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Running title: Eel hybridisation by using cryopreserved sperm

ARTIFICIAL HYBRIDIZATION OF JAPANESE AND EUROPEAN EEL (*Anguilla japonica* × *A. anguilla*) BY USING CRYOPRESERVED SPERM FROM FRESHWATER REARED MALES

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Abstract

Eggs of Japanese eel (*Anguilla japonica*) were fertilized with cryopreserved sperm of European eel (*A. anguilla*) obtained from fish reared and matured in freshwater conditions.

A new genomic PCR-RFLP marker was developed to distinguish the two species and detect maternally and paternally derived DNA fragments to genetically verify the hybrid nature of the offspring.

Keywords: propagation, fertilization, eel larvae, hybrid, PCR-RFLP, FSH

1. Introduction

Abundance of the European eel has dropped drastically from the 1980's due to climatic changes, overfishing of glass eels and adults, persistent pollutants, nematode infection, etc. (Knights, 2003). *Anguilla anguilla* is listed by the International Union for Conservation of Nature (IUCN) as critically endangered fish species [IUCN Red List of Threatened Species. Website: www.iucnredlist.org. <http://www.iucnredlist.org/apps/redlist/details/60344/0>]. Contrary to many other farmed fish species only the initial steps of successful artificial propagation of European eel are known and we have only little information on larval rearing (for review see Palstra et al., 2009). Annual production of eel farms in Europe is based on the capture of glass eels entering rivers, and this extended economic utilization is one of the major threats for the future of the eel population. Japanese scientists are in the forefront of the artificial propagation of another species of eel, Japanese eel (*A. japonica*). Since Yamamoto and Yamauchi (1974) obtained fertilised eggs and larvae from Japanese eel by hormone administration, many researchers have succeeded in obtaining eel larvae. Finally, Tanaka et al. (2003) were the first who reported about larval rearing of Japanese eel to glass eel size in captivity and its life cycle was successfully closed by using brood stock which had been artificially produced in captivity and F2 generation has been already produced (Ijiri et al., 2011). Some studies of hybridization of various eel species were published: for example, female *A. australis* with male *A. dieffenbachia* (Lokman and Young, 2000), female *A. australis* with male *A. anguilla* (Burgerhout et al., 2011) and female *A. japonica* with male *A. anguilla* (Okamura et al., 2004; Matsubara et al., 2010). The great geographical isolations (for instance the living habitat of European eel is approximately 7000-10000 km from Japanese eel habitat) are not barrier factors for eel hybridisation.

The induction of sexual maturation in captivity is a long process promoted by weekly hormone injections. The synchronisation of ovulation and spermiation is not easy, thus, the

cryopreservation of sperm would render the synchronization of spermiation and ovulation unnecessary. There are several methods described for the cryopreservation of European eel sperm (for review see Asturiano, 2008) but there are no data on successful fertilisation using European eel cryopreserved sperm so far. The objective of this study was to investigate the possibility of using cryopreserved sperm from freshwater reared European eel for fertilisation of Japanese eel eggs.

2. Materials and methods

2.1. Male maturation and sperm cryopreservation

The maturation and cryopreservation were performed in the fresh water laboratory of the University of Pannonia. Full sexual maturation and long term spermiation of farmed European eel males were induced by weekly injections of human Chorionic Gonadotropin (hCG) (250 International Unit hCG/week/fish, n=6; bodyweight 134.2±42g) in freshwater (salinity 0.05%, water temperature was maintained at 20±1.5 °C) in 2005. Sperm samples were collected at the 8th week from the first injections with an automatic pipette and distributed into plastic test tubes. Sperm motility was estimated following activation with artificial saltwater (3.5% NaCl solution) on a glass slide under 200× magnification. Sperm samples showing motility higher than 50% were chosen for cryopreservation. Sperm samples from 6 males were pooled and used for cryopreservation and fertilisation tests. A freezing dilutant was prepared in a test tube containing 3.2 mL of modified Tanaka solution (Tanaka et al., 2002) and 400 µL of methanol, and finally 400 µL of sperm was added to it. Thus sperm was diluted in a 1:9 ratio and the final concentration of the cryoprotectant was 10%. Sperm was loaded into 500 µL straws immediately after dilution (Szabó et al., 2005). Straws were frozen of each treatment in the vapor of liquid nitrogen in a styrofoam box. Straws were placed onto a styrofoam frame (height: 3 cm) floating on the surface of liquid nitrogen and

after 3 minutes they were plunged directly into liquid nitrogen. The samples were stored in a Statbourne BIO10 canister storage dewar (Statebourne Cryogenics, Washington, Tyne & Wear, UK) until fertilisation tests. Straws were thawed in a 40 °C water bath for 13 seconds.

2.2. Female maturation and fertilisation

The protocol by Ohta et al. (1997) was applied for induction of sexual maturation and ovulation of Japanese eel. Eggs, which originated from two females, were collected into dry plastic bowls by gentle abdominal pressure. The fertilisation test with Japanese eel eggs and cryopreserved sperm of European eel was performed at Hokkaido University, Japan by using dry fertilisation method (November 2010). At the same time a regular *A. japonica* reproduction was also carried out to serve as control. Eggs were distributed into 5 cm Ø Petri dishes in batches (1g) of approximately 1700-1800 eggs so that following fertilization they would form a monolayer of eggs in the Petri dish. Each batch of eggs was fertilized with one straw (500µl) of thawed sperm. Sperm was added to the eggs and then 1 mL of artificial seawater (salinity 35‰) was used to activate the gametes. After fertilisation egg samples were incubated in 50 ml Falcon tubes in a thermostat (23 °C). To follow the rhythm and phases of embryogenesis digital photos were taken of the developing embryos. All fertilization trials were conducted in triplicates.

2.3. Genetic analyses

Normal *A. japonica* and hybrid larvae were used for genetical analyses. (DNA was extracted according to the modified method of Blin and Stafford (1976). To verify genetic evidence of male contribution in the hybrid a pair of primers were designed to amplify a 100 bp fragment of the genomic Follicle Stimulating Hormone - beta subunit (FSH) coding region. For the amplification the following primers were used “FSH_Angolna_RsaI_F” 5’-

CAACAGGCCTGCAACTTCA and “FSH_Angolna_RsaI_R” 5’-

CTCAGAGCCACAGGGTAGGT. Reaction mixture contained 0.1 µL Taq polymerase and 1.2 µL of the adjacent buffer (DreamTaq, Fermentas), 0.12 µL of the primers (100 pM /µL), 1.2 µL of dNTP (2 mM/ µL; Fermentas), using 1.5 µL template DNA (30-140 µg/µL) in a 12.75 µL volume. FSH-specific PCR conditions were 2 min at 94 °C, followed by 38 cycles (30 s at 94 °C, 30 s at 64.7 °C, 30 s at 72 °C) and a final extension of one cycle 5 min at 72 °C. All amplifications were run in an Eppendorf Mastercycler (EP 384). Subsequently the PCR products were treated with *RsaI* restriction enzyme (Fermentas) applying 1 µL directly to 10 µL DNA fragments were then separated using 3% agarose gel (Serva, Germany), stained with ethidium bromide.

3. Results and Discussion

3.1. Fertilization with cryopreserved sperm from freshwater reared males

Successful fertilisation tests were carried out using sperm from freshwater reared males. In this study the hatching rate was very low because the quality of stripped eggs was poor. Three hybrid larvae and three control larvae hatched from egg batches. Hybrid embryos developed normally, compared to the control Japanese eel embryos. Both hybrid and Japanese larvae show similar somite numbers (57-58) at hatching indicating similar rate of somatogenesis, while noticeable increase in pigmentation in the caudal fin fold is detected in hybrid larvae as compared to Japanese eel larvae (Figure 1.). They were successfully hatched 34 hours after fertilization. Normal heart-beating were observed at this stage.

Full sexual maturation and long term spermiation of farmed European eel males were successfully induced by weekly hCG injections in freshwater (Boëtius and Boëtius 1967; Müller et al., 2004). Eel males reared in freshwater produced morphologically identical spermatozoa to that reported in seawater (Müller et al., 2005). Since there were no significant

differences in both the morphology and motility of spermatozoa between in freshwater and seawater matured eels, it was supposed that freshwater rearing of males is not a barrier factor for the artificial propagation of *A. anguilla* (Müller et al., 2005). The hatched viable hybrid larvae from this experiment confirmed this assumption. Additionally, advanced sexual maturation could be induced in freshwater by treating female eels, as well, with carp pituitary extract (average GSI = 9.8%, the oocytes of treated eels were in early and mid-vitellogenic phase in contrast the untreated females which had average GSI=1.0% and their gonads contained previtellogenic oocytes), thus, these results demonstrate that salinity is only a stimulating rather than a basic factor of gametogenesis of the European eel (Horváth et al., 2011).

This is the first report on successful fertilisation tests carried out using the cryopreserved sperm of European eel and Japanese eel eggs. Tanaka et al. (2002) were the first to publish and apply a practical way of cryopreservation of Japanese eel sperm and the hatchability ranged from 20.7 to 69.1% in this case. In December 2011 successful fertilisation using cryopreserved sperm was carried out using European eel eggs (Asturiano pers. comm.). There are some publications in connection with *A. anguilla* hybridisation. Okamura et al. (2004) were the first who successfully fertilised *A. japonica* eggs with *A. anguilla* sperm. Fertilisation rate was $78\pm 11.3\%$ and hybrid larvae survived up to 30 days. Matsubara et al. (2010) managed to rear *A. japonica* × *A. anguilla* larvae up to glass eel stage. Successful hybridization of female *A. australis* to male *A. anguilla* was reported previously by Burgerhout et al. (2011), however, authors did not report fertilisation and hatch results.

3.2. The fact of hybridisation was proved by genetic analyses

In order to ensure the hybrid character of the embryo concerned, we used a newly developed PCR RFLP marker. Own designed primers amplify a partial region of the eel's genomic FSH

gene coding region from both species. At first, fragments of 100 bp were amplified in each samples, using primers FSH Angolna F and FSH Angolna R (Figure 3. left). Subsequently, *RsaI* enzyme was used for restriction of PCR products, cutting only the sequence originated from the European eel, at position 40. Undigested PCR products were gained in Japanese eel samples and the hybrid, whereas the process resulted in digested fragments of 60 and 40 bp in the case of the European eel samples and the hybrid as well (Figure 3. right). Visualized with an overlapping digestion pattern, the hybrid clearly showed the two parental alleles, revealing that artificial crossing of *A. anguilla* to *A. japonica* can lead to successful fertilization. According our knowledge this is the first genomic marker that distinguish *A. anguilla*, *A. japonica*. and their hybrids from unknown parents. Mitochondrial PCR-RFLP were used (Aoyama et al. 2000; Keszka et. al., 2009; Minegishi et al. 2009, Matsubara et al., 2010) to differentiate the two species, however, information drawn from mitochondrial DNA is not suitable to identify hybrids due to its maternal inheritance. Matsubara et al (2010) proved the fact of hybridisation by using microsatellite analysis, as well, but in this case it is necessary to know the parental alleles. In addition, the computer analyses of available FSH sequences from related species showed that our marker can probably discriminate *A. anguilla* from *A. australis* and *A. marmorata*, too. However, those have not been tested on real samples. The previously described 18s rDNA based PCR-RFLP marker (Burgerhout et al. 2011) shows the same allele/sequence in *A. anguilla* and *A. japonica*, thus, this marker cannot be used to distinguish these species, only to differentiate *A. australis*, *A. rostrata* and *A. luzonensis* from them.

4. Conclusion

Freshwater rearing of males is not a barrier factor for the artificial propagation of the *A. anguilla*. Present experiments on the cryopreservation of eel sperm show that the extender originally developed for Japanese eel sperm (modified Tanaka extender) is suitable for

freezing European eel sperm together with methanol as a cryoprotectant. The novel genetic marker can provide a clear result in the detection of paternal contribution in hybridisation between the Japanese and the European eel.

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Figure and table captions

Figure 1.: Embryogenesis and hatching of *A. japonica* (a,b,c,g) and *A.japonica* × *A. anguilla* (d,e,f,h). a,d- Fertilized eggs, ~1 hour post fertilization (hpf); b,e - Stages of somitogenesis , ~ 10 somites (15.5 hpf); c,f - Stages of somitogenesis, ~ 30 somites (22.5 hpf); g,h - Hatching larvae (34hpf).

Figure 2.: Partial sequences of Follicle Stimulating Hormone - beta subunit gene coding region of European eel, Japanese eel and hybrid eels.

Figure 3.: PCR-RFLP analyses of Japan, European and a hybrid eel. Gel eletrophoresis (3% agarose gel). Amplified PCR products using FSH Angolna F and FSH Angolna R primers prior to digestion (left) and the fragments gained using restriction enzyme *RsaI* (right).

Marker = 50 bp molecule weight marker (Fermentas, EU).

Figure 1.

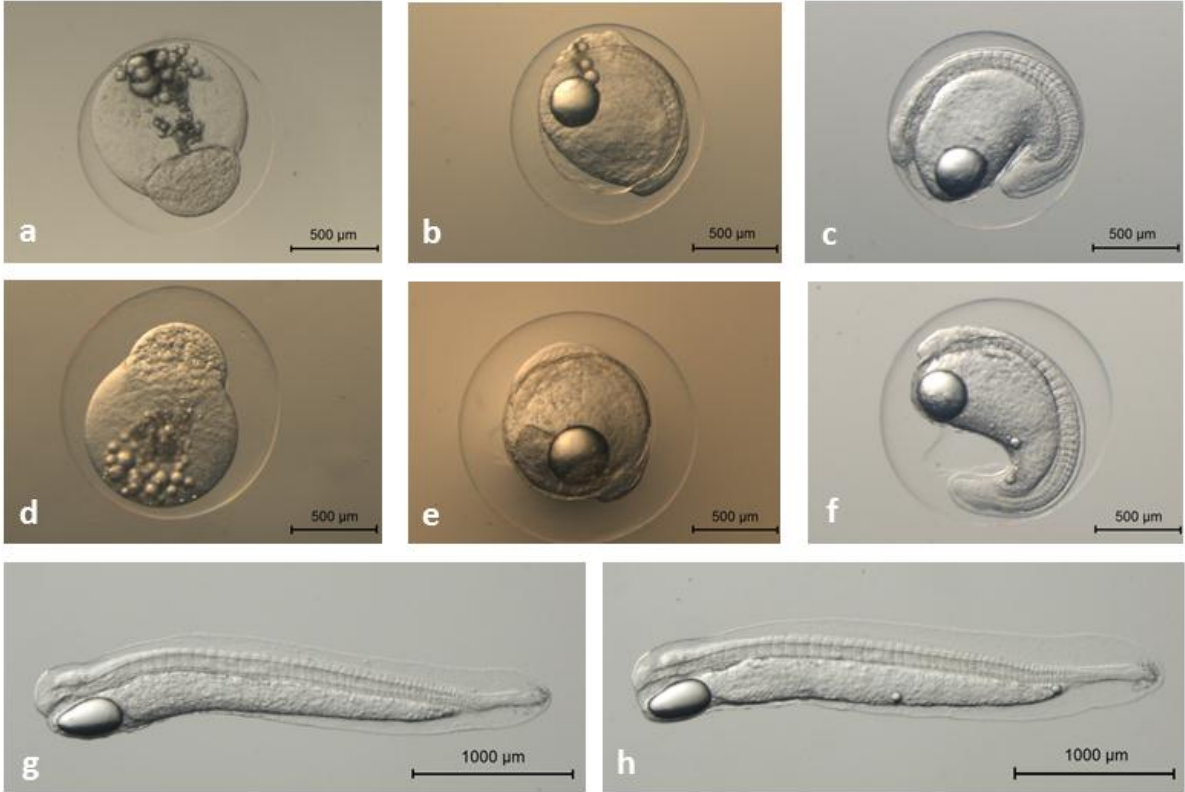


Figure 2.

