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In the intimacy of the darkness: Genetic polyandry in deep-sea luminescent lanternsharks *Etmopterus spinax* and *Etmopterus molleri* (Squaliformes, Etmopteridae)

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Abstract

Multiple paternity seems common within elasmobranchs. Focusing on two deep-sea shark species, the velvet belly lanternshark (*Etmopterus spinax*) and the slendertail lanternshark (*Etmopterus molleri*) we inferred the paternity in 31 *E. spinax* litters from Norway (three to 18 embryos per litter) and six *E. molleri* litters from Japan (three to six embryos), using 21 and 10 specific microsatellites, respectively. At least two *E. spinax* litters were sired from multiple fathers each, with highly variable paternal skew (1:1 to 9:1). Conversely, no clear signal of genetic polyandry was found in *E. molleri*.

KEYWORDS

Etmopteridae, microsatellites, paternity test, polyandry, squaliformes

Within elasmobranchs, different reproductive modes have been reported, from viviparity to oviparity (Compagno, 1990; Conrath *et al*., 2012; Wourms, 1977), and even parthenogenesis in certain captive animals (Chapman *et al*., 2007, 2008; Portnoy *et al*., 2014; Robinson *et al*., 2011). Elasmobranchs also exhibit seasonal monogamous [*e.g*., *Sphyrna tiburo* (Chapman *et al*., 2004), *Galeocerdo cuvier* (Holmes *et al*., 2018)] or polygamous [*e.g*., *Negaprion brevirostris* (Feldheim *et al*., 2001), *Carcharhinus leucas* (Pirog *et al*., 2015, 2019)] mating strategies. Indeed, multiple paternity seems common within elasmobranchs [see Rossouw *et al*. (2016) for a review]. However, data on the reproductive strategies of deep-sea elasmobranchs remains scarce, mainly due to the difficulty of observing and collecting samples from these animals.

Among deep-sea elasmobranchs, etmopterids are represented by at least 43 benthopelagic species (Straube *et al*., 2010, 2015). Some of these species are able to emit light (*i.e*., bioluminescence), such as the velvet belly lanternshark, *Etmopterus spinax* (Claes & Mallefet, 2009b; Duchatelet *et al*., 2019) and the slendertail lanternshark, *E. molleri* (Claes & Mallefet, 2015; Duchatelet *et al*., 2019). *E. spinax* is a small-bodied shark (up to 60 cm in total length) that is widespread on the eastern Atlantic continental shelf from northern Norway to the coasts of central West Africa at depths ranging from 85 to 785 m (Coelho & Erzini, 2010). This ovoviviparous species potentially has a triannual reproductive cycle, typical of squalid deep-sea sharks, with a gestation period not exceeding 1 year (Coelho & Erzini, 2008). It has a long lifespan, around 20 years (Gennari & Scacco, 2007), with sexual maturity appearing between the fourth and fifth years (Coelho & Erzini, 2008). Litter sizes vary from 1 to 18 embryos (Capape *et al*., 2001), with most litters ranging from 10 to 12 (*i.e*., five to six pups in each of the two uteri; Claes & Mallefet, 2008). Comparatively, almost no data is available for *E. molleri*. This small-bodied (up to 45 cm in total length) ovoviviparous, benthopelagic shark occurs in deep waters of

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the western Pacific Ocean from 238 to 655 m (Ebert *et al*., 2013; Weigmann, 2016). Although a relatively common species, often caught as bycatch by trawling fisheries (Coelho & Erzini, 2008; McKinnell & Seki, 1998) and the subject of many bioluminescence studies (*e.g*., Claes & Mallefet, 2008, 2009a, b, 2010, 2015; Duchatelet *et al*., 2020), data on their biology are limited.

To improve knowledge regarding the reproductive strategy in lanternsharks, we studied whether *E. spinax* and *E. molleri* present genetic polyandry (*i.e*., multiple paternity within a single litter) by inferring the paternity within 31 litters from Norway and six litters from Japan, respectively, using 21 and 10 informative specific microsatellite loci, respectively.

For *E. spinax*, individuals were collected from two different locations (130 km apart) in May 2018: the North Sea near the Norwegian coast (NOR; 60°43′03″N, 03°18′58″E) and the Raunefjord, Norway (RAU; 60°15′54″N, 05°07′46″E). All specimens were collected under the Experimental fish care permit number 17/85810 delivered to the Espegrend Marine Biological Station (University of Bergen, Norway). From each location, 22 and 12 gravid females were caught, respectively, using deep long-lines lowered at 230 m. An additional 10 adult males were captured in the North Sea and kept for further genetic analyses. For *E. molleri*, all samples were collected in November 2017 in the East China Sea near the Sesoko Island coast, Okinawa, Japan (26°28′94″N, 127°41′20″E). Seven gravid females were captured using a bottom hook-and-line method at depths ranging from 480 to 500 m. Lanternsharks were euthanized under the approval of the Animal Ethics Committee of the Catholic University of Louvain. Samples were then collected from 44 *E. spinax* and seven *E. molleri* adults (a small piece of muscle) as well as from unborn pups (the entire caudal fin) contained in the uteri of gravid females. The respective uterus (*i.e*., right or left) was noted for each embryo (except for *E. molleri*). Then, for each sample, total genomic DNA was extracted using a DNeasy Blood & Tissue kit (Qiagen) following the manufacturer's protocol and genotyping was performed using 29 and 19 specific microsatellite loci for *E. spinax* and *E. molleri*, respectively, as in Oury *et al*. (2019).

The probability of detecting multiple paternity in a litter depends on the number of microsatellite loci, the polymorphism of these loci in the population (allele frequencies), the number of offspring, the number of putative fathers and the reproductive skew (the number of embryos within a litter sired by each male). Thus, in each species, per locus diversity was estimated as the number of alleles (*Na*), the observed and expected heterozygosities (*Ho*, *He*) and the inbreeding coefficient (F_{IS}; Wright, 1931), using adult genotypes, and executed using FSTAT 2.9.3 software (Goudet, 2001). Departures from the Hardy–Weinberg equilibrium (HWE) were also tested (FSTAT). For the different litter sizes found, considering only informative loci (*i.e*., *Na* > 2), the probability of detecting multiple paternity was calculated using PrDM software (Neff & Pitcher, 2002) with allelic frequencies estimated among all adults for each species and varying (a) the number of sires from 2 to 4 and (b) the reproductive skew among sires from 1:1 (both males sired the same number of offspring) to 4:2:1:1 (one male sired two times more offspring than a second male, but four times more offspring than a third and fourth male).

Genetic polyandry was considered when at least three non-maternal alleles were found in the brood for at least two microsatellite loci to rule out genotyping errors (criterion of allelic evidence). Moreover, putative paternal sires and full and half-sibling relationships in the litters of at least three pups were inferred using COLONY 2.0.6.5 (Jones & Wang, 2010) with default parameters, except with the length of the run set to 'long' and the reproduction system set to 'polygamy' for both sexes to allow the half-sibling assignments. Allelic frequencies were estimated from the samples 'without update'. Litters of fewer than three pups were not included in the analyses as genetic polyandry cannot be detected therein and may result in overclustering (Jones & Wang, 2010).

For *E. spinax*, among the 29 loci, 21 had more than two alleles and were considered informative for genetic polyandry analyses. For these loci, *Na* varied from three for six of the loci to 15 for Et07, with a mean (±S.E.) of 5.0 ± 0.6. *Ho* and *He* (both populations pooled; see Supporting information data 1 for statistics per population) varied from 0.116 to 0.837 and from 0.112 to 0.900, respectively, with means (±S.E.) of 0.448 ± 0.045 and 0.487 ± 0.046, respectively. Four loci showed significant heterozygote deficiency (F_{IS} ranging from 0.246* to 0.447**), while one showed significant heterozygote excess (F_{IS} = −0.196*; Supporting information data 1). For *E. molleri*, among the 19 loci, 10 were informative for genetic polyandry analyses (*i.e*., *Na* > 2). Among these, *Na* varied from three for six of the loci to five for Et07 (mean ± S.E. = 3.5 ± 0.2), *Ho* from 0.143 to 1.000 (mean ± S.E. = 0.543 ± 0.092) and *He* from 0.274 to 0.821 (mean ± S.E. = 0.543 ± 0.053). Two loci showed significant deviation from HWE (Supporting information data 1). Further analyses were performed with the 21 *E. spinax* and 10 *E. molleri* loci with *Na* > 2.

According to PrDM, with the 21 informative loci, the probability of detecting multiple paternity for *E. spinax* was between 0.36 and 1.00, and increased with litter size and number of sires. For litters of eight embryos or higher (*N* = 12), the probability was greater than 80% for all scenarios (Supporting information data 2). For *E. molleri*, considering the 10 informative loci, the probability was lower, between 0.35 and 0.94 (Supporting information data 2). For both species, when broods comprised fewer than five embryos (*i.e*., five litters for *E. spinax* and one for *E. molleri*), the probability of detecting multiple paternity was lower, especially when sired by two males with high paternal skews (4:1; Supporting information data 2). Thus, for litters with few embryos, underestimation of the number of sires could be assumed, considering the low polymorphism of the loci, and especially if a high paternal skew exists.

According to the criterion of allelic evidence, genetic polyandry was clearly highlighted in two litters of *E. spinax* (ES_F04 and ES_F10; Supporting information data 3a). Furthermore, for these two litters, half-siblings were found in the same uterus (Supporting information data 3a). Three validated paternal alleles were also recorded for eight other *E. spinax* litters (ES_F03, ES_F14, ES_F16, ES_F17, ES_F18, ES_F28, ES_F29 and ES_F31), but only at one microsatellite locus (Supporting information data 3b). According to the criterion of allelic evidence only, we should not consider these litters sired from multiple

fathers, but genetic polyandry is possibly masked by the low polymorphism of some loci.

For each litter of at least three embryos, full and half-sibling relationships were inferred with COLONY (Table 1 and Supporting information data 4). For the two litters for which genetic polyandry was detected according to the criterion of allelic evidence (ES_F04 and ES_F10), COLONY indicated probabilities of 0.777 and 0.876, respectively, to be sired by two males and 0.223 and 0.124 to be sired by three males (Table 1 and Figure 1). Notably, no litter was found to be sired by more than three males. For litter ES_F04, the most probable case is to be sired by two fathers ($P_{scenario\ 1}$ = 0.777), with three embryos assigned to each father, suggesting no paternal skew (1:1; Figure 1a). Inclusion and exclusion probabilities were high for both full-sibling clusters ($P > 0.700$; Supporting information data 5). For litter ES_F10, only one embryo was issued from a different father in the most probable case (two fathers, $P_{scenario\ 1}$ = 0.812; Figure 1b), suggesting a high paternal skew (9:1). However, the exclusion probability of the cluster formed by this embryo was low (P = 0.096; Supporting information data 5), indicating potential oversplitting.

Five additional litters of *E. spinax* displayed a high probability of being fertilized by at least two males ($P_{\geq 2\ males} \geq 0.971$; Table 1 and Supporting information data 4): ES_F14, ES_F18, ES_F26, ES_F29 and ES_F31. In the most probable scenarios, these litters were fertilized by two, three, two, two and two males, respectively (Supporting information data 6), with respective paternal skews of 5:1, 2:1:1, 7:1, 5:2 and 6:1 (Supporting information data 6). For the 11 corresponding clusters, inclusion probabilities were high ($P \geq 0.581$), but exclusion probabilities were lower ($P \leq 0.421$), except for ES_F18 and ES_F26 (Supporting information data 5). For four of these five litters (all except ES_F26), one locus was previously found with at least three validated paternal alleles. Thus, these litters (a) either present genetic polyandry, but allelic evidences were found from only one locus due to low locus polymorphism, or (b) were sired by a single male, but COLONY inferences result in oversplitting.

Five litters of *E. molleri* (EM_F01, EM_F02, EM_F04, EM_F05 and EM_F07) also showed a high probability of being fertilized by two males or more ($P_{\geq 2males} > 0.860$; Table 1 and Supporting information data 7). However, most exclusion probabilities were low ($P < 0.500$; Supporting information data 5) and as no allelic evidence suggested this, the genetic polyandry in these litters cannot be argued without any doubt and potentially results from oversplitting.

Notably, COLONY inferences also grouped as half-siblings all embryos from the *E. spinax* litter ES_F12 with the embryo ES_F14a with a probability of 0.861 (Supporting information data 4), suggesting a potential case of polygyny (*i.e.*, two females fertilized by the same male) in *E. spinax*. However, these results should be taken with caution as the low polymorphism of the loci may result in overclustering within COLONY inferences. Thus, additional investigation is needed to demonstrate polygyny.

Understanding the mating strategy of deep-sea sharks is a growing topic due to the recent increasing anthropogenic exploitation of deep-sea ecosystems (Coelho & Erzini, 2008; Gaggiotti & Vetter, 1999; Koslow *et al.*, 2000; Norse *et al.*, 2012; Smith *et al.*, 1998; Stevens

TABLE 1 Summary of the 34 *E. spinax* and seven *E. molleri* sampled gravid females

Female	*N*	Right	Left	$P_{1\ male}$	$P_{2\ males}$	$P_{3\ males}$
***E. spinax* – North Sea**						
ES_F01	10	6	4	1.000	0.000	0.000
ES_F02	2	1	1	–	–	–
ES_F03	5	2	3	1.000	0.000	0.000
ES_F04	6	2	4	0.000	0.777	0.223
ES_F05	5	4	1	1.000	0.000	0.000
ES_F06	2	2	0	–	–	–
ES_F07	3	2	1	1.000	0.000	0.000
ES_F08	5	2	3	1.000	0.000	0.000
ES_F09	4	3	1	1.000	0.000	0.000
ES_F10	10	6	4	0.000	0.876	0.124
ES_F11	3	1	2	1.000	0.000	0.000
ES_F12	9	5	4	1.000	0.000	0.000
ES_F13	4	1	3	0.502	0.498	0.000
ES_F14	6	3	3	0.000	1.000	0.000
ES_F15	5	2	3	1.000	0.000	0.000
ES_F16	6	4	2	0.415	0.585	0.000
ES_F17	12	6	6	1.000	0.000	0.000
ES_F18	4	2	2	0.000	0.000	1.000
ES_F19	8	3	5	1.000	0.000	0.000
ES_F20	7	2	5	1.000	0.000	0.000
ES_F21	1	1	0	–	–	–
ES_F22	5[a]	1	3	1.000	0.000	0.000
***E. spinax* – Raunefjord**						
ES_F23	7	4	3	0.976	0.024	0.000
ES_F24	10[a]	4	4	1.000	0.000	0.000
ES_F25	6	3	3	1.000	0.000	0.000
ES_F26	8	5	3	0.000	1.000	0.000
ES_F27	13	6	7	0.919	0.081	0.000
ES_F28	18	9	9	1.000	0.000	0.000
ES_F29	7[a]	3	3	0.029	0.896	0.075
ES_F30	7	2	5	1.000	0.000	0.000
ES_F31	7[a]	3	3	0.000	0.777	0.223
ES_F32	8	4	4	1.000	0.000	0.000
ES_F33	15	7	8	1.000	0.000	0.000
ES_F34	9	5	4	1.000	0.000	0.000
***E. molleri* – East China Sea**						
EM_F01	6[a]	NA	NA	0.140	0.843	0.017
EM_F02	5[a]	NA	NA	0.000	0.395	0.605
EM_F03	6[a]	NA	NA	0.956	0.044	0.000
EM_F04	6[a]	NA	NA	0.000	0.123	0.877
EM_F05	4[a]	NA	NA	0.000	0.978	0.022
EM_F06	2[a]	NA	NA	–	–	–
EM_F07	6[a]	NA	NA	0.117	0.495	0.388

[a]Litter containing embryos not assigned to a uterus.

Note. Litter sizes (*N*) and uterus repartition of the embryos (right/left) are given for each female, as well as the probabilities of being fertilized by one, two or three males, according to COLONY inferences (noted $P_{1\ male}$, $P_{2\ males}$ and $P_{3\ males}$, respectively)

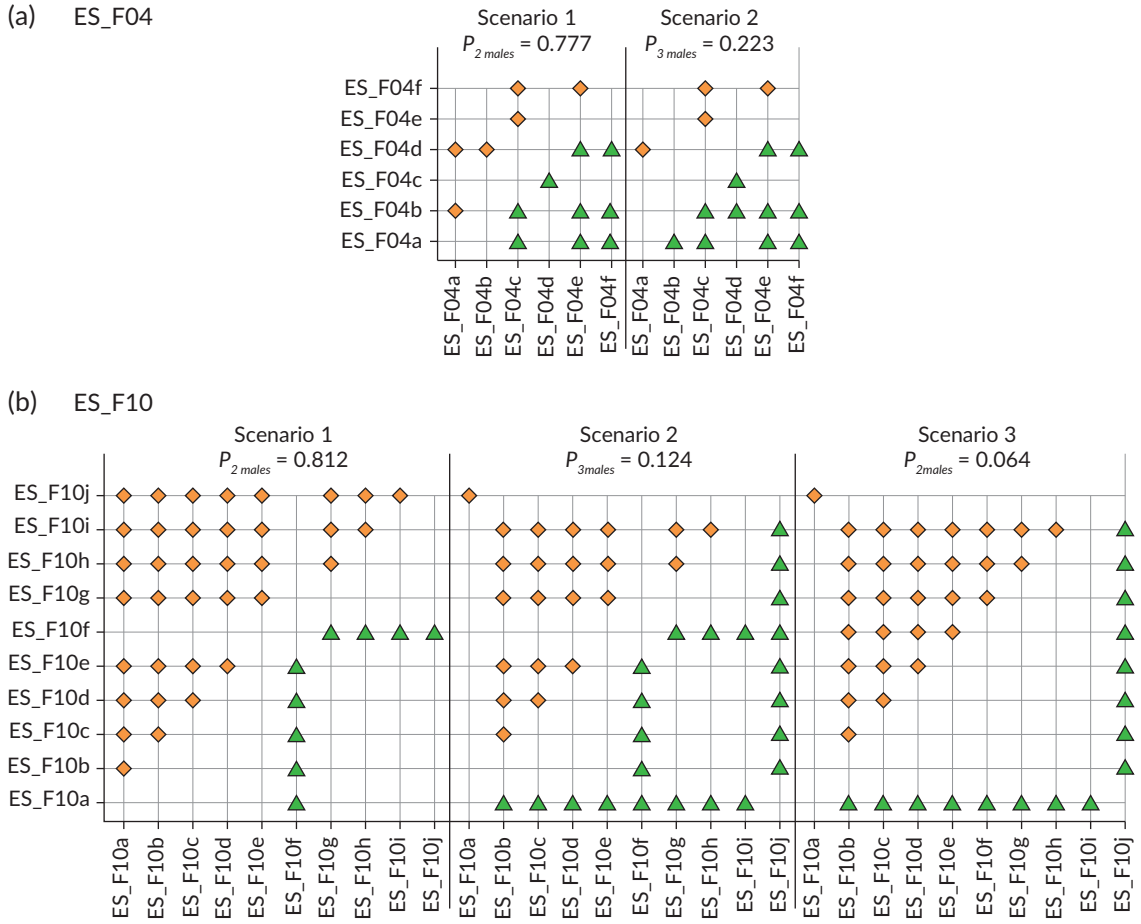

FIGURE 1 Full and half-sibling relationships inferred with COLONY for the *E. spinax* litters (a) ES_F04 and (b) ES_F10. Diamonds (upper diagonal) indicate full siblings; triangles (lower diagonal) indicate half-siblings. The probability of each scenario is indicated above

et al., 2000). Currently, few genetic studies have addressed this question (Daly-Engel *et al*., 2010; Hernandez *et al*., 2014). This work assessed for the first time genetic polyandry in the Etmopterids *E. spinax* and *E. molleri* using microsatellite loci developed for these species (Oury *et al*., 2019). Genetic polyandry was clearly detected in *E. spinax* litters (6.5%), with a highly variable paternal skew ranging from 1:1 to 9:1. Additionally, possible cases of genetic polyandry were found in both *E. spinax* and *E. molleri*, according to allelic evidence (eight *E. spinax* litters) or COLONY (five *E. spinax* litters and five *E. molleri* litters). Genetic polyandry has already be shown in other ovoviviparous sharks (*e.g*., *Squalus acanthias* (17–30% of polyandrous litters; Lage *et al*., 2008; Veríssimo *et al*., 2011), *Squalus mitsukurii* (11% of polyandrous litters; Daly-Engel *et al*., 2010), *Mustelus antarcticus* (13% of polyandrous litters; Boomer *et al*., 2013), *Mustelus lenticulatus* (24% of polyandrous litters; Boomer *et al*., 2013), *Galeorhinus galeus* (40% of polyandrous litters; Hernandez *et al*., 2014) or *Triakis semifasciata* (36% of polyandrous litters; Nosal *et al*., 2013)].

As a reproductive strategy, multiple paternity can display a large number of potential benefits, such as increasing the number of viable offspring in a single brood (Newcomer *et al*., 1999) or producing offspring with better fitness and heterozygosity (DiBattista *et al*., 2008). Multiple paternity can result from female mating choice (Whitney *et al*., 2004). As Etmopterids are known to display bioluminescent sexual dimorphism [*i.e*., male presenting sex-dependent hormonal responsive luminous claspers (Claes & Mallefet, 2009c, 2010)], female mate choice may occur in terms of male brightness or luminous courtship in the darkness of the water column, as shown for other marine bioluminescent taxa (Herring, 2000, 2007). If females do not choose the reproductive males, multiple paternity may result from some post-copulatory events, such as sperm storage and utilization (Fitzpatrick *et al*., 2012; Marino *et al*., 2015; Neff & Pitcher, 2005). Sperm retention and/or storage have been described in various elasmobranchs species [*e.g*., *Prionacea glauca* (Hazin *et al*., 1994; Pratt, 1993), *Iago omanensis* (Hamlet *et al*., 2002), *Mustelus antarticus* (Storrie *et al*., 2008), *Centroscymnus coelolepis* (Moura *et al*., 2011)]. Post-copulatory mechanisms may correspond to (a) sperm selection or competition, or (b) paternity influence by reflecting the timing of mating, such as first male to mate or male mating at the right ovulation time fathering most of the offspring (Uller & Olsson, 2008). Histological analyses of the reproductive tract in *E. spinax* females revealed the presence of sperm storage tubules in the oviducal gland giving evidence of potential post-copulatory events (Porcu *et al*., 2014).

Multiple paternity is also assumed to occur more frequently in philopatric organisms with low dispersal rates to avoid breeding

between relatives and thus inbreeding depression (Chapman *et al*., 2004; Jennions & Petrie, 2000; Sugg & Chesser, 1994). Although such a philopatric strategy was not documented for lanternsharks, schooling behaviour is suggested to occur in these species (Claes & Mallefet, 2009c, 2010; Reif, 1985; Springer, 1967). This aggregation behaviour might potentially increase (a) mate encounter probability and (b) female mate choice.

Finally, convenience polyandry (*i.e*., females mating with several males to avoid coercive breeding harassment from these males) may be the driving force behind observations of genetic polyandry (Griffiths *et al*., 2012; Nosal *et al*., 2013; Zeh & Zeh, 2001). Mating resistance is costly for female sharks, and situationally may decrease individual fitness by suboptimal mating rather than incur male harassment. If so, the contact frequency between individuals from different sexes may drive observed frequencies of genetic polyandry in sharks. For species with predictable mating aggregations, the frequency of genetic polyandry may be high due to the number of aggressive males that a female will encounter. By contrast, if females encounter fewer males, they may be less likely to engage in superfluous copulations. Here, the low inferred rate of genetic polyandry in *E. spinax* could result from low contact frequencies due to an observed segregation by sex, size and maturity stage (*i.e*., larger females living in a deeper part of the water column than males), an aggregation behaviour occurring between conspecifics of the same sex and a sex ratio in favour of females (Aranha *et al*., 2009; Coelho & Erzini, 2010; Porcu *et al*., 2014).

Further studies on the reproductive strategies of lanternsharks are necessary to determine whether luminous dimorphic attraction, sperm storage and retention, aggregative strategies or depth segregation pattern are leading motivations underlying the observed genetic polyandry.

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CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

L.D. and J.M. collected samples. L.D., N.O. and H.M. performed, analysed and interpreted the results and were major contributors in writing the original manuscript. JM and HM supervised the work and revised the manuscript. All authors read and approved the final manuscript.

ETHICAL STATEMENT

All applicable international, national and/or institutional guidelines for the care and use of animals were followed by the authors. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. *E. spinax* individuals were collected in Norway under the Experimental fish care permit number 12/14048. *E. molleri* individuals were fished and handled according to Churaumi Aquarium (Okinawa, Japan) husbandry and veterinary rules for fish experimentations. Animal procedures were conducted in compliance with the Belgian national guidelines and in agreement with the European directive 2010/63/UE, under the approval of the Animal Ethics Committee of the Catholic University of Louvain in Louvain-la-Neuve. This article does not contain any studies with human participants performed by any of the authors.

DATA ACCESSIBILITY

Microsatellite genotypes are deposited on Zenodo https://doi.org/10.5281/zenodo.3750726.

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