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1 Title: Androgenetic doubled haploids induced without irradiation of eggs in loach

2 (*Misgurnus anguillicaudatus*)

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Abbreviations: DH: doubled haploid; AA: after activation; UV: ultraviolet

Abstract

Androgenetic doubled haploids (DHs) were induced in the loach *Misgurnus anguillicaudatus* (Cobitidae) without irradiation of the eggs. The eggs of wild-type females were activated with the intact sperm of an orange-phenotype male, and treated (within 10 s of activation) at 3 ± 0.5 °C for 30 min, to eliminate the female nucleus. The eggs were then incubated in a water bath at 20 ± 0.5 °C for 35 min. Finally, diploidy was restored (65 min after activation) by heat-shock treatment at 42 ± 0.5 °C for 2 min. Under these conditions, the yield rate (mean $\pm$ SD) of putative DHs relative to the total number of eggs used was $10.43 \pm 1.69\%$, which was significantly higher ($P < 0.05$) than the yield rates obtained under the remaining heat-shock initiation conditions (55 min, 60 min, and 70 min after activation). We analyzed the ploidy status of the putative DH by using flow cytometry. All-male inheritance was confirmed by the expression of the recessive orange body color trait and microsatellite genotypes. We detected no maternally derived alleles or heterozygous genotypes at any of the 28 loci (covering 27 linkage groups) of loach, indicating the exclusively paternal inheritance and homozygosity of the obtained androgenetic DHs.

Keywords: Chromosome manipulation; Clone; Inbred line; Parthenogenesis

1. Introduction

Androgenesis is a type of induced parthenogenetic reproduction pattern with exclusively paternal inheritance. Androgenesis is considered useful for rapidly establishing clonal lines (Bongers et al., 1995; Nagoya et al., 1996; Young et al., 1996), detecting male-specific recombination rates (Singer et al., 2002), mapping quantitative trait loci (Robison et al., 2001), restoring endangered species from cryopreserved sperm (Babiak et al., 2002; Yasui et al., 2010), and investigating the effects of mitochondria or cytoplasm on development and growth (Brown et al., 2006; Fujimoto et al., 2010).

In fish, androgenesis can be induced via a 2-step process. Firstly, the nuclear DNA of unfertilized eggs is genetically inactivated by irradiation. Secondly, the irradiated eggs are activated with haploid or diploid sperm. If intact haploid sperm are used, the embryos develop as abnormal haploids, and are required to recover diploidy via pressure- or heat-shock treatment at the pro-metaphase of the first mitosis (Sakao et al., 2006). Irradiation of the eggs before activation by sperm is carried out by using gamma rays, X-rays, or ultraviolet (UV) rays. All 3 irradiation methods are effective for inactivating the egg nucleus, but require specific equipment. In particular, gamma and X-ray irradiation require special facilities for safe practice, and are therefore not suitable for routine use (Arai, 2001). UV irradiation does not require special facilities, but has low penetration ability, and therefore only eggs with a diameter of <2 mm can successfully be irradiated (Komen and Thorgaard, 2007). For all 3 types of irradiation,

specialized media are required to maintain the activation ability of the eggs, for example, water (Myers et al., 1995), synthetic ovarian fluid (Bongers et al., 1994), ovarian fluid (Lin and Dabrowski, 1998; Rothbard et al., 1999), Ringer's solution (David and Pandian, 2006), seminal plasma (Corley-Smith et al., 1996; Fujimoto et al., 2007), or Hank's solution (Yasui et al., 2010).

Morishima et al. (2011) reported that cold-shock treatment (0 °C or 3 °C for 60 min) of loach (*Misgurnus anguillicaudatus*) eggs just after activation successfully induced androgenetic development, presumably by eliminating the maternally derived egg and polar body nucleus. Most of the obtained androgenotes were abnormal haploids, and very few were normal diploids. The exclusively paternal inheritance of the putative androgenotes was confirmed by the expression of the male-specific recessive color trait and microsatellite genotypes. These findings strongly suggest the possibility of inducing androgenetic doubled haploid (DH) fish without irradiation of eggs. Thus, haploid androgenotes can readily be induced by using cold-shock treatment of newly activated eggs, and the resultant haploid embryos can be restored to diploidy by using pressure- or thermal-shock treatment at an optimum time. This new technique may enable the production of androgenetic DH solely by using temperature shocking of eggs—cold shocking for initiation of androgenesis, and heat shocking for duplication of chromosomes.

The objective of the present study was to simplify the process for androgenetic DH induction in fish. We optimized the duration of the cold-shock treatment and the

initiation timing of the heat-shock treatment, to produce an acceptable number of viable androgenetic DH from haploid sperm without irradiation of loach eggs. We used the orange male loach with orange body color and black eye phenotype (recessive trait to wild-type) as a visible paternal genetic marker. We determined the ploidy status by using flow cytometry, and genetically verified the exclusively paternal inheritance and homozygosity of the DH by using microsatellite genotyping.

2. Materials and methods

2.1. Ethics

This study was performed according to the Guide for the Care and Use of Laboratory Animals in Hokkaido University.

2.2. Fish and gamete collection

Wild-type diploid female and orange diploid male (orange body color and black eye phenotype, recessive to wild-type male; Suzuki et al., 1985) loaches were reared in the Aquarium Room of the Environment Control Experiment Building in the Faculty of Fisheries Sciences, Hokkaido University. For each batch, a single wild-type female and a single orange male loach were used. The same male loach was used for all of the crosses.

Spawning was induced by using a single injection of hCG (20 IU/g of body weight, Asuka Pharmaceutical Co. Ltd., Tokyo, Japan) as described by Suzuki and

Yamaguchi (1975). After 10–12 h of rearing at 27 ± 0.5 °C, the gametes were collected according to Morishima et al. (2011).

2.3. Optimization of the duration of the cold-shock treatment

Sperm were added to the eggs, mixed well, and activated by using ambient tap water (20 ± 0.5 °C). Within 10 s of activation, the eggs were transferred to containers (the cone base of the 50-mL centrifuge tube was cut and covered with mesh) in a Styrofoam box containing 6 L of cold tap water (3 ± 0.5 °C). The eggs were subjected to cold-shock treatment for 20 min, 30 min, 40 min, 50 min, or 60 min. The containers with the eggs were then transferred to another Styrofoam box containing 6 L ambient tap water (20 ± 0.5 °C) and incubated until 150 min after activation (AA). Subsequently, the eggs of each group were transferred to 90-mm diameter plastic dishes (1 group per plastic dish) and incubated at 22 °C until 72 AA. As an intact control group, we used just-fertilized eggs that had been transferred to a container in the Styrofoam box containing 6 L ambient tap water (20 ± 0.5 °C) and incubated until 150 min AA.

2.4. Optimization of the initiation timing of the heat-shock treatment

The containers with the eggs that had been subjected to cold-shock treatment for 30 min were incubated in a Styrofoam box containing 6 L ambient tap water (20 ± 0.5 °C) for 25 min, 30 min, 35 min, or 40 min (i.e., until 55 min, 60 min, 65 min, or 70 min

AA). The eggs were then transferred to a Styrofoam box containing 6 L hot tap water (42 ± 0.5 °C) and incubated for 2 min. After heat-shock treatment, the containers were returned to the Styrofoam box containing 6 L ambient tap water (20 ± 0.5 °C) and incubated until 210 min AA. Subsequently, the eggs of each group were transferred to 90-mm diameter plastic dishes (1 group per plastic dish) and incubated at 22 °C until 72 AA. As an intact control group, we used just-fertilized eggs that had been transferred to a container in the Styrofoam box containing 6 L ambient tap water (20 ± 0.5 °C) and incubated until 210 min AA.

2.5. Cleavage rate, somite stage rate, hatch rate, and external morphology

The cleavage rate was calculated as the proportion of cleaved eggs to the total number of eggs used, at 4 h AA. The somite stage rate was calculated as the proportion of surviving embryos to the total number of eggs used, at the somite stage. The hatch rate was calculated as the proportion of hatched larvae to the total number of eggs used. Larvae with the expression of melanophores on their bodies were designated as wild-type larvae, while larvae without melanophores on their bodies were designated as orange larvae. The orange rate was calculated as the proportion of orange larvae to the total number of hatched larvae.

2.6. Determination of ploidy, paternity, and homozygosity

At approximately 72 h AA, when color phenotypes became detectable, larvae were further analyzed for relative DNA content by using flow cytometry (PA-II, Partec GmbH, Münster, Germany), according to the procedure of Fujimoto et al. (2007). To determine the ploidy, paternity, and homozygosity of larvae from diploidization-treated groups, each larva was first digested by using 85 µL of solution A (CyStain DNA 2 step, Cod. 05-5005, Partec GmbH) for 15 min. Next, 15 µL of the digested solution was stained by using 500 µL of solution B (CyStain DNA 2 step, Cod. 05-5005, Partec GmbH), and analyzed by using flow cytometry. The remaining 70 µL of digested solution was used for DNA extraction.

Thirty putative DHs (15 normal and 15 abnormal), together with the female and male parents, were genetically analyzed for 28 microsatellite loci (covering 27 linkage groups) of loach (Table S1). DNA extraction and microsatellite genotyping were performed according to Morishima et al. (2008).

2.7. Statistical analysis

All experiments were performed in triplicate. Data are shown as mean $\pm$ SD; because of differences in egg quality, arcsine transformations were used for some groups, in order to fit the normal distribution. Data were checked for normality using the Shapiro–Wilk test, and analyzed with one-way ANOVA followed by Duncan’s multiple comparisons ($P > 0.05$). The data of microsatellite genotyping were analyzed using Chi-square goodness-of-fit ($P > 0.05$). All statistical analyses were performed

by R (R Core Team, 2012).

3. Results

3.1. Optimization of the duration of the cold-shock treatment

The cleavage rates, somite stage rates, hatch rates, and orange rates for groups subjected to different durations of cold-shock treatment are shown in Table 1. We determined no significant differences in cleavage rate among treated groups and the intact control group ($P > 0.05$). By contrast, the somite stage rate and hatch rate for the eggs subjected to cold-shock treatment for 40 min, 50 min, or 60 min, but not for 20 min or 30 min, were significantly lower than those of the intact control group ($P < 0.05$). The groups subjected to 20 min, 30 min, or 40 min of cold-shock treatment contained 3 types of larvae: (1) abnormal orange-type larvae (a recessive color marker of androgenesis); (2) abnormal wild-type larvae; and (3) normal wild-type larvae. The group subjected to 50 min of cold-shock treatment contained 2 types of larvae: (1) abnormal orange-type larvae; and (2) abnormal wild-type larvae. The group subjected to 60 min of cold-shock treatment contained only abnormal orange-type larvae (Table 2). The intact control group contained 2 types of larvae: (1) abnormal wild-type larvae; and (2) normal wild-type larvae. The orange rate was highest ($98.41 \pm 2.75\%$) in the group subjected to 50 min of cold-shock treatment, and lowest ($32.42 \pm 9.65\%$) in the group subjected to 20 min of cold-shock treatment. The yield rate of haploid androgenotes was calculated by multiplying the hatch rate by the orange rate; the

yield rate for the group subjected to 30 min of cold-shock treatment was $31.65 \pm 12.66\%$ (Figure 1).

Ploidy analysis indicated that most of the abnormal orange-type larvae in the groups subjected to cold-shock treatment were haploid, and few were diploid (Table 2). In the groups subjected to 30 min or 40 min of cold-shock treatment, diploid and triploid normal wild-type larvae were found. Among the abnormal wild-type larvae from the groups subjected to cold-shock treatment, diploid, haploid, and aneuploid larvae were found. In the intact control group, all the normal wild-type larvae were diploid. Our results indicate that cold-shock treatment just after activation of the egg can induce haploid androgenesis, as evidenced by the presence of the abnormal orange phenotype and ploidy analysis.

3.2. Optimization of the initiation timing of the heat-shock treatment

The cleavage rates, somite stage rates, hatch rates, and orange rates for groups subjected to different diploidization treatments are shown in Table 3. For all treatment groups, the cleavage rates did not differ significantly from that of the control group ($P > 0.05$). By contrast, the somite stage rates and hatch rates of all treatment groups were significantly lower than those of the control group ($P < 0.05$). The treatment group in which the heat-shock treatment was initiated at 65 min AA showed the highest somite stage rate and also the highest hatch rate ($27.64 \pm 7.08\%$ and $21.87 \pm 6.01\%$, respectively). The treatment groups in which the heat-shock treatment was

initiated at 60 min, 65 min, and 70 min AA contained 4 types of larvae: (1) normal wild-type larvae; (2) abnormal wild-type larvae; (3) normal orange-type larvae; and (4) abnormal orange-type larvae (Figure 2). The treatment group in which the heat-shock treatment was initiated at 55 min AA contained only abnormal orange-type larvae.

The flow cytometry results indicated that all of the normal orange-type larvae were diploid (Table 4). Most of the abnormal orange-type larvae were diploid; however, haploid, triploid, tetraploid, pentaploid, aneuploid, and mosaic larvae were also found. For all treatment groups, normal and abnormal wild-type larvae showed similar ploidy status to that of the abnormal orange-type non-haploid larvae; however, hexaploid larvae were also found. By contrast, all of the normal wild-type larvae in the intact control group were diploid.

We designated the orange diploid larvae as DHs, and calculated the total yield rate as the frequency of orange diploid larvae relative to the total number of eggs used. The yield rate of normal DHs was calculated as the frequency of normal orange diploid larvae relative to the total number of eggs used. Among the 4 treatment groups, the group in which the heat-shock treatment was initiated at 65 min AA showed significantly higher yield rates of total and normal DHs ($10.43 \pm 1.69\%$ and $4.39 \pm 1.46\%$, respectively) than did the remaining treatment groups and the intact control group ($P < 0.05$) (Figure 3).

3.3. Determination of paternity and homozygosity

The paternity and homozygosity of 30 putative DHs were determined by using 28 microsatellite loci from 27 linkage groups of loach (2 markers for linkage group 12, and 1 marker each for the remaining 26 linkage groups; Morishima et al., 2008). For all of the 28 loci tested, only paternal alleles were transmitted to the orange diploid progeny, and no maternally derived alleles were observed (Table 5). For example, in locus *Mac587* from linkage group 1, the male genotype was *187/261*, and the female genotype was *147/159*. Out of 30 diploid progeny, 14 gave homozygous *187/187* genotypes, while 16 gave homozygous *261/261* genotypes. Among the microsatellite loci examined, 27 were consistently segregated in the Mendelian inheritance fashion (Chi-square test, $P > 0.05$); only *Mac563* from linkage group 19 deviated significantly from the expected Mendelian segregation ratio ($\chi^2 = 4.800$, $P = 0.028$).

The 30 orange diploid progeny were completely homozygous in all of the 28 loci tested, in which the male parent was heterozygous in 18 loci, and homozygous in the remaining 10 loci. Therefore, the orange diploid progeny were DH androgenotes.

4. Discussion

Irradiation is widely used to induce androgenesis in fish (Komen and Thorgaard, 2007). Successful genetic inactivation depends upon the optimum irradiation procedure, and the use of specialized protection media to maintain the activation ability of the eggs. In the present study, we have shown that androgenetic DHs can be induced in loach without irradiation of eggs, by using a combination of cold-shock

treatment just after activation of the eggs and heat-shock treatment at 65 min AA. By eliminating the requirement for specialized protection media and irradiation, our technique simplifies the process for androgenetic induction of fish.

The yield rate of normal androgenetic DHs in our present study was $4.39 \pm 1.46\%$. Such a low yield rate is common in studies of induced androgenesis, and can be explained by a number of hypotheses. Scheerer et al. (1991) suggested that the manipulation procedure was largely responsible for the mortality of androgenetic diploids, and that genetic factors were relatively unimportant. In our present study, the cleavage rates of the groups subjected to heat-shock treatment did not differ significantly from those of the intact control group. However, development was arrested at the gastrulation stage in a large number of eggs, and this was followed by high mortality. Another possible explanation for the low yield rate of androgenetic DHs is the expression of recessive lethal genes, because of unmasking by homozygosity. However, further studies are required to clarify these hypotheses.

The mechanism by which cold-shock treatment induces androgenesis remains unclear. One possible explanation is that activation of the egg triggers the signal to proceed to the second meiosis, and thereby the release of the second polar body. The cold-shock treatment suspends these events, leading to a breakdown of the second meiosis spindles, and the enclosure of the metaphase chromosomes within a chromosome mass. After the cold-shock treatment, the interrupted meiosis restarts, but the enclosed chromosomes are unable to separate, resulting in the release of a

“whole second polar body.” Morishima et al. (2011) showed that eggs subjected to cold-shock treatment contained 4 types of second polar body extrusion, one of which was the egg nucleus released with second polar body, such that only the sperm nucleus remained in the egg. This sperm nucleus transformed into a male pronucleus, which subsequently initiated androgenetic development.

Besides androgenetic orange haploid progeny, eggs subjected to cold-shock treatment just after activation produced diploid and triploid wild-type progeny. Morishima et al. (2011) stated that wild-type triploids appear when the release of the second polar body is inhibited after the incorporation of a sperm nucleus, whereas wild-type diploids appear when cold-shock treatment has no effect on the eggs. By contrast, aneuploids and mosaics are likely produced because of effects on the cell division process (Morishima et al., 2011).

Beside androgenetic orange DHs, eggs subjected to heat-shock treatment produced various types of orange polyploid, aneuploid, and mosaic androgenotes. It is difficult to identify the precise cytological mechanisms responsible for these atypical androgenotes, but dispermy and/or polyspermy may be involved. Successful dispermic androgenesis has been performed in sturgeon species (Grunina et al., 2006, 2011), while successful androgenesis using irradiated eggs and fused sperm has been reported in salmonids (Araki et al., 1995; Nagoya et al., 2010) and cyprinids (Kirankumar and Pandian, 2004; Clifton and Pandian, 2008). Therefore, the various types of polyploid (triploid, tetraploid, and pentaploid) androgenotes found in our

present study may have resulted from dispermy or polyspermy. Another possible explanation is successful inhibition of the first cleavage, after initiation of androgenesis with 2 or more sperm—hexaploidy may appear via chromosome duplication of an androgenetic triploid embryo. However, the mechanism by which aneuploidy and mosaic androgenotes are formed remains to be elucidated. Developmental abnormalities in salmonids caused by inhibition of the first cleavage are well documented (Sakao et al., 2006). Another possible explanation for the appearance of polyploid, aneuploid, and mosaic androgenotes is suppression of the second, third, or later mitotic divisions in DHs. If these mitotic divisions are suppressed in only some blastomeres of an early embryo, different levels of polyploidy, and various types of mosaicism, may result.

In the present study, we have demonstrated for the first time the induction of androgenetic doubled haploid fish, without irradiation of eggs. By eliminating the requirement for specialized protection media and irradiation, our technique simplifies the process of androgenetic induction in fish. However, the optimum cold- and heat-shock conditions must be optimized for each target species of fish.

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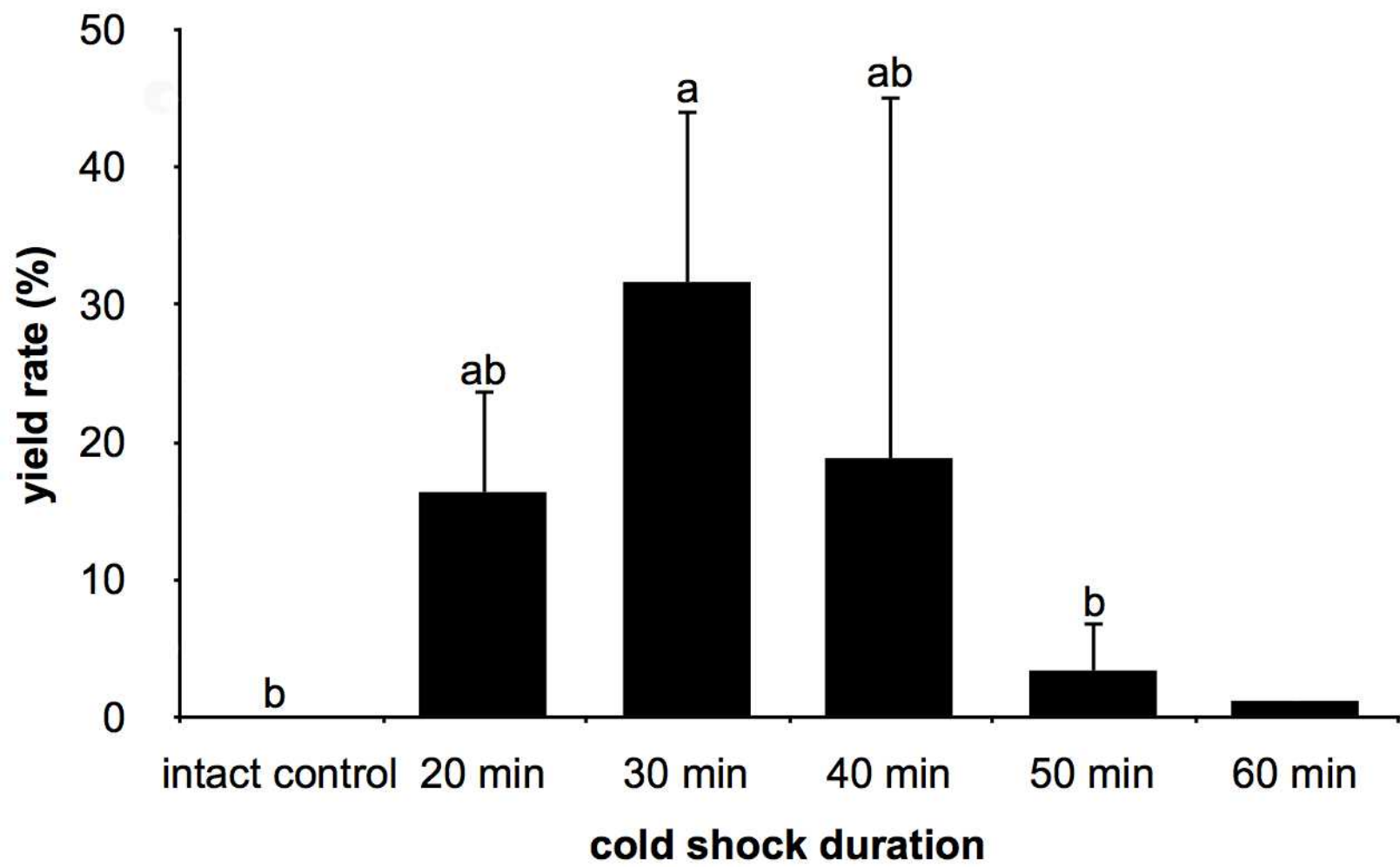
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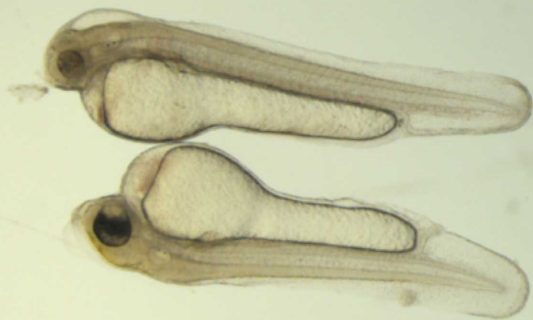
Figure captions

Figure 1. Yield rate of haploid androgenotes in loach (*Misgurnus anguillicaudatus*), calculated by multiplying the hatch rate by the orange rate, for the intact control group and groups subjected to cold-shock treatment for 20 min, 30 min, 40 min, 50 min, and 60 min. The yield rate for the group subjected to 30 min of cold-shock treatment was $31.65 \pm 12.66\%$. Different letters denote significant differences as determined by one-way ANOVA and Duncan's multiple comparisons ($P > 0.05$).

Figure 2. The 4 different types of loach (*Misgurnus anguillicaudatus*) larvae hatched from the group subjected to cold-shock treatment for 30 min, followed by heat-shock treatment at 65 min after activation (AA). (A), normal wild-type larvae; (B), abnormal wild-type larvae; (C), normal orange-type larvae; (D), abnormal orange-type larvae. Scale bar denotes 2 mm.

Figure 3. Yield rates of total and normal androgenetic doubled haploids (DHs) in loach (*Misgurnus anguillicaudatus*), relative to the total number of eggs used, for the intact control group, and groups subjected to heat-shock treatment at 55 min, 60 min, 65 min, and 70 min after activation (AA). The highest yield rates of total and normal DHs, obtained for the group subjected to heat-shock treatment at 65 min AA, were $10.43 \pm 1.69\%$ and $4.39 \pm 1.46\%$, respectively. Different letters denote significant differences as determined by one-way ANOVA and Duncan's multiple comparisons ($P > 0.05$).



A**B****C****D**

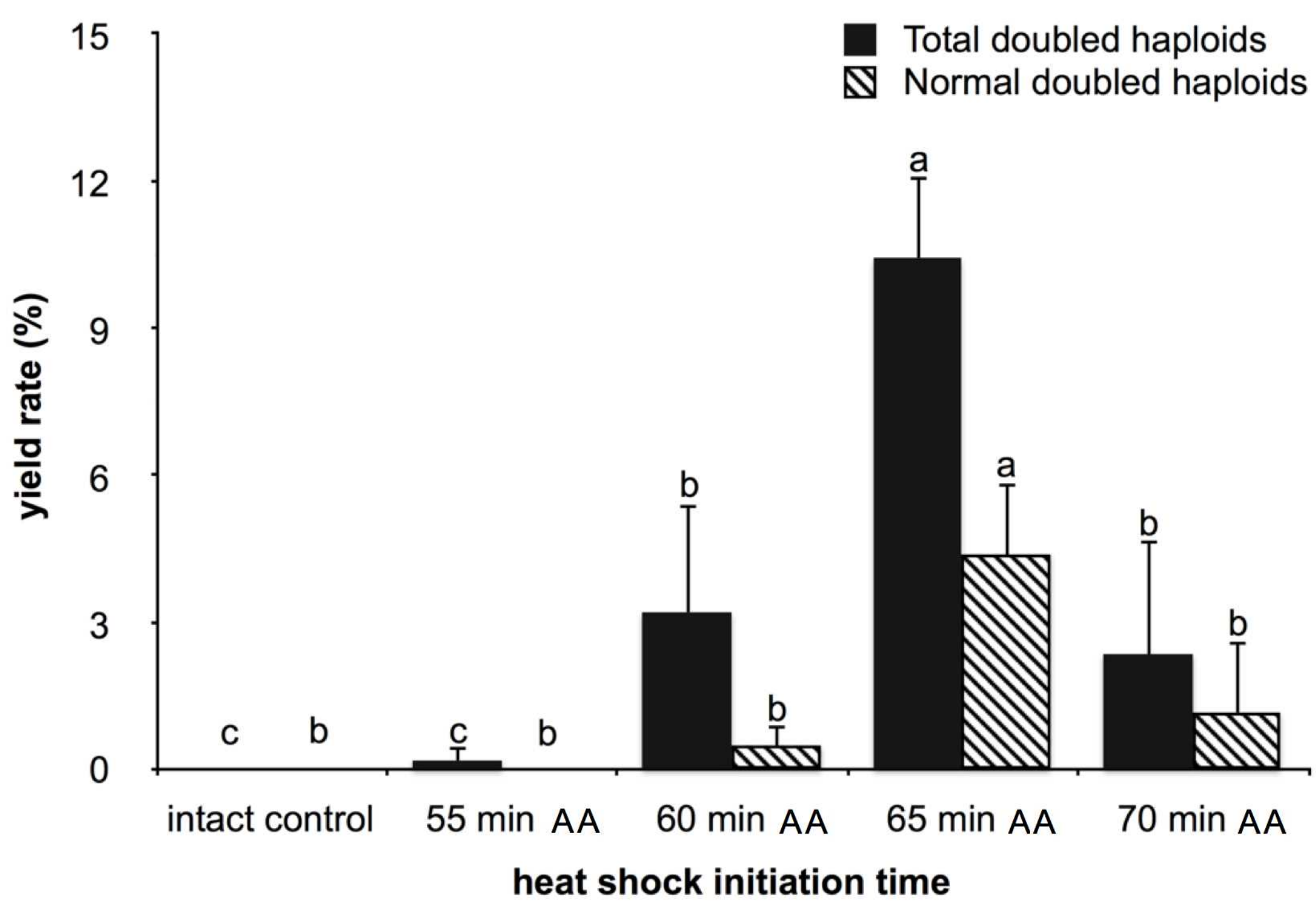


TABLE 1

Cleavage rates, somite stage rates, hatch rates, and orange rates for groups subjected to different durations of cold-shock treatment of eggs just after activation (AA) in loach, *Misgurnus anguillicaudatus*.

Cross		Duration of the cold-shock treatment ¹	Number of eggs (Total) ²	Cleavage rate (%) ³	Somite stage rate (%)	Hatch rate (%) (Total)	Orange rate (%) (Total)
Female	Male						
Wild-type 2N	Orange 2N	Intact control	156 ± 19 (468)	72.11 ± 10.74 ^a	66.80 ± 12.31 ^a	65.31 ± 12.15 ^a (66.24)	0.00 ± 0.00 ^d (0.00)
Wild-type 2N	Orange 2N	20 min	221 ± 183 (664)	57.45 ± 8.09 ^a	50.66 ± 11.55 ^{ab}	49.65 ± 11.86 ^{ab} (55.87)	32.42 ± 9.65 ^c (36.39)
Wild-type 2N	Orange 2N	30 min	317 ± 199 (952)	65.68 ± 14.97 ^a	52.06 ± 24.70 ^{ab}	49.85 ± 24.90 ^{ab} (58.61)	65.96 ± 7.68 ^b (61.65)
Wild-type 2N	Orange 2N	40 min	191 ± 115 (572)	54.67 ± 22.47 ^a	26.15 ± 33.70 ^b	24.27 ± 32.34 ^b (37.24)	69.74 ± 9.16 ^b (79.34)
Wild-type 2N	Orange 2N	50 min	196 ± 64 (589)	63.37 ± 6.92 ^a	5.64 ± 6.93 ^b	3.52 ± 3.87 ^b (4.24)	98.41 ± 2.75 ^a (96.00)
Wild-type 2N	Orange 2N	60 min	177 ± 122 (501)	62.04 ± 9.40 ^a	1.95 ± 2.35 ^b	1.19 ± 1.53 ^b (1.99)	100.00 (100.00)

¹Just after activation (within 10 s) at 20 ± 0.5 °C; subjected to cold-shock treatment at 3 ± 0.5 °C.

²Data represent mean ± SD; total number of eggs, total hatch rate, and total orange rate are shown in parentheses.

³Different superscript letters within columns denote significant differences as determined by one-way ANOVA and Duncan's multiple comparisons ($P > 0.05$).

TABLE 2

Phenotype, morphology, and ploidy status of larvae from groups subjected to different durations of cold-shock treatment (3 ± 0.5 °C) of eggs just after activation (AA) in loach, *Misgurnus anguillicaudatus*.

Treatment	Phenotype	Morphology	Number of larvae	Ploidy status				
				1N	2N	3N	Hypo-2N	Hyper-2N
Intact control	Wild-type	Normal	20	0	20	0	0	0
20 min of cold-shock	Wild-type	Normal	20	0	20	0	0	0
	Wild-type	Abnormal	19	1	16	0	2 ^a	0
	Orange	Abnormal	25	24	1	0	0	0
30 min of cold-shock	Wild-type	Normal	20	0	16	4	0	0
	Wild-type	Abnormal	12	2	7	0	3 ^b	0
	Orange	Abnormal	20	20	0	0	0	0
40 min of cold-shock	Wild-type	Normal	16	0	9	7	0	0
	Wild-type	Abnormal	23	2	20	0	0	1 ^c
	Orange	Abnormal	23	20	3	0	0	0
50 min of cold-shock	Wild-type	Abnormal	1	0	0	0	1 ^d	0
	Orange	Abnormal	20	20	0	0	0	0
60 min of cold-shock	Orange	Abnormal	9	9	0	0	0	0

^a1.4N and 1.5N; ^b1.3N, 1.4N, and 1.5N; ^c2.5N; ^d1.6N.

TABLE 3

Cleavage rates, somite stage rates, hatch rates, and orange rates for groups subjected to treatments from different initiation timing of eggs just after activation (AA) in loach, *Misgurnus anguillicaudatus*.

Cross		Treatment	Number of eggs (Total) ¹	Cleavage rate (%) ²	Somite stage rate (%)	Hatch rate (%) (Total)	Orange rate (%) (Total)
Female	Male	Initiation timing of the heat-shock treatment					
Wild-type 2N	Orange 2N	Intact control	174 ± 38 (523)	85.85 ± 7.79 ^a	82.01 ± 8.01 ^a	81.96 ± 8.09 ^a (83.94)	0.00 ± 0.00 ^b (0.00)
Wild-type 2N	Orange 2N	55 min AA	176 ± 38 (527)	71.46 ± 11.85 ^a	1.33 ± 2.30 ^c	0.76 ± 1.31 ^c (0.76)	100.00 (100.00)
Wild-type 2N	Orange 2N	60 min AA	185 ± 34 (556)	66.19 ± 12.54 ^a	8.91 ± 3.71 ^c	5.94 ± 2.15 ^c (6.29)	68.84 ± 8.89 ^a (65.71)
Wild-type 2N	Orange 2N	65 min AA	197 ± 27 (591)	67.96 ± 15.66 ^a	27.64 ± 7.08 ^b	21.87 ± 6.01 ^b (23.35)	61.95 ± 16.25 ^a (59.42)
Wild-type 2N	Orange 2N	70 min AA	233 ± 97 (699)	61.17 ± 22.81 ^a	10.54 ± 12.11 ^c	7.07 ± 7.95 ^c (5.87)	56.65 ± 11.52 ^a (63.41)

¹Data represent mean ± SD; total number of eggs, total hatch rate, and total orange rate are shown in parentheses.

²Different superscript letters within columns denote significant differences as determined by the one-way ANOVA and Duncan's multiple comparisons ($P > 0.05$).

TABLE 4

Phenotype, morphology, and ploidy status of larvae from groups subjected to different treatments from different initiation timing of eggs just after activation (AA) in loach, *Misgurnus anguillicaudatus*.

Treatment (Heat-shock initiation time)	Phenotype	Morphology	Number of larvae ^a	Ploidy status							
				1N	2N	3N	4N	5N	6N	Aneuploidy	Mosaic
Intact control	Wild-type	Normal	60	0	60	0	0	0	0	0	0
55 min AA	Orange	Abnormal	4	0	2	0	0	0	0	2 ^b	0
60 min AA	Wild-type	Normal	3	0	0	1	0	0	1	0	1 ^c
	Wild-type	Abnormal	8	0	0	3	0	0	4	0	1 ^d
	Orange	Normal	3	0	3	0	0	0	0	0	0
	Orange	Abnormal	21	2	16	0	0	0	0	1 ^e	2 ^f
65 min AA	Wild-type	Normal	8	0	1	1	1	0	3	0	2 ^g
	Wild-type	Abnormal	48	0	3	2	2	6	31	2 ^h	2 ⁱ
	Orange	Normal	26	0	26	0	0	0	0	0	0
	Orange	Abnormal	56	1	40	0	6	0	0	4 ^j	5 ^k
70 min AA	Wild-type	Normal	2	0	0	0	0	0	2	0	0
	Wild-type	Abnormal	13	0	1	3	3	2	4	0	0
	Orange	Normal	6	0	6	0	0	0	0	0	0
	Orange	Abnormal	20	0	8	3	4	4	0	1 ^l	0

^aThe number of larvae represent the additive data of triplicates for each treatment group.

^b2.5N and 2.8N; ^c3N-6N; ^d3N-6N; ^e2.5N; ^f2N-2.3N and 2N-4N; ^g3N-6N and 3.5N-4N; ^h5.3N and 5.6N; ⁱ3.4N-4N and 4.4N-6N; ^j2.5N, 2.8N, 3.5N, and 3.8N; ^k1.7N-2N, 1.9N-2.2N, 1.9N-2.8N, 2.6N-3N, and 2N-4N; ^l4.7N.

TABLE 5

Genotyping of putative androgenetic doubled haploids showing the orange phenotype in loach, *Misgurnus anguillicaudatus*.

Locus (LG)	Female	Male	Diploid progeny genotype: <i>n</i>	χ^2 (df= 1) <i>a/a:b/b</i> = 1:1	<i>P</i> value
<i>Mac587</i> (1)	147/159	187/261	187/187:14 261/261:16	0.133	0.715
<i>Mac375</i> (2)	325/343	339/351	339/339:16 351/351:14	0.133	0.715
<i>Mac47</i> (3)	164/164	138/144	138/138:16 144/144:14	0.133	0.715
<i>Mac291</i> (4)	226/250	254/254	254/254:30	—	—
<i>Mac382</i> (5)	272/282	294/294	294/294:30	—	—
<i>Mac353</i> (6)	446/446	462/462	462/462:30	—	—
<i>Mac43</i> (7)	192/192	124/124	124/124:30	—	—
<i>Mac425</i> (8)	120/130	138/142	138/138:13 142/142:17	0.533	0.465
<i>Mac73</i> (9)	118/118	204/222	204/204:15 222/222:15	0.000	1.000
<i>Mac37</i> (10)	109/109	95/105	95/95:17 105/105:13	0.533	0.465
<i>Mac477</i> (11)	144/144	134/152	134/134:11 152/152:19	2.133	0.144
<i>Mac2</i> (12)	128/128	114/122	114/114:15 122/122:15	0.000	1.000
<i>Mac251</i> (12)	160/160	178/186	178/178:18 186/186:12	1.200	0.273
<i>Mac235</i> (13)	440/447	427/453	427/427:18 453/453:12	1.200	0.273
<i>Mac287</i> (14)	245/270	206/209	206/206:15 209/209:15	0.000	1.000
<i>Mac357</i> (15)	137/143	157/157	157/157:30	—	—
<i>Mac458</i> (16)	245/270	208/208	208/208:30	—	—
<i>Mac596</i> (17)	296/320	292/316	292/292:16 316/316:14	0.133	0.715
<i>Mac380</i> (18)	176/176	206/220	206/206:14 220/220:16	0.133	0.715
<i>Mac563</i> (19)	286/292	302/308	302/302:9 308/308:21	4.800	0.028
<i>Mac4</i> (20)	612/626	638/638	638/638:30	—	—
<i>Mac523</i> (21)	344/344	310/350	310/310:13 350/350:17	0.533	0.465

TABLE 5
Continued.

Locus (LG)	Female	Male	Diploid progeny genotype: <i>n</i>	χ^2 (df= 1) <i>a/a:b/b</i> = 1:1	<i>P</i> value
<i>Mac612</i> (22)	228/242	222/236	222/222:15 236/236:15	0.000	1.000
<i>Mac310</i> (23)	374/386	370/370	370/370:30	—	—
<i>Mac658</i> (24)	236/236	226/266	226/226:13 266/266:17	0.533	0.465
<i>Mac263</i> (25)	260/300	278/278	278/278:30	—	—
<i>Mac362</i> (26)	384/388	386/386	386/386:30	—	—
<i>Mac637</i> (27)	333/385	363/369	363/363:14 369/369:16	0.133	0.715

Table S1. Microsatellite primer information.

Locus	Core sequence	Primer sequence 5'→3'	Linkage group	Accession no.
<i>Mac587</i>	(CA) ₁₅	F: CGGGAAGAATGGGACCTACA R: GCCCCATAATCTCCCGTTCA	1	AB303598
<i>Mac375</i>	(GA) ₃₇	F: TTACTTCTGCCAGTAGCTGT R: ACATGTGTACACGTTTGGTC	2	AB303500
<i>Mac47</i>	(CA) ₁₆	F: AACAAAACAACAGAAACGCCTGC R: TCCGGGACAGCAGCAAAATT	3	AB081625
<i>Mac291</i>	(GA) ₆	F: TGGAAATCCCTCGGTCTGTC R: CGCAAGAGATGGGTCAAGTC	4	AB303477
<i>Mac382</i>	(CT) ₁₆	F: CATTTGTTTTTCGGCCTCTGT R: GCCGCTATTTGCATCTGAAG	5	AB303504
<i>Mac353</i>	(GA) ₁₉	F: AAGCANAAGTGGTGAGAG R: CAGCTGTTATCTTTCTAGG	6	AB303491
<i>Mac43</i>	(CA) ₈ -TG- (CA) ₃	F: ATAATGAGAAGCAGAGCGGT R: TGCATTGCTTTGGTTCAGTG	7	AB081624
<i>Mac425</i>	(GT) ₁₄	F: CTACGGAAGTGCTGCAGACA R: CATCCTAGGCTCAGGCCAAA	8	AB303516
<i>Mac73</i>	(GT) ₂₈	F: ATTCAAACAGGCCAGCAGAT R: GAATTCGGGAGGGAAATGTT	9	AB303430
<i>Mac37</i>	(CA) ₁₅	F: GCAAGTACATGCTCATCCT R: CACCTGCATTCTTACATCT	10	AB060181
<i>Mac477</i>	(CA) ₃₃	F: GCTGAGACTCTTTATGTCTCAC R: GCTATCAAGGGAAGTGAATGG	11	AB303575
<i>Mac2</i>	(CA) ₁₄	F: CAGGTGTCCTCATAGACTGA R: TGGGATCTCTGCGGGACAGA	12	AB060172
<i>Mac251</i>	(GT) ₁₂	F: TCAAGGGTTTGACATTTGCTT R: CCCCACAACGTGATAATTCC	12	AB303466
<i>Mac235</i>	(GA) ₂₇	F: CTCTCCGACTCATCCCATCT R: CCGGATGACCATTCAAAGTC	13	AB303461
<i>Mac287</i>	(CT) ₂₃	F: CCCTGGACTAACTCTGCTCT R: GGAGGTGCTTTCGTACACCA	14	AB303476
<i>Mac357</i>	(GA) ₁₂	F: AGAAGTTATGGTGTGTTGAG R: GCTTTTGAAACNCCCCAAAG	15	AB303494
<i>Mac458</i>	(CA) ₂₁	F: TATCTGACCAATGCCTGCAA R: CTGAGGGGAAAACACTACCCA	16	AB303523
<i>Mac596</i>	(CA) ₁₆	F: TAAATGGATACTGCACGCTT R: CTTAGAATTCGGAGGATGGAC	17	AB303602
<i>Mac380</i>	(GA) ₁₆	F: GTTTTCCTTGGTGTGCATGA R: TTATGCAGCCAATGAGACGA	18	AB303502
<i>Mac563</i>	(CA) ₁₅	F: GCATGTCTTATGGTTCACAC R: AGTCACTGAAGTTCAGCATC	19	AB303593

Table S1. Continued.

Locus	Core sequence	Primer sequence 5'→3'	Linkage group	Accession no.
<i>Mac4</i>	(CA) ₄₉	F: GCTTTGTTACCTAGAGATCCA R: CACCCCCGTACCAAACCTTTT	20	AB060174
<i>Mac523</i>	(GT) ₂₆	F: GAAATGCATCCTCTCCAGTT R: ATTCCTGATGGATCAGGTCA	21	AB303585
<i>Mac612</i>	(GT) ₄₉	F: TAGCCACTAGAAGATGCTGA R: ATGTTCAAACCTACCAGCTGT	22	AB303607
<i>Mac310</i>	(GA) ₁₆	F: CTCAGAGTTCATGTCAGTGT R: CACCTTCGGATTAGATCCAG	23	AB303480
<i>Mac658</i>	(GT) ₉	F: ACAGCTGTGTGGGTCAGTGT R: TGGTCCAGAAGCAGGGGTAA	24	AB303615
<i>Mac263</i>	(GA) ₂₇	F: AGACTCCAATTTTGGCTCTT R: TCAACAATGCTGTTGAGGTT	25	AB303469
<i>Mac362</i>	(GA) ₇	F: AAGGAATAAAGCGAGAAGAG R: TCACAGATGACATAACTTCC	26	AB303497
<i>Mac637</i>	(CA) ₁₉	F: AGCTGCATTACTTCAGCTTC R: AATATTGTGCCATGTTCCCT	27	AB303612