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Note

Characterization of the early-stages of the wolffish hybrid *Anarhichas minor* × *Anarhichas lupus*: conservation and aquaculture applications

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Abstract – This is the first report of early-life developmental characteristics (coloration pattern, growth and survival) and genetic identification of the wolffish interspecific hybrid between *Anarhichas minor* and *A. lupus*, both endangered species in Canadian coastal water and of interest for cold-water aquaculture diversification. A first growth trial at 8 °C featuring pure strains *A. minor* and the hybrid *A. minor* × *A. lupus* in triplicates was conducted in 2006 during the period from 0 to 140 days post-hatch. A second growth trial was runned in 2007 featuring *A. minor*, *A. lupus* and the reciprocal hybrids *A. minor* × *A. lupus* and *A. lupus* × *A. minor*. Egg development indicators and early-hatching characteristics are reported.

Key words: Hybridization / *Anarhichas* / Wolffish / Genetic characterization / Microsatellite markers

1 Introduction

The Atlantic and spotted wolffish (*Anarhichas lupus* Linnaeus and *Anarhichas minor* Olafsen) are arctic-boreal bottom-dwelling species distributed in the North Atlantic Ocean. Due to severe declines in their abundance and biomass, they have been identified as threatened (*A. minor*) or of special concern (*A. lupus*) in Canadian coastal waters (O'Dea and Haedrich 2000, 2001; Kulka et al. 2002). Habitat degradation and by-catches are possible factors involved in wolffish population status (Collie et al. 2000), as well as elevated temperatures during egg incubation or ovarian maturation (Tveiten et al. 2001; Lamarre et al. 2004) possibly caused by climatic changes occurring in the nordic environments of the Northwest Atlantic (Guelpen et al. 2005). Both species are included in the recovery strategy recently proposed by Kulka et al. (2007) for the reestablishment of wolffishes in the east coast waters of Canada. They are also the focus of aquaculture research as they present an excellent potential for the diversification of cold-water aquaculture (Le François et al. 2002; Foss et al. 2004).

Distributions of the spotted and the Atlantic wolffishes strongly overlap (Kulka et al. 2007) suggesting some degree of competition for the same resources (i.e. feeding and habitat) (Scott and Scott 1988). They also appear to share the same reproduction area and timing (Keats et al. 1985; Falk-Petersen et al. 1999). The available information, as well as the recent work of Le François et al. (2008), suggest a fair level of heterospecific gamete compatibility between these two species. In addition, Johnstone et al. (2007) determined phylogenetic relationships among mitochondrial genome of *Anarhichas* species and indicated that *A. minor* and *A. lupus* are each other's closest relative. As a consequence, a weak prezygotic isolation among those wolffish species is foreseeable and heterospecific mating in nature is in all probability occurring and could very well contribute to the observed decline of their natural populations. Based on morphological observations, the occurrence of wolffish hybrids in the wild has been suggested by Luhmann (1954). Later, Templeman (1986) described intermediate forms of wolffish (possibly *A. minor* × *Anarhichas denticulatus* Krøyer) in the North Atlantic Ocean, whereas Imsland et al. (2008) based on population genetic structure investigations, suggest their possible presence in Canadian waters.

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Hybridization can affect the fitness of inter-population and inter-specific hybrids (McClelland and Naish 2007; Ellison and Burton 2008) and in the case of small effective populations and endangered species, the evolutionary and biological consequences of hybridization e.g. increase in genetic diversity, reinforcement of reproductive isolation and genetic extinction (reviewed by Dowling and Secor 1997) requires baseline information. This information might also be useful in an aquaculture context in which hybrids could considerably improve the economics of wolffish cultivation (see Desjardins et al. 2007; see Gaudreau 2009). Furthermore, in the eventuality of efforts for the restoration of the wild populations of the Saint-Lawrence estuary (QC, Canada) (see Larocque et al. 2008), the assessment of available methods for identification of purebred wild fish specimens from the hybridized population is a valuable contribution (Meraner et al. 2008).

Since not much more information can be found in the literature on wolffish hybridization, this paper proposes a first assessment of early-life developmental characteristics (i.e. color pattern, growth and survival) of *A. minor* × *A. lupus* hybrids produced under controlled conditions and their genotypic identification. Considering that phenotypic identification of hybrids can be difficult and unreliable (Kingston and Gwilliam 2007), wolffish microsatellite markers could confirm their advantages in the detection of wolffish hybrids, as suggested by McCusker et al. (2008), since genetic markers have been used in several studies for hybrid identification (Kingston and Gwilliam 2007). This is the first report of wolffish hybrid production in captivity supported by genetic characterization.

2 Materials and methods

During two spawning seasons, 2005-2006 and 2006-2007, three ♀*A. minor* × ♂*A. lupus* hybrid families (SH1, SH2, SH3) and three pure *A. minor* families (S1, S2, S3) were produced by artificial fertilization (Moksness and Pavlov 1996) by mating one or two male(s) per female. All families originated from wild captive broodstock of spotted and Atlantic wolffish (9–13 years old) held at the facilities of the Centre Aquacole Marin (QC, Canada). S3 and SH3 are conspecific and heterospecific crosses from the same female; other families are not related in any way. Fertilized eggs were incubated for approximately 1000 degree-days in modified Heath tray incubators at 6.0 ± 0.2 °C. Hatching occurred in January 2006 for S1, SH1 and SH2, and in January-Febuary 2007 for S2, S3 and SH3. Hatching rate and the time (in degree-days) between fertilization and 50% of hatched fry, was recorded for each family (i.e. length of the incubation period, LIP). Two ♀*A. lupus* × ♂*A. minor* hybrid families (AH1, AH2) and one *A. lupus* family (A1) were also produced (A1 and AH2 are half siblings), but were only briefly morphologically characterized.

Immediately after hatching, small-scale growth trials were conducted. Triplicates of 2006 families S1, SH2 and SH3 were reared in low water level units in a recirculating rearing system (Aquabiotech Inc., QC, Canada) at 8 °C for a period of 140 days post-hatch (DPH) (150 × 3 = 450 fish; temperature 8.1 ± 0.3 °C; salinity 30.0 ± 0.3; oxygen saturation 102.3 ± 6.8%; pH 7.9 ± 0.2; nitrate level 0.187 ± 0.166 mg L^{-1}). Twenty fish per unit were weighed (wet weight, g) and measured (total length, cm) at 0, 10, 20, 35, 50, 70, 90, 120 and 140 DPH. In 2007, families S2, S3 and SH3 were reared without replication in flow-through low water level raceways (temperature 8.1 ± 1.2 °C; oxygen saturation 89.7 ± 4.4%; salinity 29.8 ± 1.3) and their growth was monitored from 0 to 300 DPH. Fishes were fed enriched *Artemia* nauplii for the first two weeks post-hatching and weaned onto a marine fish feed formulae (Gemma Skretting Canada, N.-B.). Condition factor (*CF*) was calculated using: $CF = 100(W/L_T^3)$, where W is weight of the fish (g) and L_T is the total length (cm). Growth rates (*GR*) were calculated as follow: *GR* (% day^{-1}) = 100 × (LnW_{tf} – LnW_{ti})/experimental time in days, where W_{ti} is mean weight (g) of the fish at hatching and W_{tf} is mean final weight of the fish. Missing hatching data for S2 unable the assessment of its GR.

Forty fish of the 2006 families (S1, SH1, SH2) at 140 DPH and 2007 families (S3, SH3, AH2, A1) at 479-533 DPH inclusively were subjected to a gross morphological characterization (i.e. color pattern, caudal and pectoral fins shape) from high-quality photographs taken using a DSC H50 digital camera (Sony Canada Ltd., ON).

Genotyping at seven microsatellite markers (*Alu*7, *Alu*10, *Alu*23, *Alu*25, *Alu*26, *Alu*29 and *Alu*31) was performed to confirm hybrid status of fish from SH1 and SH2 and to evaluate genetic differentiation between pure or hybrid fish (See McCusker et al. 2008 for microsatellites' sequences). DNA was extracted from adipose fins from parental species (*A. lupus*, n = 10; *A. minor*, n = 58) and from five newly-hatched fish from each family S1, SH1 and SH2 using the DNeasy DNA extraction kit (QIAGEN, Mississauga, ON, Canada). Primers (5′) were end labelled with HEX, FAM or TET dye. Polymerase chain reaction (PCR) amplifications were run on a TouchGene®Gradient thermal cycler (Techne, UK) in 15 µl reactions containing 500 mM KCl, 100 mM Tris HCl (pH 8.3), 25 mM $MgCl_2$, 10 mM each dNTPs, 10 µM of each primer, 4 µl genomic DNA and 1 unit *Taq* Polymerase. Amplification conditions were the following: 3 min of denaturation at 94 °C; 20 cycles of the following: denaturing at 94 °C for 45 s, 55 °C (–1 °C every 4 cycles) for *Alu*10, 23, 25, 26, and 29, 6 °C (–1 °C every 4 cycles) for *Alu*7 and 31 for 45 s and extension at 72 °C for 45 s; then 25 cycles of the following: 94 °C for 45 s, 50 °C for *Alu*10, 23, 25, 26, and 29, 55 °C for *Alu*7 and 31 for 45 s and 72 °C for 45 s; followed by a final extension at 72 °C for 15 min. PCR products were electrophoresed on 6% polyacrylamide gels and scanned with a FMBio III scanner (Hitachi Software Engineering America Ltd, CA, USA). Allele sizes in base pair (bp) were assigned using a CRX fluorescent ladder (Promega Corp., WI, USA). A factorial correspondence analysis (FCA) was performed using microsatellites genotypes of all individuals processed (n = 58, 15 and 10 for pure *A. lupus*, pure *A. minor* and hybrids from families SH1 and SH2 obtained in 2006 respectively), using GENETIX software (Belkhir et al. 1996). FCA graphically projects the individuals on the factor space defined by the similarity of their genotypes.

Statistical analyses were performed with SYSTAT (version 11.0, SPSS, 2004, Chicago, IL, USA). Independent sample t-tests were used to test for possible differences in egg diameter, hatching rate, LIP, weight, length and CF at hatching

Table 1. Egg diameter (mm), hatching rate (%), length of the incubation period (degree-days), weight (g), length (mm) and condition factor at hatching of pure strain and hybrid crosses of spotted wolffish based on three different families (mean ± SD). Different letters indicate significant differences between crosses, $p < 0.05$. *A. minor*, spotted wolffish; *A. lupus*, Atlantic wolffish.

	Cross (♀× ♂)	
Variable	*A. minor* × *A. minor*	*A. minor* × *A. lupus*
n	3	3
Egg diameter (mm)	5.8 ± 0.1	5.8 ± 0.1
Hatching rate (%)	22.1 ± 18.6	17.1 ± 10.7
Length of the incubation period (degree-days)	932 ± 62	909 ± 25
Weight at hatching (g)*	0.135 ± 0.007[a]	0.111 ± 0.002[b]
Length at hatching (mm)*	25.0 ± 2.4	23.9 ± 2.7
Condition factor at hatching*	0.92 ± 0.19	0.86 ± 0.28

* $n = 2$ for pure strain cross, missing data for S2.

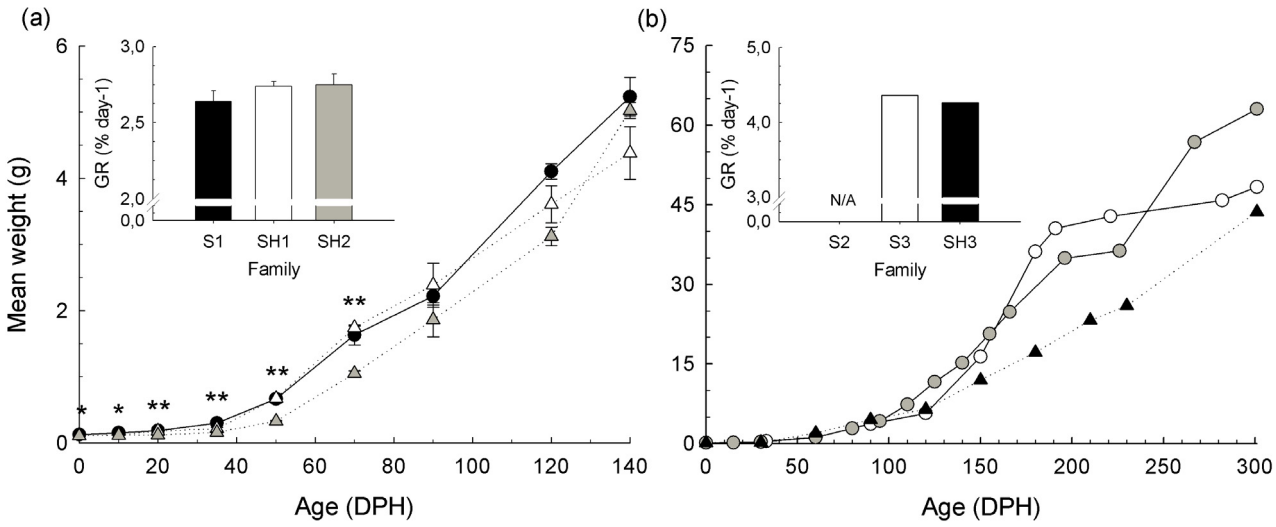

Fig. 1. (a) Growth in weight (g) from hatching to 140 DPH of 2006 families (S1 (•), *A. minor*; SH1 (Δ), SH2 (▲), *A. minor* × *A. lupus*). Inset shows their growth rate (GR, % day^{-1}) from 0 to 140 DPH. ($n = 3$). (b) Growth in weight (g) from hatching to 300 DPH of 2007 families (S2 (•), S3 (○), *A. minor*; SH3 (▲), *A. minor* × *A. lupus*). Inset shows their growth rate (GR, % day^{-1}) from 0 to 300 DPH. ($n = 1$). Different symbols identified hybrid and pure strain crosses and colors different families. Asterisks indicate significant differences between 2006 families, $p < 0.05$ (* S1 > SH1 = SH2; ** S1 = SH1 > SH2) (mean ± SD).

between purebred and hybrid crosses of spotted wolffish. Normality of data was tested using Kolgomorov-Smirnov test with Lilliefors correction and homogeneities of variance determined with F-tests. One-way ANOVA were used to test for possible differences in weight, length, CF, survival rate and GR between 2006 families. Normality of residual was tested using Kolgomorov-Smirnov test with Lilliefors correction and homogeneities of variance determined with Levene. Tukey *post-hoc* test was used when significant differences were detected ($p < 0.05$).

3 Results

Considering both production years, no significant differences appear between purebred and hybrid crosses for egg diameter (*t*-test, $df = 4$, $F = 1$, $p > 0.5$), hatching rate (*t*-test, $df = 4$, $F = 3.1$, $p > 0.5$) or the length of the incubation period (*t*-test, $df = 4$, $F = 7.1$, $p > 0.5$) (Table 1). A hybridization response featuring mean weight at hatching day is however observed, S > SH (*t*-test, $df = 3$, $F = 12.3$, $p < 0.01$) (Table 1). No differences in mean length (*t*-test, $df = 3$, $F = 0.8$, $p > 0.5$) and condition factor at hatching (*t*-test, $df = 3$, $F = 0.5$, $p > 0.5$) were detected between crosses, however, high variation between families possibly concealed possible effect of hybridization status (Table 1).

During the first growth trial (0-140 DPH in 2006), the differences in weight at hatching between the hybrid and pure strain crosses were maintained until 10 DPH (S1>SH1=SH2). Thereafter, SH1 achieved weight equivalent to the pure family (S1=SH1>SH2) and from 70 DPH no further significant differences were found among these families (Fig. 1, Table 2). GR values were in accordance with these results (Fig. 1a inset, ANOVA, $df = 2$, $F = 0.901$, $p > 0.05$). For length and CF values, no particular trends were observed among families (Table 2). Percent mortalities for S1, SH1 and SH2 stabilized respectively at 42, 42 and 49 DPH. No significant differences between families were observed for survival throughout the experiment, except from 35 DPH for family SH2 (Table 2). SH2 displayed a significantly lower final survival rate (11.0 ± 1.9%)

Table 2. General linear model for weight, length, condition factor (CF) and survival rate for 2006 families (S1, SH1, SH2) at different age in DPH. Significant probabilities are bold faced ($p < 0.05$).

Age	Source		Weight		Length		CF		Survival rate	
(DPH)		*df*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
0	Family	2	118.7	**0.000**	26.4	**0.001**	50.3	**0.000**	-	-
10	Family	2	129.6	**0.000**	5.22	**0.049**	12.1	**0.008**	2.49	0.178
20	Family	2	171.1	**0.000**	153.2	**0.000**	35.5	**0.000**	3.23	0.126
35	Family	2	9.57	**0.014**	11.2	**0.009**	3.66	0.091	16.2	**0.007**
50	Family	2	29.5	**0.001**	31.6	**0.001**	6.44	**0.032**	16.1	**0.007**
70	Family	2	16.2	**0.004**	9.43	**0.014**	7.46	**0.024**	15.7	**0.007**
90	Family	2	1.10	0.393	1.34	0.330	0.15	0.866	21.1	**0.004**
120	Family	2	4.81	0.068	2.35	0.191	1.20	0.375	19.8	**0.004**
140	Family	2	1.60	0.309	0.56	0.613	2.51	0.197	19.8	**0.004**

compared to S1 and SH1 (53.7±17.5% and 69.2±2.6% respectively) (Table 2). During the second growth trial (0–300 DPH in 2007), the available information suggest lower or similar growth performance of SH3 compare to its half sib cross S3 (Fig. 1b and inset) and no specific trends were observed for final survival rate (>80%).

Based on coloration patterns and caudal and pectoral fins structures, it was not possible to clearly distinguish reciprocal hybrids: *A. minor* × *A. lupus* (at 140 DPH and ≈ 500 DPH) or *A. lupus* × *A. minor* (at ≈ 500 DPH) from pure *A. minor* (Fig. 2). *A. lupus* was, however, clearly discernible from both the hybrids and pure *A. minor*.

The eight microsatellites markers used allowed the confirmation of the hybrid status of families SH1 and SH2 and allowed a perfect discrimination of hybrids from pure fish (Fig. 3). They all inherited in a mendelian way (all offspring tested have one allele of each parent). The allele range observed for *Alu*25 was markedly different for *A. minor* and *A. lupus* (120 to 132 bp and 156 to 176 bp respectively).

Discussion

Our results demonstrate that the spotted and Atlantic wolffish can easily interbreed to produce viable hybrid progeny and that morphologically, the reciprocal hybrids cannot be distinguished from pure *A. minor* by simple visual observations. Reciprocal hybrid juveniles (*A. minor* × *A. lupus* and *A. lupus* × *A. minor*) distinctively display color patterns with a predominance of dark spots in opposition to the spotless Atlantic wolffish juveniles (adults are vertically dark stripped, Barsukov 1972). Hybrid maternal as well as paternal predominance of various phenotypic traits have been reported for other fish species (Park et al. 2003). In our study, newly-hatch *A. minor* × *A. lupus* hybrids displayed a smaller size at hatching than pure *A. minor* indicating a certain degree of hybridization response as *A. lupus* larvae are generally smaller than *A. minor* (Le François et al. 2008). However, no unambiguous conclusions can be made on hybrid growth and survival as our incomplete mating design and low number of families produced seriously limit the scope of our findings. Nevertheless, our hatching data (Table 1) were in accordance with previous reports on spotted and Atlantic wolffish (Falk-Petersen et al. 1999; Hansen and Falk-Petersen 2001; Falk-Petersen and Hansen 2003) as well as the early growth performances (Mokness and Pavlov 1996; Hansen and Falk-Petersen 2002; Le François et al. 2004; Lamarre et al. 2004; Savoie et al. 2006).

In addition to coloration patterns, species and hybrid identification can rely on a collection of unique morphological traits, characteristic of each species, such as vertebrae and fin rays number, teeth distribution, caudal fin structure and skull forms (see Barsukov 1972). However, morphological variability introduced by hybridization, further increases the complexity of their identification (Loy et al. 1999). Hybrids can involve two distinct species, strains or populations within a single species (Duchesne and Bernatchez 2007) and in order to successfully identify the hybrid forms, it requires a certain level of genetic information, especially when parental species are phylogenetically closely related, such as the Atlantic and the spotted wolffish. Fish genotypic hybrid identification for field studies is frequently used (Metcalf et al. 2008; Papousek et al. 2008). Our results, based on the utilisation of seven loci (i.e. *Alu*7, *Alu*10, *Alu*23, *Alu*25, *Alu*26, *Alu*29 and *Alu*31) were found to be adequate for the detection of wolffish hybrids originating from our broodstock populations.

Increase of genetic diversity induced by hybridization could facilitate adaptations to novel environments (Seehaussen 2004) and provide opportunities for the expansion of wolffishes distribution range. Indeed, the Atlantic wolffish, which is the southeast and more coastal wolffish species (Barsukov 1959 cited by Barsukov 1972; Scott and Scott 1988), is believed to be adapted to a wider range of temperature and salinity conditions, while the spotted wolffish is more stenothermal as it inhabits deeper ocean areas (Foss et al. 2001; Le François et al. 2004). Further investigations in captivity within a wide range of environmental conditions (temperature, salinity, etc. and possible interactions) will be important to fully understand the eco-physiological consequences of wolffish hybridization and their aquaculture relevance. At present, the mitochondrial functionality impairment that could be generated by hybridization (Blier et al. 2006) and severely limit the adaptability of the hybrids exposed to sub-optimal conditions is currently being assessed as well as the evaluation of the heritability of antifreeze protein synthesis capacities (present in *A. lupus* but not *A. minor* (Desjardins et al. 2006, 2007).The evaluation of the reproductive abilities of the hybrids based on histological, hormonal and functionality assessments is also planned.

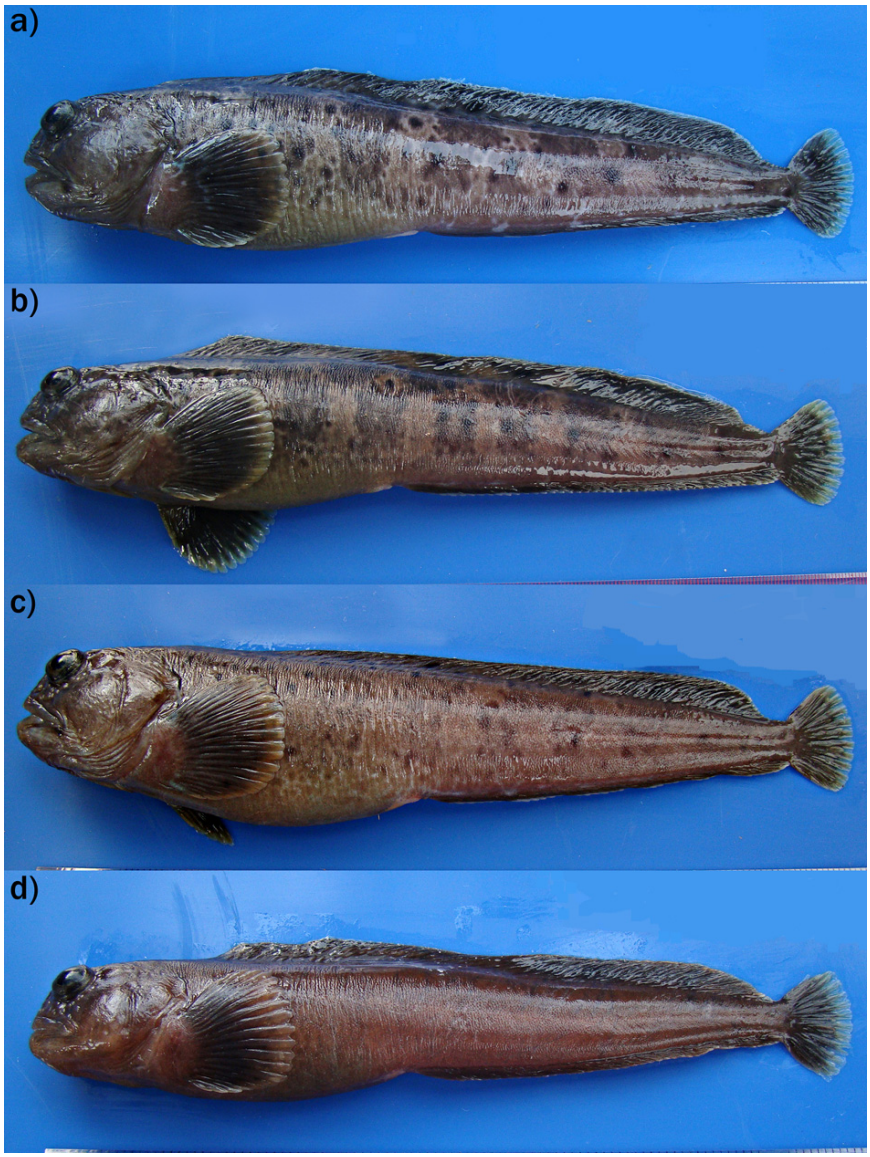

Fig. 2. (a) S3, spotted wolffish (*A. minor*), 500 DPH. (b) SH3, *A. minor* × *A. lupus* hybrid, 492 DPH. (c) AH2, *A. lupus* × *A. minor* hybrid, 533 DPH. (d) A1, Atlantic wolffish (*A. lupus*), 479 DPH. S3 and SH3 are conspecific and heterospecific crosses from the same female as well as A1 and AH2. DSC H50, Sony Canada Ltd., ON, Canada.

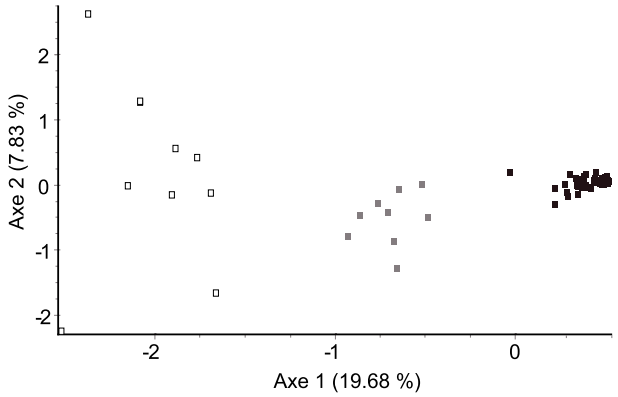

Fig. 3. Factorial correspondence analysis (FCA) based on microsatellites genotyping (pure *A. lupus* (□); *A. minor* × *A. lupus* hybrid (■); pure *A. minor* (■).

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