

Full Length Research Paper

Analysis of the early development in first and backcross generations between *Paralichthys olivaceus* and *Paralichthys dentatus*

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This study investigated the performance difference of reciprocal hybrids and backcrosses between *Paralichthys olivaceus* and *Paralichthys dentatus*. The fertilization and hatching rates, combined fitness measure, early developmental characteristics and chromosome number were analyzed. The crosses of *P. olivaceus* ♀ × *P. dentatus* ♂ (F1), F1♀ × *P. olivaceus* ♂ and F1♀ × *P. dentatus* ♂ could normally fertilize (the fertilization rate: 93.2 to 97.7%), hatch (the hatching rate: 84.4 to 93.1%) and develop during pre-larvae stage. However, the fertilization rate (41.7%) and combined fitness measure (0.327) of *P. dentatus* ♀ × *P. olivaceus* ♂ was significantly reduced compared with other crosses. And the embryos of *P. dentatus* ♀ × *P. olivaceus* ♂ hatched out with flexural spine and poor vitality and died within 3 days post hatching. In addition, the result of cytogenetics analysis showed two chromosomes were missing in *P. dentatus* ♀ × *P. olivaceus* ♂, which provided evidence of postzygotic barriers in the two species. The results would be useful for better understanding the genetic phenomena of the distant hybridization in fish species.

Key words: *Paralichthys olivaceus*, *Paralichthys dentatus*, early development, inter-specific hybridization, backcross.

INTRODUCTION

Interspecies hybridization has been widely used in the development of aquaculture in many fish species (Bartley et al., 2000), like cyprinids (Reddy, 2000), trout and salmon (Galbreath and Thorgaard, 1995; Scheerer and Thorgaard, 1983), tilapia (Verdegem et al., 1997), catfish (Dunham and Argue, 1998) and so on. Many of them constitute a significant portion of global aquaculture production because of their enhanced characteristics: fast growth, robustness, environmental tolerance and disease resistance and so on. On the other hand, many produced inter-specific hybrids are of no applied

importance (Hulata, 1995), for their inviability or infertility after fertilization (Bolnick and Near, 2005). This reduction in hybrid fitness is caused by postzygotic barriers, one category of reproductive isolation, which prevents introgression between the parental species and plays an important role in speciation (Coyne and Orr, 2004).

The hybridization practice is less prevalent in flatfish culture, only a few crosses have been done (Purdom and Lincoln, 1974; Kim et al., 1996; Park et al., 2003), which are without much impact on actual production. Japanese flounder *Paralichthys olivaceus* is one of the most commercially important species, along the coastal waters of China, southwest of the Korean peninsula and Japan. The spawning season is from April to June in Bohai and Huanghai Sea in north China. Summer flounder *Paralichthys dentatus*, a high temperature tolerance species, is also a commercially important flatfish, which inhabits coastal waters from Maine to Florida in north

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Atlantic (Bengtson, 1999). The spawning season begins in September and continues to the next February (Morse, 1989). Both *P. olivaceus* (Po) and *P. dentatus* (Pd) belong to the family Paralicththyidae (Balart, 1984; Walsh et al., 1999).

Manipulations of environmental cues (such as photoperiod and water temperature) and nutrient supply have been used to induce Po and Pd reproduction synchronously and the fertilized eggs of reciprocal hybridization and backcrosses were obtained in our laboratory. Earlier researches on interspecific hybridization in Po and Pd were quite limited, most focused on embryo development and growth of Po ♀ × Pd ♂ (Guan et al., 2007a, b; Yu et al., 2010). The performance of Pd ♀ × Po ♂ and backcrosses in early development stage have not been reported. The aim of this study was to systematically investigate the performance of reciprocal hybrids and backcrosses between Po and Pd in fertilization rate, hatching rate, combined fitness parameters (Hatfield and Schluter, 1999; Jasminca and Gabriele, 2008), early developmental characteristics and chromosome number and to clarify the phenomena of postzygotic barriers in the distant hybridization in fish species. The results would provide evidence of postzygotic barriers between these species and be useful for better understanding genetic phenomena of the distant hybridization in fish species.

MATERIALS AND METHODS

The Po was a local population and cultured in Qingdao hatchery for years. The juvenile Pd (8 to 10 g) was introduced into China in 2002 from USA and cultured to 1.5 to 2.0 kg in Qingdao for three years. The hybrid experiment between Po and Pd began in January 2005. The Po ♀ × Pd ♂ females were found matured and began to spawning in 2008, but no males matured. So, an extensive crossbreeding program among Po and Pd was carried out.

The Po and Pd, and their F1 hybrids (Po ♀ × Pd ♂) (females weighed 3.5 kg and males weighed 1.5 kg) were reared at a hatchery in Lian San-dao, Qingdao, China. Po and Pd were exposed to 14L:10D and 16L:8D photoperiod, 16 to 18 and 11.5 to 12.5°C water temperature, respectively, to induce the maturation of gonads. The whole reproductive control period lasted for 5 months till the next April. Adults of F1 hybrids were stocked in laboratory tank with aerated sea water and natural light. Males with more than 85% active sperm and females with healthy looking eggs (right visual characteristics) were chosen for the following experiments. Six crosses were carried out, including two parental controls: Po × Po, Pd × Pd; two inter-specific hybrids: Po × Pd (F1), Pd × Po and two backcrosses: F1 × Po, F1 × Pd (the maternal species are named first). Each cross repeated three times. Totally, five Po females, ten Po males, two Pd females, ten Pd males and two F1 females were used.

The artificial insemination experiment for each cross was carried out by using a dry method. The sperm applied in the bowl containing eggs and seawater was added 2 min after the eggs and sperm were mixed well. The eggs and sperm were incubated together for 10-15 min at room temperature (18 ± 1°C) to allow fertilization. The sperm suspension was washed out using seawater. The suspended eggs were collected from the bowl. About 500 treated eggs were taken from each cross and incubated in a 500 ml beaker to determine fertilization and hatching rates. Each

experiment was repeated three times. The remaining was incubated in six cylindrical static rearing tanks (160 L) for embryo development observation and chromosome preparation. The culture condition was as follows: water temperature (18 ± 1°C), dissolved oxygen (8.0 ± 0.2 mg/l) and salinity (33 ± 0.5%).

The fertilization rate was determined by calculating the percentage of gastrula stage embryos among the initial number of eggs. The hatching rate was calculated as the percentage of hatched larvae in relation to the fertilized eggs. To determine hybrid fitness, a combined fitness measure (CFM) was used by multiplying fertilization rate and hatching rate. Embryonic development was recorded under a stereoscopic microscope equipped with a digital still camera (Nikon-YS100). Chromosome preparation was obtained from gastrula stage embryos as described by Shao et al. (2010).

RESULTS AND DISCUSSION

Early development of six crosses except for Pd × Po was documented to establish a frame of reference during the embryonic and pre-larvae period (Figure 1). There were no significant differences of fertilization rates among Po × Po (94.7%), Pd × Pd (85.2%), Po × Pd (97.7%), F1 × Po (93.2%) and F1 × Pd (94.2%) ($P > 0.05$) (Table 1). The hatching rates of Po × Pd (84.4%), F1 × Po (85.8%) and F1 × Pd (93.1%) were even higher than Po × Po (72.5%) and Pd × Pd (74.5%) ($P < 0.05$), indicated some transgressive inheritance appeared in Po × Pd and two backcrosses in hatching rate. While, in the Pd × Po cross, considerable embryos appeared irregular cleavage, especially from 8 cell to multicellular stage (Figure 2) and died before gastrula stage; the fertilization rate of Pd × Po (41.7%) was significantly lower than the control parents ($P < 0.05$). Once survived gastrula stage, the embryos developed normally as other groups until hatching under microscope, so the hatching rate of Pd × Po (78.1%) was similar to parental controls ($P > 0.05$). However, all the newly hatched larvae were deformed with flexural spine and poor vitality (Figure 1, D5) and died within 3 days post hatching. Similar results were previously reported in hybrids between *E. marginatus* × *E. aeneus* (Glamuzina et al., 1999) and *Epinephelus costae* × *Epinephelus marginatus* (Glamuzina et al., 2001).

The combined fitness measure differed significantly in the six crosses (Table 1). The CFM of F1 × Pd was the highest (0.877), followed by Po × Pd (0.825), F1 × Po (0.800), Po × Po (0.687), Pd × Pd (0.637) and Pd × Po (0.327). The CFM of Pd × Po was significantly lower than that of Po × Pd and other crosses. The results indicated that the viability between the two hybrid crosses differed depending on which species was the maternal parent. An obvious postzygotic barrier occurred in Pd × Po. Similar results have been observed in other fishes, such as different hybrid combinations in the family of Centrarchidae (Bolnick and Near, 2005), *Etheostoma caeruleum* × *E. zonale* (Mendelson et al., 2006) and reciprocal hybridizations between two populations of Eurasian perch (*Perca fluviatilis* L.) (Jasminca and Gabriele, 2008). A broad consensus that intrinsically low hybrid fitness could be explained by “Dobzhansky-Muller incompatibilities”

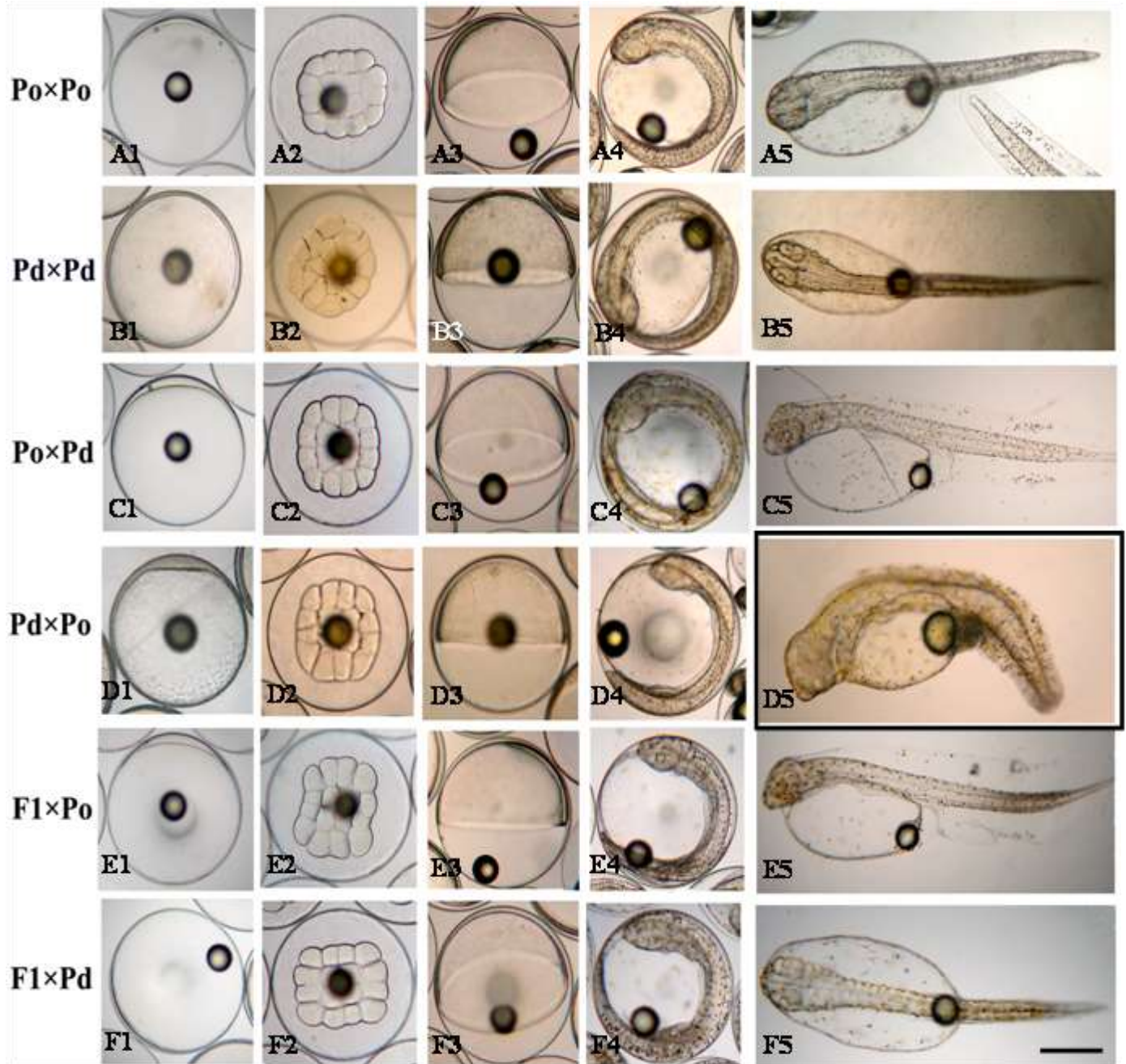


Figure 1. The embryo development of Po x Po, Pd x Pd, Po x Pd, Pd x Po, F1 x Po, F1 x Pd. A1-F1: the fertilized eggs, A2-F2: 16-cell stage; A3-F3: gastrula stage; A4-F4: pre-hatching stage; A5-F5: newly hatched larva. D5 showed newly hatched larva of Pd x Po with flexural spine. Scale bar= 0.5 mm

Table 1. Level of fertilization and hatching performances of *P. olivaceus* and *P. dentatus*, their F1 hybrids and two backcrosses.

Parameter	Po x Po	Pd x Pd	Po x Pd	Pd x Po	F1 x Po	F1 x Pd
Fertilization rate (%)	94.7±2.6 ^a	85.4±2.5 ^a	97.7±2.1 ^a	41.7±17.8 ^b	93.2±3.5 ^a	94.2±3.7 ^a
Hatching rate (%)	72.5±2.5 ^a	74.5±4.0 ^a	84.4±2.1 ^b	78.1±2.7 ^a	85.8±1.6 ^b	93.1±2.7% ^c
CFM	0.687±0.037 ^{a,c}	0.637±0.046 ^a	0.825±0.033 ^{a,c}	0.327±0.143 ^b	0.800±0.039 ^{a,c}	0.877±0.052 ^c

Table values are the means ± S.D. for the indicated number of samples. Means in a line with the same letter are not significantly different ($P = 0.05$) in the general linear models procedure and SNK test.

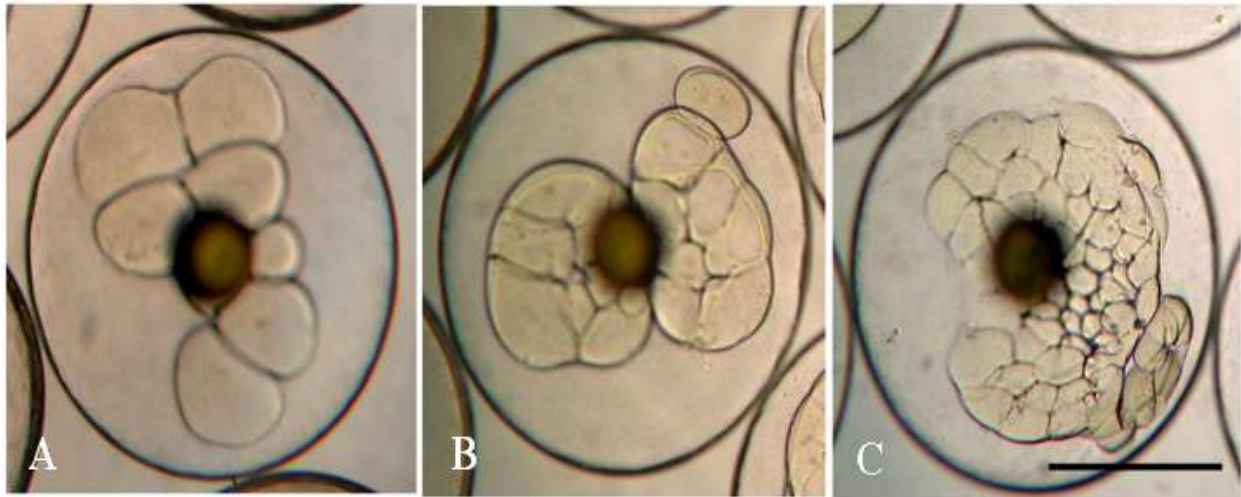


Figure 2. Abnormal cleavage stage embryos of Pd x Po. A. 8-cell stage; B.16-cell stage; C. multicellular stage. Scale bar= 0.5 mm.

Table 2. Examination of chromosome number in Po x Po, Pd x Pd, Po x Pd and Pd x Po.

Spread number		Distribution of chromosome number					
		<44	45	46	47	48	>49
Po x Po	100	5	4	4	11	70	6
Pd x Pd	100	2	2	7	8	77	4
Po x Pd	100	4	6	5	8	75	2
Pd x Po	100	8	11	68	5	7	1

(DMIs) (Bolnick et al., 2008), which is often the result of deleterious epistatic interactions between genes from divergent parental genomes (Noor and Feder, 2006). In particular, such interaction could reduce hybrid fitness if populations that have diverged in allopatry met again secondarily (Turelli and Orr, 2000; Payseur et al., 2004). The phenomenon of postzygotic barriers has also been reported in other species, like sea urchins (Strathmann, 1981; Uehara and Shingaki, 1984; Lessios and Cunningham, 1990), drosophila (Coyne and Orr, 1989) and plants (Tiffin, 2001).

In this study, cytogenetics analysis showed the chromosome numbers among parents and the two hybrid crosses were markedly different (Table 2). Of 100 Po x Pd metaphases examined, 75% possessed 48 acrocentric chromosomes, so that offspring of Po x Pd were diploids with 48 chromosomes (Figure 3c). This result is in accordance with Po x Po and Pd x Pd (Figure 3a, b). However, in Pd x Po, 68% of the metaphase showed 46 acrocentric chromosomes (Figure 3d). Two chromosomes were found to be missing and these individuals were aneuploids. Similar results were also obtained by Xu et al. (2009). The poor vitality, irregular blastomere and deformed newly hatched larvae in the cross of Pd x Po were probably due to incompatibilities between the

mitochondrially encoded genes in *P. dentatus* cytoplasm and nuclear genes in *P. olivaceus* sperm as suggested in DMIs. Genes in the cytoplasmic organelles may contribute to more or stronger DMIs, so the viability asymmetries aroused (Bolnick et al., 2008). The influence of *P. dentatus* cytoplasm might be quite strong before gastrulation, so the cleavage stage presented a big obstacle to development.

This result is consistent with the observation in euteostomatine fishes that maternal influence was typically strong before gastrulation and slowed afterward (Hubbs, 1967).

Chromosomal aneuploidy is one of the most significant factors which may adversely influence embryo survival (Leitao et al., 2001; McCombie et al., 2005). The embryos of Pd x Po could not avoid dying, although, part of them could survive gastrulation. As reported in salmonid hybrids, the mito-nuclear incompatibility accounted for chromosomal elimination and thus, led to asymmetric viability (Fujiwara et al., 1997).

It should be noted that for F1 x Po, three batches of fingerlings died nearly at the same time (about 18 dph), but we have no satisfactory explanation for this phenomenon. Further experiments on molecular and cell genetics should be performed.

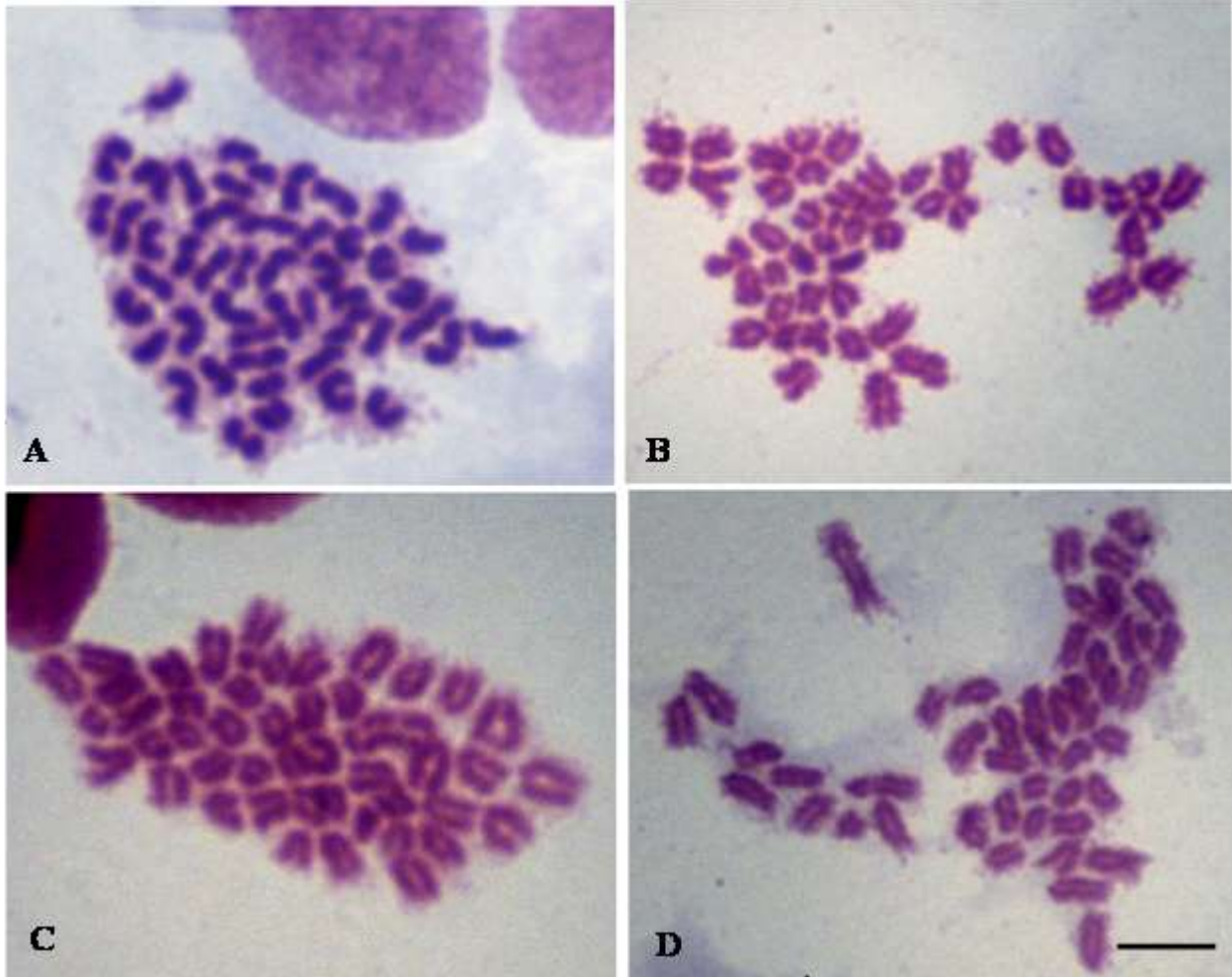


Figure 3. Metaphase chromosome spreads in intraspecific and interspecific crosses. A. The metaphase spread of Po x Po, 2n = 48 t; B. the metaphase spread of Pd x Pd, 2n = 48 t; C. the metaphase spread of Po x Pd, 2n = 48 t; D. the metaphase spread of Pd x Po, 2n = 46 t. Two chromosomes were found to be missing in this cross. Scale bar = 5 μ m.

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