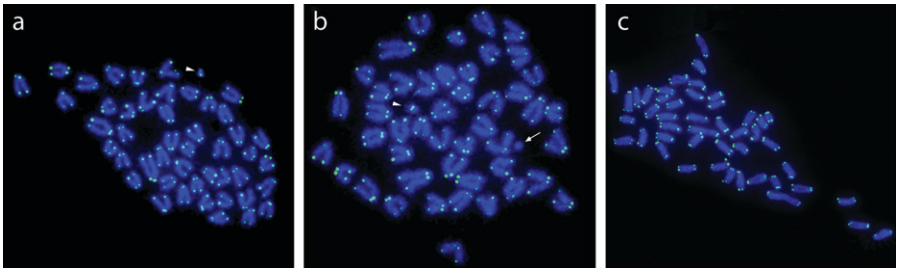

FIGURE 5.—Telomere staining of metaphase chromosomes of black Amazon line II (WLC 922-25/IV) and the host species (black molly) with FITC labeled $(CCCTAA)_3$ PNA oligonucleotide. (a) Metaphase with one microchromosome (arrowhead). (b) Metaphase from the same fish with two microchromosomes, only one of them being labeled with the telomere probe (arrowhead). (c) Mitotic chromosomes of a black molly male displaying specific telomere signals on each end of all chromosomes.

The presence of telomeres is obviously responsible for the stable inheritance of a microchromosome. It can be assumed that microchromosomes that show overtransmission in lines I and II are those that have functional telomeres. This may also explain the persistence of microchromosome-carrying clones in certain river systems in the wild (LAMATSCH *et al.* 2004). So far all microchromosome-bearing fish collected from natural populations had only one microchromosome. The stable lines obtained in the laboratory also carry the single telomere-containing microchromosome, although the original introgression event contributed two or even three chromosomes. The reason why the fish have only a single stable microchromosome is completely unclear.

Our study also indicates that further introgression events can happen as seen by the appearance of a new microchromosome. However, the new introgression became unstable rapidly. Surprisingly, in both the pedigrees (III/4 and III/9) the unstable marker chromosomes arising through the latest introgression are extremely tiny. This is consistent with a comparative study on the stability of minichromosomes in different species, which revealed a lower size as a limiting factor for a stable chromosome transmission (SCHUBERT 2001).

The question of whether certain parts of the genome (*e.g.*, telomere- or centromere-near regions) are more prone to end up in a microchromosome cannot be answered. From our data of laboratory-bred fish, it appears that the regions containing the pigmentation gene are more often represented. However, due to the conspicuous phenotype, fish with such microchromosomes are much more readily identified. In the black Amazon lines I and II, stable microchromosomes do not carry the pigmentation locus, whereas the unstable second microchromosome leads to pigment spots and is easily maintained in the stock populations because of selective breeding. In the three pedigrees analyzed, 4 of 39 individuals had additional microchromosomes of paternal origin. Two of the four new microchromosomes carried a pigmentation gene. Given the fact that only two of the $n = 23$ chromosomes of the black molly harbor a pigmentation locus, it can be argued that the chromosomal fragments from the pigmentation regions show a predisposition to escape elimination, which is not shown by other regions of the genome. To answer these questions and to understand the relationship between the pigmentation mosaicism and the number of microchromosomes, isolation of microchromosomes by microdissection is required. Such material can then be used for cloning and sequencing as well for in-situ hybridization.

The fact that telomere-containing microchromosomes are stably inherited over at least eight generations (and probably much longer) becomes interesting when using a probabilistic approach to calculate the genetic contribution of a male to its offspring. In a large sexually reproducing population, the genetic contribution of a certain individual male is rapidly diluted out: children share ~50% of their DNA with their father, the grandchildren 25%, great-grandchildren 12.5%, and so on. In G_7 certainly <1% of a particular male's genome is left in any individual offspring. However, the paternally derived microchromosomes in *P. formosa* constitute ~0.5–1% of the whole genome. Once a microchromosome is stably integrated into the germline, it may be transmitted unchanged. After G_7, theoretically, more paternal DNA will be present in the offspring of an asexual female with a microchromosome than in a sexual lineage. Of course, selection cannot favor males that introduce genes into the Amazon mollies genome, just as this scenario cannot explain why host males court and copulate with the asexuals who are the "wrong" females, because there is no evidence of a "genetic feedback" mechanism into the host species. A microchromosome can be seen as an introduction of selfish genetic elements, which is consistent with a common view that B chromosomes are selfish DNA elements (JONES 1985; SHAW and HEWITT 1990; MCVEAN 1995; CAMACHO *et al.* 2000). To determine whether microchromosomes increase the genetic diversity of *P. formosa* and/or compensate for mutation in genes that cannot be purged due to the absence of recombination (Muller's ratchet), analysis of microchromosomal gene content and expression has to be performed. Whether the microchromosome-carrying individuals are under natural selection and may enjoy some selective advantages can, however, be inferred from comparing fitness components of clones with and without microchromosomes.

Another important point for evaluating the importance of the introgression of paternal DNA from host species is the frequency of the event. So far it is unknown whether the microchromosomes found in natural populations of *P. formosa* in the Río Purificación river system go back to a few introgression events or if they are the result of many independent events. The differences in size of the microchromosomes of the lines analyzed here could be the result of independent introgression events or of clonal diversity due to loss or gain of sequences on the microchromosome. However, in the laboratory, introgression events are quite frequent (up to 10%) and the transmission of newly acquired independent microchromosomes has been observed several times. This speaks for a relatively frequent introgression of paternal DNA through B chromosomes.

In summary, more than initially supposed, microchromosomes can be inherited in the clonal Amazon molly for many generations. We show here that microchromosome mitotic instability does not tend to decrease its frequency considerably since, in most cases, the spotted phenotype associated with it showed only a low chance to be lost during reproduction. However, to complete the scenario on the biological role of these extra elements, the analysis of possible fitness differences associated with microchromosome presence is necessary to ascertain whether the potential increase in genetic diversity expected from microchromosome introgression is actually a selective advantage in Amazon molly natural populations.

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LITERATURE CITED

Bakkali, M., F. Perfectti and J. P. M. Camacho, 2002 The B-chromosome polymorphism of the grasshopper *Eyprepocnemis plorans* in North Africa: II. Parasitic and neutralized B-1 chromosomes. Heredity **88:** 14–18.

Bougourd, S. M., and R. N. Jones, 1997 B chromosomes: a physiological enigma. New Phytol. **137:** 43–54.

Camacho, J. P. M., T. F. Sharbel and L. W. Beukeboom, 2000 B-chromosome evolution. Philos. Trans. R. Soc. Lond., B, Biol. Sci. **355:** 163–178.

Camacho, J. P. M., M. W. Shaw, M. D. Lopez Leon, M. C. Pardo and J. Cabrero, 1997 Population dynamics of a selfish B chromosome neutralized by the standard genome in the grasshopper *Eyprepocnemis plorans*. Am. Nat. **149:** 1030–1050.

Djahanbakhch, O., M. Ezzati and A. Zosmer, 2007 Reproductive ageing in women. J. Pathol. **211:** 219–231.

Gisselsson, D., and M. Hoglund, 2005 Connecting mitotic instability and chromosome aberrations in cancer—Can telomeres bridge the gap? Semin. Cancer Biol. **15:** 13–23.

Green, D. M., 1990 Muller ratchet and the evolution of supernumerary chromosomes. Genome **33:** 818–824.

Hemann, M. T., M. A. Strong, L. Y. Hao and C. W. Greider, 2001 The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. Cell **107:** 67–77.

Jones, R., 1985 Are B chromosomes selfish?, pp. 397–425 in *The Evolution of Genome Size*, edited by T. Cavalier-Smith. Wiley, London.

Jones, R., and H. Rees, 1982 *B-chromosomes*. Academic Press, New York.

Kallman, K. D., 1975 The platyfish, *Xiphophorus maculatus*, pp. 81–132 in *Handbook of Genetics*, edited by K. D. Kallman. Plenum Press, New York.

Kondrashov, A. S., 1988 Deleterious mutations and the evolution of sexual reproduction. Nature **336:** 435–440.

Lamatsch, D. K., I. Nanda, I. Schlupp, J. T. Epplen, M. Schmid *et al.*, 2004 Distribution and stability of supernumerary microchromosomes in natural populations of the Amazon molly, *Poecilia formosa*. Cytogenet. Genome Res. **106:** 189–194.

Liu, L., M. A. Blasco and D. L. Keefe, 2002 Requirement of functional telomeres for metaphase chromosome alignments and integrity of meiotic spindles. EMBO Rep. **3:** 230–234.

McVean, G. T., 1995 Fractious chromosomes—hybrid disruption and the origin of selfish genetic elements. Bioessays **17:** 579–582.

Muller, H. J., 1932 Some genetic aspects of sex. Amer. Nat. **66:** 118–138.

Nanda, I., M. Schartl, W. Feichtinger, I. Schlupp, J. Parzefall *et al.*, 1995 Chromosomal evidence for laboratory synthesis of a triploid hybrid between the gynogenetic teleost *Poecilia formosa* and its host species. J. Fish Biol. **47:** 619–623.

Nur, U., J. H. Werren, D. G. Eickbush, W. D. Burke and T. H. Eickbush, 1988 A selfish B-chromosome that enhances its transmission by eliminating the paternal genome. Science **240:** 512–514.

Östergren, G., 1945 Parasitic nature of extra fragment chromosomes. Botaniska Notiser **2:** 157–163.

Palestis, B. G., R. Trivers, A. Burt and R. N. Jones, 2004 The distribution of B chromosomes across species. Cytogenet. Genome Res. **106:** 151–158.

Schartl, M., I. Nanda, I. Schlupp, B. Wilde, J. T. Epplen *et al.*, 1995 Incorporation of subgenomic amounts of DNA as compensation for mutational load in a gynogenetic fish. Nature **373:** 68–71.

Schröder, J., 1964 Genetische Untersuchungen an domestizierten Stämmen der Gattung Mollienesia (Poeciliidae). Zoologische Beiträge **10:** 369–463.

Schubert, I., 2001 Alteration of chromosome numbers by generation of minichromosomes—Is there a lower limit of chromosome size for stable segregation? Cytogenet. Cell Genet. **93:** 175–181.

Schultz, R. J., and K. D. Kallman, 1968 Triploid hybrids between the all-female teleost *Poecilia formosa* and Poecilia sphenops. Nature **219:** 280–282.

Shaw, M. W., and G. M. Hewitt, 1984 The effect of temperature on meiotic transmission rates of the B-chromosome of *Myrmeleotettix maculatus* (Orthoptera, Acrididae). Heredity **53:** 259–268.

Shaw, M. W., and G. M. Hewitt, 1990 B chromosomes, selfish DNA and theoretical models: Where next? Oxf. Surv. Evol. Biol. **7:** 197–223.

Sumner, A. T., 1972 Simple technique for demonstrating centromeric heterochromatin. Exp. Cell Res. **75:** 304–306.

Thomson, R. L., 1984 B-chromosomes in *Rattus fuscipes*. 2. The transmission of B-chromosomes to offspring and population studies - support for the parasitic model. Heredity **52:** 363–372.

White, M. J. D., 1973 *Animal Cytology and Evolution*. Cambridge University Press, London.

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