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Genetic verification of induced gynogenesis and microsatellite-centromere mapping in the barfin flounder *Verasper moseri*

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Abstract

Primer sets were newly developed for 34 polymorphic microsatellite loci in the barfin flounder *Verasper moseri*. Mendelian inheritance was confirmed by examining the genotypic segregation in 2 normal diploid full-sib families. All the 34 loci showed genotypic segregation according to the Mendelian manner of inheritance; in some cases, null alleles were assumed. The genotypes at 27 loci were also examined in 4 meiotic gynogenetic diploid lines produced by fertilizing eggs with UV-irradiated sperm, followed by inhibition of the second meiotic division by cold shock. The absence of paternal alleles verified the success of gynogenetic development in all 4 meiotic gynogenetic diploid lines; the proportion of heterozygous progeny of a heterozygous mother, i.e., the frequency of second division segregation (y), was used to estimate the map distance of each microsatellite locus in relation to the centromere. Marker-centromere distances were estimated to be in the range of 0 to approximately 50 centiMorgan (cM) under the assumption of complete interference. Using 8 diagnostic loci located at the telomeric region of the chromosome, complete homozygosity was confirmed in 1 mitotic gynogenetic diploid line that was produced by suppressing the first cleavage via hydrostatic pressure shock.

Keywords: *Verasper moseri*, microsatellite, gynogenesis, genetic map, half-tetrad analysis.

1. Introduction

The barfin flounder *Verasper moseri* is a large flatfish species that inhabits cold sea basins near the east coast of Hokkaido, Japan. This species is a promising candidate for aquaculture and stocking due to its high commercial value and stable growth in cold water. Similar to the growth of other flatfishes, the barfin flounder exhibits sexually dimorphic growth, and the females grow to adult size faster than males (Mori et al., 1999). Thus, sex manipulation is required in order to achieve an all-female population, especially in aquaculture. The induction of gynogenetic diploids is considered a useful technique to identify the sex determination system in the barfin flounder and to generate an all-female population in a male heterogametic species. Meiotic gynogenetic diploids can be produced by inhibiting the extrusion of the second polar body, while completely homozygous mitotic gynogenetic diploids are produced by the inhibition of the first cleavage after the activation of eggs with genetically inert UV-irradiated sperm.

In order to produce an all-female population and for future cloning of the barfin flounder, meiotic and mitotic gynogenetic diploids have been experimentally produced after the optimization of treating conditions to induce gynogenesis and duplicate the chromosomes (Mori et al., 2004). In other species, gynogenesis is usually induced using UV-irradiated sperm of a different species in order to eliminate sporadic or occasionally appearing diploids by inviable hybridization (Arai, 2001). However, in barfin flounders, meiotic gynogenesis has been induced using irradiated sperm from the same species because it is difficult to collect heterospecific sperm during the spawning season (Mori et al., 2004). Thus, the possible contamination of the

putative gynogenetic progeny with sporadic diploids due to insufficient genetic inactivation of the irradiated sperm cannot be ruled out. Genetic verification of gynogenesis, i.e., all-female inheritance, is an important step in chromosome manipulation in order to evaluate the success of induction.

At the level of homozygosity, meiotic and mitotic gynogenesis differ genetically (Arai, 2001; Nagy and Csani, 1984; Palti et al., 2002). Complete homozygosity can be achieved by mitotic gynogenesis in a single generation. Thus, mitotic gynogenesis is an effective technique for producing inbred lines of fish in a substantially shorter time. Such completely homozygous fish that spawn genetically uniform eggs are indispensable for cloning via a second cycle of gynogenesis. However, contamination by meiotic gynogenetic diploids due to the spontaneous inhibition of the release of the second polar body should be eliminated (Arai, 2001). Therefore, precise separation of the 2 kinds of gynogenetic diploids is necessary for successful cloning of a target aquaculture species. In the meiotic gynogenetic diploids of a heterozygous mother, the locus at the distal portion of the chromosome is considered heterozygous because of its high gene or marker-centromere recombination rate, as reported for other fish species (Thorgaard et al., 1983; Guyomard, 1984; Estoup et al., 1993; Kauffaman et al., 1995; Lindner et al., 2000; Matsuoka et al., 2004). Thus, putative mitotic gynogens must be identified by their complete homozygosity at such diagnostic markers (Suwa et al., 1994; Morishima et al., 2001; Ezaz et al., 2004).

Codominant microsatellite markers are superior for use in parentage studies because of their numerous polymorphisms, ubiquitous distribution in the genome, and simple sampling and preservation requirements (Ferguson

et al., 1995). In the present study, new microsatellite markers were developed, and their Mendelian inheritance was examined by observing genotypic segregation in 2 normal diploid full-sib families generated by normal fertilization. We used 27 microsatellite markers to confirm the success of gynogenetic development in 4 meiotic gynogenetic diploid lines. The microsatellite-centromere map distance for a polymorphic locus was estimated from the frequency of second division segregation (y) as the proportion of heterozygous meiotic gynogenetic diploid progeny of a heterozygous mother. Microsatellite loci with high marker-centromere recombination frequencies, located in the telomeric region of chromosomes, were then used to verify complete homozygosity of the mitotic gynogenetic diploids.

2. Materials and methods

2.1. Induction of gynogenetic fish

A barfin flounder broodstock was transported from the Hokkaido Institute of Mariculture, Shikabe (reorganized in 2006 to the Hokkaido Mariculture Fisheries Experiment Station, Muroran), to the Hokkaido Central Fisheries Experiment Station, Yoichi, and was reared in 4-kL tanks. Eggs and sperms were obtained from 5 different females and males (Table 1), respectively. In this study, the parental fishes and their progenies from 2 normal diploid full-sib families (ND-1 and ND-2), 4 meiotic gynogenetic diploid lines (MEI-1, MEI-2, MEI-3, and MEI-4), and 1 mitotic gynogenetic diploid line (MIT-1) were used for the genetic analyses. Meiotic gynogenetic diploids were obtained by fertilizing the eggs with UV-irradiated sperm

(40–45 mJ/cm^2) and subsequent cold shock (–1.5 °C) for a duration of 70 min at 7 min after insemination (Mori et al., 2004). The same set of parents (F1 and M1) were used to produce the normal diploid full-sib family ND-1 and the meiotic gynogenetic line MEI-1. The female F2 and male M2 parents were used to produce the full-sib family ND-2 and the meiotic gynogenetic line MEI-2. The meiotic gynogenetic lines MEI-3 (parents: F3 and M3) and MEI-4 (parents: F4 and M4) comprised only meiotic gynogenetic diploids, and no counterpart normal diploids were produced. The mitotic gynogenetic diploid line MIT-1 was produced from F5 and M5 by inhibiting the first cleavage via hydrostatic pressure treatment (650 kg/cm^2) for a duration of 6 min at 180–240 min after fertilization (Mori et al., 2004).

2.2. Isolation of microsatellite markers

Diploid barfin flounders cultured at the Hokkaido Central Fisheries Experiment Station were the source of DNA for the isolation of microsatellite regions. DNA was isolated and purified using the routine phenol-chloroform procedure. Microsatellite regions were isolated in a manner similar to that used for the loach *Misgurnus anguillicaudatus* (Morishima et al., 2001). Forward and reverse primers were designed based on the unique regions flanking each microsatellite repeat. Sequence data were deposited in DDBJ, and the accession numbers are shown in Table 2.

2.3. Microsatellite genotyping

DNA was extracted from the blood or muscle and purified by the phenol-chloroform method. The DNA samples were adjusted to a

concentration of 100 ng/μL and used for PCR. Microsatellite genotyping was performed using an automated sequencer and M13-tailed primers (Zhou et al., 2002). The reaction mixture (10 μl) used for the amplifications contained 100 ng template DNA, 40 μM dNTPs, 0.3 pM M13-tailed forward primer, 3.0 pM reverse primer, 3.0 pM fluorescence-labeled M13 primer, and 0.025 U *Taq* polymerase (TaKaRa, Otsu, Japan), and the amplification conditions were as follows: denaturation for 1 min at 94 °C; 30–35 cycles of denaturation for 15 s at 94 °C, annealing for 15 s at 56 °C, and extension for 30 s at 72 °C; followed by final extension for 1 h at 72 °C. Following PCR, 1 μl of each product and 0.1 μl of LIZ size standard (Gene Scan 500 LIZ size standard, Applied Biosystems, Foster City, CA, USA) were added to 10 μl of HiDi Formamide for electrophoresis on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Electrophoretograms were analyzed using the Gene Mapper 3.7 software (Applied Biosystems). The Mendelian inheritance at each locus was verified by observing the genotypes of the parent fish and their progenies in the 2 full-sib families ND-1 and ND-2, and it was statistically confirmed using the chi-square (X^2) test ($\alpha = 0.05$). The significance level for multiple tests was adjusted to confirm the overall statistical significance ($\alpha = 0.05/n$; n = number of tests performed) (Cooper, 1968). The microsatellite loci in which the paternal alleles could be distinguished from the maternal ones were useful for the genetic confirmation of successful meiotic gynogenesis. For microsatellite-centromere mapping, microsatellite loci showing heterozygosity with regard to the female parent were screened in the 4 meiotic gynogenetic lines (MEI-1—MEI-4).

2.4. Microsatellite-centromere mapping

The microsatellite-centromere (M-C) recombination rate (frequency of second meiotic division segregation = y) was estimated from the proportion of recombinant heterozygous genotypes in the half-tetrad meiotic gynogenetic progeny of the heterozygous mother at a specific locus. Assuming complete chiasma interference (only a single cross-over event between non-sister chromatids), the gene (marker)-centromere distance in centiMorgans (cM) may be obtained as $100(y/2)$, where y is the percentage of heterozygous gynogenetic diploid progeny observed in the total number of samples (Thorgaard et al., 1983.)

3. Results

3.1. Development of microsatellite markers

A total of 3290 colonies from the genomic library were screened by hybridization with the probe $(GT)_{25}$. Consequently, a positive signal was obtained from 172 clones, and 124 of them were sequenced. These clones contained at least one $(GT)_n$ /$(CA)_n$ repeat. However, many such sequences were not used due to the proximity of the microsatellite to the cloning site, degenerate repetitive sequences in one of the flanking regions, extremely small or large microsatellites, and/or unreadable sequences. Finally, specific primers were designed for 34 polymorphic microsatellite regions at a temperature of 56 °C, and fragments of 100–400 base pairs were amplified. Table 2 shows the core sequences, forward and reverse primer sequences, annealing temperatures, number and size of alleles, and the DDBJ accession

numbers of these loci.

3.2. Verification of Mendelian segregation

Genotypic segregation was examined at 34 microsatellite loci in both full-sib families (Table 3). Among these, 30 exhibited good conformation with Mendelian segregation. For example, in the full-sib family ND-2, a cross between the female (*152/154*) and male (*150/154*) at *the Vemos1* locus produced 4 genotypes (*152/150*, *152/154*, *150/154*, and *154/154*) in the progeny, and the genotypic frequencies were in good accordance with the expected ratio (1:1:1:1); this indicated Mendelian inheritance. Significant deviation from the expected Mendelian ratio was detected at the *Vemos1*, *Vemos13, Vemos26,* and *Vemos31* loci in ND-1 or ND-2 (χ^2 test, $\alpha = 0.05$). However, after multiple test corrections ($\alpha/30$), this deviation was not significant.

At *Vemos44* and *Vemos66*, inconsistent genotypic segregations were observed between the parents and progenies in ND-1. The observed parental genotypes at *Vemos44* in ND-1 were *262/262* in the female and *262/264* in the male. Therefore, the genotypic frequencies in the progeny were expected to be equal between these genotypes. However, in addition to these 2 expected genotypes, genotype *264/264* was also observed in the progeny. This result was explained by the involvement of a null allele in the female genotype (*262/null*). By considering this null allele, the genotypic frequencies fit well to the Mendelian inheritance model (Table 3). A similar distortion observed at *Vemos66* in ND-1 was also explained by the assumption of a null allele in the male parent (Table 3).

3.3. Verification of meiotic gynogenesis

In order to examine all-female inheritance in the gynogenetic diploid lines, 27 loci in which the paternally derived alleles were distinguished from the maternally derived ones were selected. The lack of contribution by the paternal alleles was confirmed in all the offspring from all 4 meiotic gynogenetic diploid lines examined in the present study. Thus, successful meiotic gynogenesis was achieved at least in the 4 meiotic gynogenetic lines examined (Table 4).

3.4. M-C map distance

The proportions of the heterozygous genotypes at the 27 microsatellite loci in the 4 meiotic gynogenetic diploid lines (MEI-1, MEI-2, MEI-3, and MEI-4) are given in Table 4. Unequal frequencies of the 2 homozygous classes were significant in 6 loci in the four meiotic gynogenetic diploid lines analyzed (Table 4; *Vemos6* in MEI-3, *Vemos11* in MEI-1, *Vemos29* in MEI-1, *Vemos39* in MEI-2, *Vemos60* in MEI-1, and *Vemos62* in MEI-4). The deviation from the equal number of homozygotes remained significant at *Vemos*11, *Vemos*39, and *Vemos60*, although multiple test corrections were considered (n = 30; n, number of tests performed). We also tested for homogeneity in the proportion of heterozygotes between the gynogenetic lines at each locus based on the contingency χ^2 analysis. *Vemos43* (χ^2 = 4.83; df = 1), *Vemos57* (χ^2 = 4.31; df = 1), *and Vemos76* (χ^2 = 10.42; df = 1) showed significant differences between the gynogenetic diploid lines (Table 4). However, none of these differences were significant when the number of tests

(n = 19) was considered. Thus, pooled results from different families were used to estimate the recombination frequency (y). The M-C recombination frequencies for the 27 loci ranged from y = 0 at *Vemos6* and *Vemos29* to y = 0.99 at *Vemos68.* Therefore, the marker-centromere map distance was estimated to be between 0 and 49.5 cM in the microsatellite loci of the barfin flounder. Of the 27 loci, 14 were estimated to be located in the telomeric region; 10, in the centromeric region; and 3, in intermediate region of the chromosome.

3.5. Verification of mitotic gynogenesis

Vemos1, *Vemos10, Vemos18, Vemos25*, *Vemos26, Vemos42, Vemos44, Vemos47, Vemos57, Vemos65, Vemos66, Vemos68, and Vemos76* were used as diagnostic loci because they showed a very high M-C recombination rate, and they were selected to identify mitotic gynogenetic diploids produced by the inhibition of the first cleavage. Putative mitotic gynogenetic progeny were completely homozygous at all the 8 diagnostic loci (*Vemos10, Vemos25*, *Vemos26, Vemos42, Vemos57, Vemos65, Vemos66, and Vemos68*), in which the mother fish showed heterozygosity (Table 5).

4. Discussion

4.1. Polymorphism and inheritance

The 34 microsatellite loci analyzed were polymorphic because 2 to 10 alleles per locus were detected in the 9 barfin flounder parents used in this study. Genetic analyses of the control crosses of the 2 normal diploid full-sib families ND-1 and ND-2 indicated that genotypic segregation at all 34 loci

conformed to Mendelian expectations. Although the allelic segregation at *Vemos11, Vemos39*, and *Vemos60* in the control normal diploid full-sib family (Table 3) conformed to Mendelian expectations, the unequal frequencies of 2 homozygotes were observed in 1 of the 4 meiotic gynogenetic diploid lines even after multiple test corrections (Table 4). The aberrant segregation in these loci might have resulted from the reduced viability of homozygous individuals, probably due to the unmasking of a recessive lethal or semi-lethal gene tightly linked to a specific locus.

From the analyses of the normal diploid full-sib families, the presence of null alleles was considered at the *Vemos44* and *Vemos66* loci based on the fact that the genotypic segregation was well explained by this assumption. The null alleles detected in the present study might have resulted from mutations such as substitutions, insertions, or deletions in one or both priming sites, thus preventing the binding of DNA strands and oligoprimers, as suggested by Callen et al. (1993).

4.2. Confirmation of meiotic gynogenetic development

Meiotic gynogenesis is generally easier to induce than mitotic gynogenesis, and it has been widely used to study sex determination and manipulation (Arai, 2001). If the male heterogametic (XX female-XY male) sex determination system is involved, the gynogenetic diploid progeny will be all female because there is no contribution of the sperm with the Y chromosome. Based on the sex ratio in gynogenetic diploid progeny, the involvement of male heterogamety has been suggested in the Japanese flounder (Yamamoto, 1999), marbled sole (Kakimoto et al., 1994), and other fish species (see review Pandian et al., 1998; Felip et al., 2001; Arai, 2001). In

contrast, if the female heterogamety (ZW female-ZZ male) system is involved, the gynogenetic diploids will exhibit various proportions of male (ZZ), super female (WW), and sometimes female (ZW) progeny depending on the cross-over or recombination between the centromeres and the sex-determining element. Based on the sex ratios in gynogenetic diploid progeny, the female heterogametic sex model has been strongly suggested in the rosy bitterling (Kawamura et al., 1998) and some species of sturgeons (Van Eenennaam et al., 1999; Omoto et al., 2005; Flynn et al., 2006).

To elucidate sex determination, complete success of gynogenetic development is required because sporadic diploids, due to the inadequate inactivation of sperm, will contaminate the gynogenetic lines and cause a deviation from the true sex ratio. In particular, in the barfin flounder, sex was reported to fluctuate depending on the water temperature at the stage of gonadal differentiation, and such spontaneous sex reversal from genetic females to physiological males is normally inhibited by rearing the fish at 14 °C during the critical period (Goto et al., 1999). The microsatellite analyses showed no genetic contribution of the paternal parent in all the induced gynogenetic diploid progeny examined in this study. The sex ratio in genetically verified gynogenetic diploids must be examined in order to identify an accurate temperature-dependent sex determination system in the barfin flounder.

4.3. Mapping of microsatellite loci in relation to the centromere

Using the meiotic gynogenetic diploids, the recombination rates of the microsatellite loci were estimated in relation to the centromere, and they

were found to range from y = 0 to 0.99. At a locus, the maximum recombination frequency in relation to the centromere is theoretically expected to be 0.667 (2/3) if there is no chiasma interference (Perkins, 1962). However, the recombination rates of 25 loci were higher than the theoretical value (Table 4). This strongly suggests the existence of positive chiasma interference after a single chiasma in the barfin flounder. Such a complete interference has been also reported in other fish species (Thorgaard et al., 1983; Guyomard, 1984; Arai et al., 1991; Liu et al., 1992; Estoup et al., 1993; Suwa et al., 1994; Kauffman et al., 1995; Lindner et al., 1999; Morishima et al., 2001; Matsuoka et al., 2004). Thus, in the barfin flounder, the M-C map distances varied between 0 and 49.5 cM. These mapping data are useful to differentiate the type of gynogenetic diploids for cloning using the chromosome manipulation technique, as described in the next section. In addition, gene (marker)-centromere mapping data are also informative for the determination of the centromere location in a linkage group (Johnson et al., 1996; Sakamoto et al., 2000).

4.4. Complete homozygosity of mitotic gynogenetic diploids

Mitotic gynogenesis is the most important chromosome manipulation technique because it produces a completely homozygous generation that is indispensable for the generation of a clone lineage (Arai, 2001). In the present study, all the mitotic gynogenetic progeny were confirmed to be genetically homozygous at the diagnostic loci, which yielded high recombination rates in relation to the centromere. Thus, this result indicated the successful induction of mitotic gynogenesis in the barfin flounder. These

completely homozygous gynogenetic progeny will be available as mother fish for the production of cloned barfin flounders by the second cycle of gynogenesis in the next generation. Cloned fish have been successfully produced in Japan, for example, the fresh water fish species *ayu Plecoglossus altivelis* (Han et al., 1991) and the amago salmon *Oncorhynchus rhodurus* (Kobayashi et al., 1994) as well as the marine fish species the Japanese flounder *Paralichthys olivaceus* (Yamamoto, 1999) and the red sea bream *Pagrus major* (Kato et al., 2001). Thus, cloning the barfin flounder in the near future appears promising.

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Table1. Full-sib families and gynogenetic diploid lines analyzed in the present study.

Family/ Line	Abbreviation	Parental fish		No.of progeny
		Female	Male	
Normal diploid full-sib family	ND-1	F1	M1	48
	ND-2	F2	M2	30
Meiotic gynogenetic diploid line	MEI-1	F1	M1	40
	MEI-2	F2	M2	40
	MEI-3	F3	M3	40
	MEI-4	F4	M4[a]	40
Mitotic gynogenetic diploid line	MIT-1	F5	M5	10

[a] = unknown due to the loss of sample.

Table2. Core sequences, forward and reverse primer sequences, annealing temperatures, number and size of allele, allele sizes and DDBJ accession numbers of 34 microsatellite regions newly developed

Microsatellite locus	Core sequence	Primer sets (5'-3') (F: forward;R: reverse)	Annealing temperature (℃)	Number of alleles [a]	Allele size(bp)	DDBJ access number
Vemos1	$(GT)7GA(GT)_{12}$	F:TAAATGTGCTGTAGGGTGGA R:TTGGACCACTGACTCATCTG	56	4	138-154	AB237644
Vemos4	$(GT)_{12}$	F:GGAGCACCACATTCTTCTCA R:CTCCCAGCCAAATGCACTAA	56	8	159-199	AB237645
Vemos6	$(TG)_{38}$	F:CGTGATACTGTGCTGATTGC R:CTTTGGTAGTTCACTCTCCC	56	7	238-254	AB237646
Vemos7	$(GT)_{12}$	F:TATCTGCCAAACCCCCCACT R:CCAGCACATTTGCGACAGCT	56	2	214-216	AB237647
Vemos10	$(TG)_{18}$	F:CGGGCATCCGTGTGATATTA R:AGCTGTTGGTATTTCGCTCC	56	5	179-196	AB237648
Vemos11	$(GT)_9GC(GT)_{10}$	F:CCTTATCTCTGCTGTGCAGT R:CATGTGATTGATTCGTGTCC	56	6	125-159	AB237649
Vemos13	$(CA)_{19}$	F:AGAAGGCAACCGCTGATATC R:AACGCTTACAGCAGCTGGGA	56	7	199-249	AB250358
Vemos18	$(CA)_8$	F:CTCATTCACCCCAAACCCTG R:CACTCGATACCTGCAAGGCA	56	2	136-138	AB237650
Vemos19	$(CA)_8GCACG(CA)_{19}$	F:CCACCATCTCACTCTCTCAC R:GGAGAGGGGGTCAAAAGAAA	56	4	181-203	AB237451
Vemos25	$(GT)_{14}TT(GT)_6$	F:CCGTTCTCTAAGCAGTTTCC R:AAGCTCGGCGCAGCACAAAT	56	4	161-165	AB237652
Vemos26	$(GT)_8GG(GT)_{12}$	F:CTCTATCTCTCTTTCCCGTC R:GGATAAGCTGAGAAGTGTGG	56	4	151-159	AB237453
Vemos 29	$(GT)_{22}$	F:AAGTCACCTCCTATGGTAAG R:TAACCTAAATCCAGACTCCA	56	2	216-218	AB241059
Vemos 31	$(GT)_{26}$	F:GAGGATACATGTGCTGGTTG R:GCTCATCTGGATGATGGAC	56	5	283-311	AB241254
Vemos 35	$(TG)_5$	F:AGCAGCCATGTAAGAGACAC R:CCACCAGAAACAGGATCCAC	56	3	351-381	AB241255
Vemos 37	$(TG)_{16}$	F:CCACTAAATCACCCTGTCACT R:GATGAAAGGCAACGTTGCT	56	3	158-164	AB241256
Vemos 39	$(TG)_{29}$	F: GAAGCAGAGCTTATAGGAAC R:GACGATCAATAACCTGAGAG	56	7	244-264	AB241257
Vemos42	$(TA)_2TCA(TG)_{12}$	F: GAATGTCTCAGCTCTGGAAG R: GAAACAATCTGGGGAAGGAG	56	5	136-142	AB250359
Vemos 43	$(CA)_4CG(CA)_6$	F:CCTCACTCTAATGAAGGACA R:TAAGAGGAAAAGGGGAAGAC	56	3	255-259	AB241258
Vemos 44	$(GT)_8AAGTCCATGG(GT)_8$	F:CCGTGTTCATAATAGTGTGTAG R:CCGTGTTCATAATAGTGTGTAG	56	2	198-200	AB241259
Vemos 46	$(CA)_{17}$	F:GTTGGGGATGAATCCAGT R:GCATGATAAAGGCAGAGGT	56	2	176-180	AB241260
Vemos 47	$(CA)_{15}$	F:TTGTCCCCACGTTACACAAC R:CAAGAGGTGCTGGTACTCTG	56	3	157-168	AB241261
Vemos 55	$(CA)_{17}$	F:CATAACCTGTTTCTTAGCAC R:CGCTGAGATTAAAGAGGAA	56	4	323-405	AB241262
Vemos 57	$(CA)_{28}$	F:CAAACAGAGATACACTGATGG R:TACTGCTTCCTGTGACAAG	56	7	242-299	AB241263
Vemos 60	$(TG)_{23}$	F:AAACCCTCTGTAAGACGACA R:GCAACATGCATCTTTACCAC	56	3	320-324	AB241264
Vemos 61	$(GT)_{21}$	F:AGACAGGGTTGTTTGTCTCA R:CTTCTGTGACCTTGGTCTCT	56	5	164-204	AB241265
Vemos 62	$(GT)_{18}$	F:TGTTGCCCGTAACTCTCT R:GGAGGAATAACTATGATCACCA	56	7	223-226	AB241266
Vemos 64	$(CA)_5CG(CA)_8$	F:CAATTGTTGATGACTGCTGA R:CATCAAAGAGCTTGCAGAA	56	2	186-188	AB241267
Vemos65	$(GT)_{25}$	F: CATGAGTTCCAAGCAAACC R: CGTTTACTGTTTGATCACCA	56	10	162-208	AB290199
Vemos 66	$(CA)_{16}$	F:TCCCAGCTAACGTCATGTC R:TCTGAGCTGATGATCACTTAGG	56	4	340-344	AB241268
Vemos 68	$(TG)_{10}$	F:CCAGTGTGTCACTTCTACAC R:AAAACACAGCTGATGGAAAC	56	3	363 372	AB241270
Vemos 69	$(CA)_4AA(CA)_8$	F:CTAAAGGGAACAAAAGCTG R:AGACAATTCTCCCTTTCC	56	2	267-271	AB241269
Vemos 71	$(GT)_{16}$	F:TTCATTCAGGTACGACCACA R:TTCATATCCAGAGCGGGTTT	56	4	338-350	AB241271
Vemos 76	$(CA)_{18}$	F:GACAAAAGGCTGTCTGCT R:CACAACATGTCGATTTTCTGTC	56	4	178-182	AB241272
Vemos 77	$(CT)_8(CA)_{10}$	F:GCCTCAAAGATCAGCAGTT R:GCTCCTTCATGTTCAGACTC	56	4	245-255	AB241273

[a] In nine individuals (5 females, 4males)

Table 3. Genotypic segregation in two full-sib families ND1 and ND2 of normally fertilized diploids at 34 microsatellite loci.

Locus	Full-sib Family	Parents		Progeny							Total	df	χ2
		Female	Male	*aa*	*ab*	*ac*	*ad*	*bb*	*bc*	*bd*			
Vemos1	ND-1	154/154(*aa*)	138/152(*bc*)	-	31	16	-	-	-	-	47	1	4.78*
	ND-2	152/154(*ab*)	150/154(*cb*)	-	7	6	-	9	2	-	24	3	4.33
Vemos4	ND-1	159/185(*ab*)	199/199(*cc*)	-	-	23	-	-	17	-	40	1	0.90
	ND-2	185/189(*ab*)	159/165(*cd*)	-	-	9	2	-	8	7	26	3	4.46
Vemos6	ND-1	244/246(*ab*)	238/238(*cc*)	-	-	25	-	-	21	-	46	1	0.35
	ND-2	246/254(*ab*)	246/250(*ac*)	4	10	6	-	-	9	-	29	3	3.14
Vemos7	ND-1	214/214(*aa*)	214/214(*aa*)	43	-	-	-	-	-	-	43	-	-
	ND-2	214/214(*aa*)	214/216(*ab*)	10	20	-	-	-	-	-	30	1	3.33
Vemos10	ND-1	180/180(*aa*)	180/180(*aa*)	45	-	-	-	-	-	-	45	-	-
	ND-2	180/182(*ab*)	186/196(*cd*)	-	-	7	8	-	9	6	30	3	0.67
Vemos11	ND-1	125/157(*ab*)	157/159(*bc*)	-	8	14	-	11	4	-	37	3	5.94
	ND-2	145/151(*ab*)	155/157(*cd*)	-	-	5	6	-	7	6	24	3	0.33
Vemos13	ND-1	200/210(*ab*)	200/238(*ac*)	6	11	12	-	-	15	-	30	3	3.82
	ND-2	228/249(*ab*)	200/244(*cd*)	-	-	5	13	-	3	8	29	3	7.83*
Vemos18	ND-1	136/138(*ab*)	136/136(aa)	23	23	-	-	-	-	-	46	1	0.00
	ND-2	136/138(*ab*)	136/136(*aa*)	16	13	-	-	-	-	-	29	1	0.31
Vemos19	ND-1	180/180(*aa*)	180/202(*ac*)	22	-	21	-	-	-	-	43	1	0.02
	ND-2	180/194(*ab*)	184/184(*cc*)	-	-	13	-	-	17	-	30	1	0.53
Vemos25	ND-1	163/165(*ab*)	161/165(*cb*)	-	7	16	-	7	12	-	41	3	5.43
	ND-2	163/165(*ab*)	161/161(*cc*)	-	-	16	-	-	11	-	27	1	0.93
Vemos26	ND-1	155/159(*ab*)	153/153(*cc*)	-	-	17	-	-	25	-	42	1	1.52
	ND-2	155/159(*ab*)	151/153(*cd*)	-	-	5	3	-	4	12	24	3	8.33*
Vemos29	ND-1	216/218(*ab*)	216/218(*ab*)	13	25	-	-	5	-	-	43	2	4.12
Vemos31	ND-1	283/311(*ab*)	283/299(*ac*)	17	11	11	-	-	4	-	43	3	7.88*
Vemos35	ND-1	373/373(*aa*)	351/381(*bc*)	-	18	22	-	-	-	-	40	1	0.40
Vemos37	ND-1	158/158(*aa*)	162/164(*bc*)	-	26	17	-	-	-	-	43	1	1.88
Vemos39	ND-1	256/264(*ab*)	244/264(*ca*)	9	10	11	-	-	12	-	42	3	0.48
Vemos42	ND-1	138/142(*ab*)	136/138(*ca*)	11	6	14	-	-	7	-	26	3	4.32
Vemos43	ND-1	255/257(*ab*)	257/259(*bc*)	-	7	17	-	10	11	-	45	3	4.69
Vemos44	ND-1	262/n(*ab*)	262/264(*ac*)	(18) [a]		15	-	-	5	-	38	2	5.37
Vemos46	ND-1	176/176(*aa*)	176/180(*ab*)	24	22	-	-	-	-	-	46	1	0.09
Vemos47	ND-1	157/159(*ab*)	168/168(*cc*)	-	-	21	-	-	19	-	40	1	0.10
Vemos55	ND-1	323/393(*ab*)	391/405(*cd*)	-	-	13	10	-	10	9	42	3	0.86
Vemos57	ND-1	263/299(*ab*)	242/260(*cd*)	-	-	16	5	-	9	7	37	3	7.43
Vemos60	ND-1	322/324(*ab*)	320/320(*cc*)	-	-	17	-	-	22	-	39	1	0.64
Vemos61	ND-1	164/204(*ab*	172/178(*cd*)	-	-	10	13	-	11	11		3	0.42
Vemos62	ND-1	226/226(*aa*)	222/224(*bc*)	25	-	-	-	-	17	-	42	1	1.52
Vemos64	ND-1	186/186(*aa*)	186/188(*ab*)	28	17	-	-	-	-	-	40	1	2.69
Vemos65	ND-1	172/182(*ab*)	202/202(*cc*)	-	15	-	-	-	23	-	38	1	1.68
Vemos66	ND-1	340/344(*ab*)	344/n(*bc*)	7	11	-	-	(24) [b]		-	42	3	1.62
Vemos68	ND-1	363/371(*ab*)	363/371(*ab*)	11	21	-	-	12	-	-	44	3	2.36
Vemos69	ND-1	267/267(*aa*)	267/271(*ab*)	19	22	-	-	-	-	-	41	1	0.22
Vemos71	ND-1	338/350(*ab*)	340/340(*cc*)	-	20	-	-	-	23	-	43	1	0.21
Vemos76	ND-1	178/184(*ab*)	182/182(*cc*)	-	-	23	-	-	19	-	42	3	0.38
Vemos77	ND-1	245/255(*ab*)	247/253(*cd*)	-	-	11	9	-	8	8	36	3	0.67

* $p < 0.05$

[a] =(aa+an), [b] =(bb+bn); n = null allele

Table4. Microsatellite genotypes at 27 microsatellite loci in four meiotic gynogenetic diploid lines.

Locus	Gynogenetic line	Parent genotype		Genotypes of gynogens							Total	Recombination frequency	M-C distance	df	X^{2a}
		Female	Sperm donor	*aa*	*ab*	*bb*	*ac*	*ad*	*be*	*bd*		(y)	(cM)		
Vemos1	MEI-2	152/154 (*ab*)	150/154 (*cb*)	2	33	0	-	-	-	-	35	0.94	47.1		
	MEI-3	152/154 (*ab*)	154/154 (*bb*)	6	27	1	-	-	-	-	34	0.79	39.5		
	Total			**8**	**60**	**1**					**69**	**0.87**	**43.5**	**1**	**3.36**
Vemos4	MEI-1	159/185 (*ab*)	199/199 (*cc*)	15	2	17	-	-	-	-	34	0.06	2.9	1	0.13
	MEI-2	185/189 (*ab*)	159/165 (*cd*)	18	8	11	-	-	-	-	37	0.22	10.8	1	1.69
	MEI-3	157/175 (*ab*)	181/185 (*cd*)	18	6	10	-	-	-	-	34	0.18	8.8	1	2.28
	MEI-4	175/199 (*ab*)	U	17	9	10	-	-	-	-	36	0.25	12.5	1	1.81
	Total			**68**	**25**	**48**					**141**	**0.18**	**9.0**	**3**	**4.96**
Vemos6	MEI-1	244/246 (*ab*)	238/238 (*cc*)	18	0	21	-	-	-	-	39	0.00	0.0	1	0.23
	MEI-2	246/254 (*ab*)	246/250 (*ac*)	20	0	20	-	-	-	-	40	0.00	0.0	1	0.00
	MEI-3	232/238 (*ab*)	244/246 (*cd*)	23	0	10	-	-	-	-	33	0.00	0.0	1	5.12*
	Total			**61**	**0**	**51**					**112**	**0.00**	**0.0**		
Vemos10	MEI-2	180/182 (*ab*)	186/196 (*cd*)	0	34	1	-	-	-	-	35	0.97	48.6		
Vemos11	MEI-1	125/157 (*ab*)	157/159 (*bc*)	0	3	35	-	-	-	-	38	0.08	3.9	1	35 .00*[b]
	MEI-2	145/151 (*ab*)	155/157 (*cd*)	13	2	20	-	-	-	-	35	0.06	2.9	1	1.48
	Total			**13**	**5**	**55**					**73**	**0.07**	**3.5**	**1**	**0.13**
Vemos13	MEI-1	200/210(*ab*)	200/238 (*ac*)	13	1	21	-	-	-	-	35	0.03	1.4	1	1.88
	MEI-2	228/249(*ab*)	200/244 (*cd*)	10	0	12	-	-	-	-	22	0.00	0.0	1	0.18
	MEI-3	200/204(*ab*)	200/228 (*ac*)	13	2	19	-	-	-	-	34	0.06	2.9	1	1.12
	Total			**26**	**3**	**42**					**91**	**0.03**	**1.5**	**2**	**1.48**
Vemos18	MEI-1	136/138 (*ab*)	136/136 (*aa*)	0	39	1	-	-	-	-	40	0.98	48.8		
	MEI-2	136/138 (*ab*)	136/136 (*aa*)	1	38	1	-	-	-	-	40	0.95	47.5		
	MEI-3	136/138 (*ab*)	136/136 (*aa*)	0	34	1	-	-	-	-	35	0.97	48.6		
	MEI-4	136/138 (*ab*)	U	0	40	0	-	-	-	-	40	1.00	50.0		
	Total			**1**	**151**	**3**					**155**	**0.97**	**48.5**	**3**	**2.00**
Vemos19	MEI-2	180/194 (*ab*)	184/184 (*cc*)	8	19	11	-	-	-	-	38	0.50	25.0	1	0.47
Vemos25	MEI-1	163/165 (*ab*)	161/165 (*ca*)	0	36	3	-	-	-	-	39	0.92	46.2		
	MEI-2	163/165 (*ab*)	161/161 (*cc*)	0	34	4	-	-	-	-	38	0.89	44.7		
	MEI-3	161/165 (*ab*)	163/165 (*cb*)	0	35	1	-	-	-	-	36	0.97	48.6		
	MEI-4	163/167 (*ab*)	U	1	39	0	-	-	-	-	40	0.97	48.6		
	Total			**1**	**144**	**8**					**153**	**0.94**	**47.0**	**3**	**3.16**
Vemos26	MEI-1	155/159 (*ab*)	153/153 (*cc*)	1	35	1	-	-	-	-	37	0.95	47.3		
	MEI-2	155/159 (*ab*)	151/153 (*cd*)	0	35	0	-	-	-	-	35	1.00	50.0		
	MEI-3	153/153 (*aa*)	159/159 (bb)	0	38	0	-	-	-	-	38	1.00	50.0		
	Total			**1**	**108**	**1**					**110**	**0.98**	**49.0**	**2**	**4.02**
Vemos29	MEI-1	216/218 (*ab*)	216/218 (*ab*)	24	0	9	-	-	-	-	33	0.00	0.0	1	6.82*
Vemos31	MEI-1	283/311 (*ab*)	283/299 (*ac*)	21	3	13	-	-	-	-	37	0.08	4.1	1	1.88
	MEI-3	275/299 (*ab*)	289/311 (*cd*)	19	3	12	-	-	-	-	34	0.09	4.4	1	1.58
	Total			**40**	**6**	**25**					**71**	**0.08**	**4.0**	**1**	**0.01**
Vemos39	MEI-1	256/264 (*ab*)	244/264 (*ca*)	17	7	14	-	-	-	-	38	0.18	9.2	1	0.29
	MEI-2	252/260 (ab)	242/254 (cd)	25	7	2	-	-	-	-	34	0.20	10.3	1	19.59*[b]
	MEI-3	256/260 (*ab*)	256/264 (*ac*)	12	13	14	-	-	-	-	39	0.33	16.6	1	0.15
	Total			**54**	**27**	**30**					**111**	**0.24**	**12.0**	**2**	**2.70**
Vemos42	MEI-1	138/142 (*ab*)	136/138 (*ca*)	1	37	1	-	-	-	-	39	0.95	47.4		
	MEI-2	130/138 (*ab*)	138/140 (*bc*)	0	38	0	-	-	-	-	38	1.00	50.0		
	Total			**1**	**75**	**1**					**77**	**0.97**	**48.5**	**1**	**2.00**
Vemos43	MEI-1	255/257 (*ab*)	257/259 (*bc*)	15	8	15	-	-	-	-	38	0.21	10.5	1	1.00
	MEI-2	255/257 (*ab*)	259/259 (*cc*)	11	17	10	-	-	-	-	38	0.45	22.4	1	0.05
	Total			**26**	**25**	**25**					**76**	**0.33**	**16.5**	**1**	**4.83***

Table4 (continued)

Locus	Family	Parent genotype		Genotypes of gynogens							Total	Recombination frequency	M-C distance	Df	X^2 aa:bb =1:1
		Female	Sperm donor	*aa*	*ab*	*bb*	*ac*	*ad*	*be*	*bd*		(*y*)	(cM)		
Vemos44	MEI-4	262/264 (*ab*)	U	1	32	1	-	-	-	-	34	0.94	47.1		
Vemos47	MEI-1	157/159 (*ab*)	168/168 (*cc*)	1	33	0	-	-	-	-	34	0.97	48.5		
Vemos55	MEI-1	323/393 (*ab*)	391/405 (*cd*)	14	8	9	-	-	-	-	31	0.26	12.9	1	1.09
Vemos57	MEI-1	263/299 (*ab*)	242/260 (*cd*)	3	24	2	-	-	-	-	29	0.83	41.3		
	MEI-3	242/260 (*ab*)	274/299 (*cd*)	0	37	1	-	-	-	-	38	0.97	48.5		
	Total			**3**	**61**	**3**					**67**	**0.91**	**45.5**	**1**	**4.31***
Vemos60	MEI-1	322/324 (*ab*)	320/320 (*cc*)	0	21	12	-	-	-	-	33	0.64	32.3	1	12.00*[b]
Vemos61	MEI-1	164/204 (*ab*)	172/178 (*cd*)	9	17	8	-	-	-	-	34	0.50	25.0	1	0.06
	MEI-3	158/178 (*ab*)	164/204 (*cd*)	9	22	8	-	-	-	-	39	0.56	28.2	1	0.06
	Total			**18**	**39**	**16**					**73**	**0.53**	**26.5**	**1**	**0.30**
Vemos62	MEI-3	212/226 (*ab*)	220/228 (*cd*)	15	15	9	-	-	-	-	39	0.38	19.0	1	1.50
	MEI-4	208/222 (*ab*)	U	18	13	5	-	-	-	-	36	0.36	18.0	1	7.35*
	Total			**33**	**28**	**14**					**75**	**0.37**	**18.5**	**1**	**0.04**
Vemos65	MEI-1	172/182 (*ab*)	202/202 (*cc*)	5	29	4	-	-	-	-	38	0.76	38.0		
	MEI-2	190/200 (*ab*)	162/208 (*cd*)	4	29	1	-	-	-	-	34	0.85	42.6		
	MEI-3	162/206 (*ab*)	170/170 (*cc*)	3	34	1	-	-	-	-	38	0.89	44.5		
	Total			**12**	**92**	**6**					**110**	**0.84**	**42.0**	**2**	**2.50**
Vemos66	MEI-1	340/344 (*ab*)	344/n (*bc*)	1	33	0	-	-	-	-	34	0.97	48.5		
	MEI-4	344/346 (*ab*)	U	0	29	0	-	-	-	-	29	1.00	50.0		
	Total			**1**	**62**	**0**					**63**	**0.98**	**49.0**	**1**	**0.87**
Vemos68	MEI-1	363/371 (*ab*)	363/371 (*ab*)	1	32	0	-	-	-	-	33	0.97	48.5		
	MEI-2	365/371 (*ab*)	371/371 (*bb*)	0	38	0	-	-	-	-	38	1.00	50.0		
	MEI-4	363/371 (*ab*)	U	0	29	0	-	-	-	-	29	1.00	50.0		
	Total			**1**	**99**	**0**					**100**	**0.99**	**49.5**	**2**	**2.05**
Vemos71	MEI-1	338/350 (*ab*)	340/340 (*cc*)	12	11	7	-	-	-	-	30	0.36	18.3	1	1.31
	MEI-3	338/340 (*ab*)	338/344 (*ac*)	7	20	10	-	-	-	-	37	0.54	27.0	1	0.53
	MEI-4	338/340 (*ab*)	U	5	19	9	-	-	-	-	33	0.58	28.8	1	1.14
	Total			**24**	**50**	**26**					**100**	**0.50**	**25.0**	**2**	**3.13**
Vemos76	MEI-1	178/184 (*ab*)	182/182 (*cc*)	9	24	0	-	-	-	-	33	0.72	36.4		
	MEI-4	180/182 (*ab*)	U	0	33	0	-	-	-	-	33	1.00	50.0		
	Total			**9**	**57**	**0**					**66**	**0.86**	**43.0**	**1**	**10.42***

U= unknown due to the loss of sample

[a] Chi square test for equal numbers of homozygotes (1d.f.) is performed when number of homozygotes is superior to10. Chi-square value in the total row is contingency chi-square for differences in *y* between gynogenetic lines. *p<0.05

[b] Significance after multiple test corrections

Table5. Verification of mitotic gynogenetic MIT-1 line at 8 microsatellite loci with high recombination rates.

Locus	*y*	Parents		Progeny			Total (N)
		Female	Male	*aa*	*ab*	*bb*	
Vemos10	*0.97*	174/182(*ab*)	174/182(ab)	9	0	1	10
Vemos25	*0.94*	163/165(*ab*)	165/165(*bb*)	2	0	6	8
Vemos26	*0.98*	153/155(*ab*)	155/155(*bb*)	6	0	2	8
Vemos42	*0.97*	136/138(ab)	136/138(ab)	6	0	4	10
Vemos57	*0.91*	263/265(*ab*)	289/289(*cc*)	2	0	8	10
Vemos65	*0.84*	162/180(*ab*)	180/202(*bc*)	5	0	5	10
Vemos66	*0.98*	340/344(*ab*)	340/344(*ab*)	7	0	3	10
Vemos68	*0.99*	363/371(*ab*)	363/371(*ab*)	4	0	5	9

(*y*) = Meiotic second division segregation frequency (see Table 4)