Polyandry is extremely rare in the firefly squid, *Watasenia scintillans*

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Abstract

Although polygamy has versatile benefits for both sexes, many species favor monogamy for reasons with the clarity or unclarity. In cephalopods, all species are regarded to be polygamous, which could be attributed to their common life-history traits. Contrary to this prediction, we show empirical evidence for monogamy in the firefly squid, *Watasenia scintillans*. The peak spawning season comes after male disappearance owing to long-reserved spermatangia deposited by male at exact locations (bilateral pouches under neck collar) on female with a symmetric distribution. Such a non-random placement of spermatangia prompted us to hypothesize that females engage in lifetime monoandry. Hence we assigned genotypes of female-stored spermatangia and offspring. We found that in 94.7 % females, the spermatangia were delivered from a single male and all embryos in the same egg string sired by sperm from stored spermatangia. Throughout the season, relative testes mass was much smaller in *W. scintillans* than all other cephalopods previously examined. The mean number of male-stored spermatophores was approximately 30, the equivalent to 2.5 mates. Our demographic and morphometrical data agree with the prediction that monogyny is favored when potential mates are scarce such as absence of female remating. Together, these results suggest the likelihood of mutual monogamy.

Keywords: monogamy, monoandry, cephalopods, squids, deep-sea, spermatangium

1. Introduction

In recent decades, research on behavioral and molecular ecology identified polyandry (a female mate with multiple males), a prerequisite for postcopulatory sexual selection, as a prevalent mating pattern across animal taxa [1-5]. In this pattern, females are able to receive direct or indirect benefits to a greater extent by mating with multiple males than with a male, resulting in offspring with ‘good genes’ or higher genetic diversity [6, 7]. Conversely, costs of polyandry (or polygamy) are borne on both sexes such as increased risks of predation, disease/virus infection, harassment during courtship or copulation, and therefore often shortening their lifespan [8-10]. Yet, cost-benefit trade-offs in choosing a mating pattern cannot be easily anticipated due to the underlying social and population complexities combined with various life-history and reproductive strategies. Furthermore, even though polygamy bears high costs on each sex, it would not necessarily be transitioned to the contrasting mating pattern, monogamy (both male and female mate with one partner). Because monogamy, as a whole, should meet the criteria of either ‘mutual benefits’ for both sexes [7, 11, 12] or ‘unilateral benefit’ to one sex over the other (e.g., postcopulatory mate guarding). The latter could also generate sexual conflict or energetic costs on the guarders [13, 14]. Hence, monogamy is favored only in environmental conditions where the opportunity or benefit to monopolize mates does not exist [15]. For examples, birds and mammals have entailed biparental care for young and long-term (prolonged) pairing, setting a precondition for evolution of monogamy [14, 16, 17]. Species that are under constraints of feeding or breeding habitat often choose the monogamous relationship to protect these resources [18, 19]. Female disperse could be a reason for monogamy because searching for extra-pair mates is costly and risky for males [20]. However, monogamy could have arisen in species without these cases, therefore still posing an evolutionary puzzle [21].

Most cephalopod species have a short lifespan (approximately one year), reproduce semelparously and display a diverse array of mating behaviors in favor of adaptive or alternative consequences of intrasexual reproductive competition [22-25]. Behavioral and anatomical observations of some coastal species have uncovered the coherent reproductive tactics and traits thereof in the context of promiscuous mating [22, 26-29]. Notably, all cephalopod species reported are regarded to be polyandrous [30-33], except that the diamond squid, *Thysanoteuthis rhombus* appears to form the long-term pair-bond during migration and are therefore possibly monogamous [34]. Although this phenomenon needs confirmation with genetic analysis, many other species also require further investigations to determine whether the influence of observed female promiscuity is limited to their mating behavior, or even reached to their offspring paternity.

Since it is now widely recognized that apparent mating behavior, regardless of whether it is promiscuous or monogamous, does not always coincident with, or proportional to, genetic parentage [3, 35].

In cephalopods, the main factors that could possibly influence to this inconsistency are cryptic female choice (CFC), last-male sperm precedence (LMSP) and extra-pair copulation as a consequence of alternative reproductive tactic (ART) [24, 25, 33]. CFC is the postcopulatory mechanism by which females bias storage or usage of spermatozoa [36]. In the Japanese pygmy squid *Idiosepius paradoxus*, females strip off the spermatangia handed on from unfavorable males [37]. In addition, as females have smaller sperm-storing capacity (within the seminal receptacle) relative to sperm number in the spermatangia and sperm use from the seminal receptacle is under the female control, it is possible that CFC could also operate during these processes [38]. LMSP would be in practice if most recent mate takes a disproportionate share in the paternity [39]. In the cuttlefish, *Sepia lycidas* [40] and perhaps the dumpling squid, *Euprymna tasmanica* [41], males replace previously stored rival ejaculates for their own. Such the sperm displacement as well as widely observed mate guarding behavior must have evolved through LMSP [39]. ART refers to behavioral traits selected to maximize fitness in two or more alternative ways in the context of intraspecific and intrasexual reproductive contest [42]. Several loliginidae species show ART with distinct male morphs in mating posture and ejaculate traits [24, 43, 44]. Males with larger body mass ('consorts') deposit the spermatophores near the oviduct thereby sperm gain higher accessibility to eggs, whereas smaller males ('sneakers') take an alternate route to fertilize eggs by depositing sperm at either distal or proximal time from egg spawning [28, 45, 46].

Regardless of the presence of these male- or female-driven strategies that can facilitate post-mating paternity skew, it is uncommon, but not impossible, that one male can monopolize exclusive paternity of a brood if polyandry underlies the mating practices in a given species. In general, most cephalopods may have developed life-histories and reproductive modes suitable for pursuing polyandry; they are short lived, semelparous and capable of long-term storing multiple sperm packages in the female [33]. So far there has been no report, to our knowledge, for paternal care or provisioning by males after mating. Postcopulatory mate guarding is a possible cause of mutual monogamy, if it can deprive from both sexes the opportunities for seeking additional mates. Mate guarding by males is often seen in squids [44, 46, 47], however it is either temporal or incomplete, and sometimes interrupted by extra-pair copulations. Some organisms living under photic zone may suffer from low mate availability, another possible cause

of monogamy, due to low population density [48], however mating pattern of any deep-sea species yet to be determined. Although there are many untested scenarios or conditions where monogamy is preferably selected, in the current consensus view, monogamy is seemingly unlikely to be a prevalent strategy in cephalopods [31, 32].

To date, however, most knowledge of reproductive ecology in cephalopods has gained from studies with limited representatives, mainly those with coastal habitats, because behavioral observations are possible in field or aquarium. Besides these approaches, the DNA finger printing has been a promising technique to track down the outcomes of mating events. To gain global insights into cephalopod reproductive systems, in this study, we chose the firefly squid, *Watasenia scintillans* because their ecological characteristics are rather clear [49], yet their reproductive mode remains largely equivocal. Furthermore, they are commercially important resources, which allows the fishery to catch massively on a daily basis during the reproductive season [50]. Here, we show genetic, morphometrical and demographic evidence for a substantial trend in monogamous mating in *W. scintillans*, which provides first reported case in cephalopods.

2. Methods

(a) Animal and embryo collection

The squid, *W. scintillans* was obtained from fishery catches by bottom trawls towed around the Oki island (Shimane Prefecture) and Sakai-port off (Tottori Prefecture), or by fixed net set around and near the shelf break in the innermost part of the Toyama bay (Toyama Prefecture), Japan. The commercial fishing period of this species is approximately from January to May in Shimane/Tottori, and from March to May in Toyama. All the morphometric measurements were undertaken on specimens collected in Shimane/Tottori during 2016-2019, whereas for microsatellite analysis of the spermatangia and the parentage analysis, live animals caught at Toyama bay were used. At the Uozu Aquarium, spawning was induced at 14°C in dark. Spawning occurred spontaneously in the aquarium tank when the field-caught females were transported immediately after the catch. Each egg-string retrieved in isolation was then cultured for 4-5 days at 17°C until hatching and thereafter, paralarvae being picked at random (15~45 specimens/egg-string, n=4) were genotyped.

(b) Measurements of growth and reproductive indices

The squid specimens were measured (mostly within one day after fishing) for dorsal mantle length (ML), total wet weight (body weight; BW), testis weight (TW), spermatophoric complex weight (SCW), number of spermatophore stored in the Needham's sac and the terminal organ, ovary weight (OW) and number of spermatangium on the left and right side of the female seminal receptacle. The number of spermatangium was counted by viewing under a stereomicroscope. Testicularsomatic index (TSI) and ovariansomatic index (OSI) were calculated as $TSI = 100 \times TW \times BW^{-1}$ and $OSI = 100 \times OW \times BW^{-1}$, respectively. To score the number of spermatophore, spermatophoric complex was fixed in 10% formalin in seawater, thereafter dissected under a stereoscope. All males of *W. scintillans* were mature (stage V or VI according to classification by [51]). The data for other squid species were extracted from the literature (Table S1). Average spermatophore weight (ASW) was estimated from the regression lines of Figs. 2B and 2C and in each individual, gonadal investment (resource allocation) to spermatophore was calculated as $100 \times (SpN \times ASW) \times (TW + SCW)^{-1}$, where SpN indicates number of spermatophore stored in the spermatophoric sac. Resource allocation to testis was calculated as $100 \times TW \times (TW + SCW)^{-1}$. TSI and other morphometric data of *I. paradoxus* [37], *H. bleekeri* [52] and *Doryteuthis plei* [53] were provided from the corresponding authors of previous reports.

(c) Development of microsatellite markers

Microsatellite makers (GenBank Acc. LC514114 - LC514117) are developed from partial genome sequences of *W. scintillans* obtained by next-generation sequencing shared by National Center for Child Health and Development in Tokyo. The MISA pipeline [54] was used to identify reads containing microsatellite repeats and primer sets to amplify the repeats. We searched for trimers, tetramers, pentamers and hexamers with sufficient flanking sequences to develop PCR primers. A total of 1632 putative SSRs with repeat size > 60bp and flanking regions at the both ends were detected and for SSR screening, thereafter we chose 20 primer pairs flanking tetra- and trinucleotide SSR motif with a minimum of 20 repetitions and the expected product size between 140 and 280 bp. Primer3 [55] was implemented to predict primers pairs targeting the flanking regions. PCR amplification was carried out with genomic DNAs isolated from five different individuals and validated by agarose gel electrophoresis. The primer pairs that gave no band or no obvious polymorphism in band size were eliminated. The remains were further analyzed by acrylamide gel electrophoresis with a dozen of DNA samples obtained from different individuals. Consequently, we selected four SSR loci that were fully characterized by fragment

length analysis (see below). Nucleotide sequences of microsatellite makers and their characteristics are shown in Table S2.

(d) Paternity analysis

A mass of spermatangia was removed from the female seminal receptacle, soaked in 70% ethanol for 10 min and disassembled into single spermatandium with fine forceps. Each spermatangium was placed in the 1.5ml test tube, and digested with 50 µl of lysis buffer (10 mM Tris-Cl (pH 8), 500mM NaCl, 50mM EDTA, 2% dithiothreitol, 1 mg/ml Proteinase K (Sigma-Aldrich)) at 52°C for overnight with constant agitation (120 rpm). After centrifugation (14,000 rpm, 10min, 4°C), the supernatant was transferred into new test tube followed by phenol/chloroform extraction. Extracted DNA was precipitated by adding 1/10 volume of 3 M sodium acetate (pH 5.2) and x2.5 volume of 99.5% ethanol, and centrifuged (14,000 rpm, 10 min, 4°C). After removing the supernatant, the precipitant was washed with 70% ethanol, air-dried, dissolved in 50 µl of water and stored at -20°C. Isolation of genomic DNAs from mantle tissue, hatched paralavæ were carried out as well. A parentage DNA analysis was carried out by genotyping the mother, her storing spermatangia, and spawned eggs, using the software program COLONY v.2.0.6.5 [56].

All genomic DNA samples were run on 1% agarose gel electrophoresis for evaluating quality and quantity. It should be noted that in rare but certain occasion, no DNA was recovered from the spermatangium, which could be explained by the absence of spermatozoa in the spermatangium. This lack of complete recovery of genomic DNA was reflected on the result that the percent of success in PCR amplification with microsatellite markers was 99.0% (1172/1184) and overall success of paternity identification was 98.9% (270/273).

Amplification of microsatellite loci was performed with polymerase chain reaction with fluorescently tagged forward primer and non-labeled reverse primer, and approximately 20 ng of genome DNA in 10 µl of reaction mixture (TaKaRa Ex Taq system) with heat denaturing (95°C, 1min) followed by 30 cycles of denaturing (95°C, 30 sec), annealing (56°C, 30 sec) and extension (72°C, 20 sec) and terminated by 15 min incubation at 72°C. Each PCR product was run on 8% mini-slab polyacrylamide gel electrophoresis (Bio-Rad, 130 V constant, 60 min), and gels stained with 0.1 µg/ml ethidium bromide and image processed by ImageQuant LAS 500 (GE Healthcare). After validation of quality, four PCR products (labeled with FAM, Hex, Cy3 and PET) amplified with the same genome DNA were mixed and subjected to fragment length analysis (3130xl Genetic Analyzer, 500LIZ, FASMAC Co. Japan). Then, by using Cervus 3.0

201 ([57], the combined non-exclusion probabilities for all microsatellite loci were calculated to be <
202 0.0001, which is sufficiently low value for correctly identifying the real sire [58].

204 3. Results

205 (a) Seasonal dynamics of population and reproduction in *W. scintillans*.

206 Females of the firefly squid, *W. scintillans* come to the shallow water to spawn in the spring.
207 While males disappear much earlier from the coastal zones than do females [50], which is
208 accounted for sex difference in the lifespan being approximately one month shorter in males
209 than females [49, 59]. The coincidental disappearance of males and maiden females, occurring
210 from mid-February to mid-March (hereafter designated as the estimated mating period, EMP),
211 supports this scenario as being plausible (Fig. 1A). Hence, females must internally preserve
212 spermatozoa for a considerable time until spawning. Sperm storage occurs in the male-derived
213 spermatangia, which are affixed to the female seminal receptacle located under the collar on the
214 bilateral sides of the nuchal cartilage (Figs. 1B–1E). Noteworthy, an equal number of
215 spermatangia, approximately six (left side, 5.89 ± 1.58 ; right side, 5.96 ± 1.65 ; paired t test, $t =$
216 -1.02 , $df = 582$, $p = 0.31$), was stored on each side of the seminal receptacle (Fig. 1F). This
217 pattern remained unaltered with a constant and gradual decrease in the number of spermatangia
218 throughout the reproductive season (days required to lose one spermatangium from either the
219 left- or right side was 175.4 or 192.3, respectively; Fig. 1G), suggesting a lifelong preservation
220 of the spermatangia once attached to the female seminal receptacle.

222 (b) Evidence for behavioural and genetic monogamy

223 This well-organized pattern of sperm storage with a left–right symmetry and an approximately
224 fixed number led us to test the hypothesis that females receive spermatozoa by a single
225 copulation attempt from the male (i.e., behavioral monogamy). By genotyping with validated
226 microsatellite markers (Table S2), we found that in 94.7% (18/19) of females, all the
227 spermatangia stored were delivered by a single male. In one case, where the number of
228 spermatangia stored was extraordinarily large (left, 13; right, 12), three males were involved in
229 copulation (Fig. 2).

230 To ascertain whether females use spermatozoa from the storing spermatangia to fertilize
231 their eggs (i.e., genetic monogamy), a parentage DNA analysis was carried out with SSR loci of
232 the mother, her storing spermatangia, and her brood. The COLONY analysis [56] indicated that
233 all paralarvae analyzed were assigned to the single paternity derived from the storing

spermatangia (Datasheet S1, combined non-exclusion probability < 0.001 ; paternity rate by a candidate male = 100%, $n = 4$). These results also rule out the possibility that females commit cryptic sperm storage following extra-pair copulations.

(c) Estimation of male mating opportunities

We next performed morphometric and quantitative measurements of maturity and fecundity of *W. scintillans* individuals before, during, and after EMP. We found that males continue to accumulate spermatophores in their storage organ (Needham's sac) throughout the season except during EMP (Fig. 3A), when the spermatophores are being used. Such a continuous increase in stored spermatophore can be explained by a higher manufacturing rate than their consumption rate, suggesting that males would have lost their copulation opportunities after EMP. The mean number of male-stored spermatophores just before EMP (pre-EMP) was ~ 30 , which estimates that males can copulate no greater than 2~3 times (because the mean number of female-received spermatophores is 12). At pre-EMP, males become fully mature with the highest testicular somatic index (TSI), an indicator of sperm-producing capacity or promiscuity (Figs. 3B–D), whereas females have just begun maturing and require several more weeks to become more fecund (Fig. 2E). However, the TSI was, to our current knowledge, extremely lower in *W. scintillans* (0.15 ± 0.09 , $n = 408$) than in other investigated cephalopods (Fig. 3F). From another point of view, *W. scintillans* males invest most gonadal expenditure to production of the spermatangia rather than sperm, giving a stark contrast to the case of *I. paradoxus* (Figs. 3G–L) that copulates multiple times throughout the reproductive season [37]. Collectively, we assume that males of *W. scintillans* are incapable of attempting multiple mates, hence resulting in monogynous mating pattern, because of low sperm production capacity and limited mating opportunities due to male-biased operational sex ratio and absence of female remating.

4. Discussion

One of the hallmarks of reproduction in most coleoid cephalopods is their copulation behavior by which males deposit sperm packages (spermatophores) on female using the heterocotylated arm(s). Because females are primarily promiscuous, the spermatangia are delivered simultaneously or sequentially from multiple males, resulting in random distribution of mixed spermatangia around the deposition site on the female. However, we contingently found that females of *W. scintillans* store masses of the spermatangia that are evenly distributed at exact locations on bilateral sides of the nuchal cartilage with approximately six on each side. Such the

extraordinary regular pattern of spermatangium placement was unusual in cephalopods, thus prompted us to test whether all these spermatangia were derived from a single male.

Our current genetic data (paternity of each spermatangium stored on a female) clearly demonstrated that most females of squid *W. scintillans* mate with only one male (Fig. 2), therefore suggesting behavioral monoandry (females mate with only one partner). Furthermore, parentage analysis identified no DNA mismatch between the spermatangia and embryos from the same females, confirming genetic monogamy (Fig. S1). During reproductive season, females may spawn eggs several times at certain intervals [49], however other males would not be involved in replenishing the spermatangia during the intervals because 1) males disappear before females being fully fecund (Figs. 1A, 3E), and 2) spontaneous loss of once-attached spermatangium to female seldom occurs (Fig. 1G). Accordingly, male mating opportunities would be limited due to infrequent female remating and male-biased sex ratio. Both tertiary (adult) sex ratio and operational sex ratio [60] were largely male-biased in the beginning of and throughout EMP, respectively (Fig. 1A). Under these conditions, mathematical modeling predicts that monogyny (males mate with only one partner) can become fixed as evolutionary stable strategy (ESS)[60, 61]. So, we speculate that female monoandry had first established followed by male monogyny, which together brought into mutual monogamy [60]. If male monogyny becomes ESS, then evolution could favor males who invest more energy on something other than fecundity (sperm production) [62, 63], which explains the observed least investments on testis (sperm production) and spermatangium (copulation opportunity) in this species. Instead of allocating their reproductive investment toward greater fecundity, males would have chosen a “live-fast-die-young” life-history strategy to adapt to a mechanism of first-male sperm precedence [64] (Fig. 1A). We speculate that the low level of male fecundity had occurred as the consequence of female-driven monoandry [62, 63].

If females and males are predominantly pursuing a monogamous mating strategy, what could be the benefit they reap in exchange for eroding the genetic diversity of the offspring? And in doing so, how do they physiologically evade remating attempts despite physically being together? Therefore, the first question arises as to what was a feasible cause or causes that drove their mating system to be primarily, if not exclusively, monoandrous. It is unambiguous that some factors known to involve monogamy in other taxa do not work for this species such as long-term pair-bond, biparental care, low population density, habitat limitation, time constraint for reproduction and enforcement (mate guarding) [16, 18, 21, 65], simply because they make condensed spawning migrations and males disappear entirely before peaking at egg laying.

Alternatively, considering that this species suffers from high predation pressures and serves as a dominant prey for demersal fishes [66], a time-consuming courtship behavior could potentially be deleterious to their survival; hence, multiple mating would reduce reproductive success for both sexes [8, 67, 68]. There are ample examples to support the prediction that a predation risk serves as an evolutionary force to monoandry [8]. For example, in the cicada *Subpsaltria yangi*, when males emit an advertising call, only virgin females make a response call, thereafter males fly to them to mate (hence monoandry). These flights by males are at high risk – only 25% of the second flights were successful and the remaining failures were mostly attacked by the robber fly, *Philonicus albiceps* [69].

It is a widespread phenomenon across taxa that non-virgin females lose sexual attractiveness or responsiveness to male-specific courtship signaling, and the underlying mechanisms may vary with physiological, physical and behavioral changes upon mating [70-72]. Nevertheless, their fitness consequences would be mostly against the risk of predation. Although the actual mating behaviors of deep-sea organisms are largely concealed, mating of any species should be well adopted in the dark environment [48]. The communication-like bioluminescence signaling executed by the Octopodiformes squid, *Taningia danae* was regarded as a potential courtship behavior [73]. *W. scintillans* has special eyes (photoreceptor cells) containing three visual pigments ($\lambda_{\max}$ ~471, ~484 and ~500 nm), possibly allowing them to distinguish conspecific illumination (green) from environmental down-welling light (blue) [74]. It is therefore feasible that bioluminescence of the firefly squid could play a role for courtship signaling or mate search in a once-in-a-lifetime fashion. In this study, the fine-scale analysis of seasonal dynamics in demographics, mating status and reproductive indices of each individual from fishery catches sculptured the reproductive landscape of this species. In summary, we provide, for the first time in cephalopods, genetic evidence for virtually monoandrous mating pattern and its adaptive significance awaits deciphering in the light of ecological niche of this species.

Data accessibility

All datasets for information of the SSR markers and the paternity analysis are available in the electronic supplementary material files and newly developed SSR markers have been deposited at the DDBJ/GenBank with accession no. LC514114 - LC514117.

Authors' contributions

NH conceived this study. NS, SIT, NEA, SK, TS and NH performed experiments. NS, YI, OI,

MAY and NH analyzed data. NH wrote the paper.

Competing interests

The authors declare no competing financial interests.

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Figure legends

Fig. 1 The mode of and change in sperm storage throughout the reproductive season in *W. scintillans*. (A) Population dynamics of males and mated females from January to May. A cyan box indicates the estimated mating period. (B) A dorsal view of the female around the neck. Arrowheads point to the spermatangia visible through the transparent mantle. (C) A single mass of spermatangium with unidirectionally oriented ejaculatory ducts (arrowheads). (D) Spermatozoa stored in the spermatangia. (E) An anatomical illustration of the female seminal receptacle along the body axis (A, anterior; P, posterior). (F) The frequency of female individuals having different numbers of spermatangia in bilateral storage sites. (G) A seasonal change in spermatangia number in females.

Fig. 2 Paternity analysis of each spermatangium stored on the females. Using a total of nineteen females, every single spermatangium was isolated and genotyped. Each color represents

paternity share by the first (*blue*), second (*green*) and third (*red*) male. *Cyan* indicates unidentified paternity.

Fig. 3 Allocation of male reproductive resources in *W. scintillans* and other squids. (A-E) In *W. scintillans*, seasonal changes in the number of male-storing spermatophore (A), mantle length (B), body weight (C), TSI (D) and OSI (E) are plotted as the mean $\pm$ SE. Data points represent males in *blue* and females in *red*. *Cyan* boxes indicate the estimated mating period. (F) A graph showing a comparison of male testicularsomatic indices (TSI) among previously reported cephalopod species and *W. scintillans* found in this study (*). Data were extracted from the literature indicated by reference # (Table S1). The columns indicate the mean (*blue column*) or minimum (*gray column*) values specified in the literature, otherwise mean values were estimated (*gradient column*) from presented data points in the graphs. (G-H) The GSI values from male individuals are plotted against number of spermatophore (G, H) or testis weight (I, J) in *W. scintillans* (G, I) and highly promiscuous *Idiosepius paradoxus* (H, J). (K, L) In each individual, allocation of male reproductive resources to testis (K) or spermatophore (L) is expressed in percentage.

References

1. Brockmann H.J., Colson T., Potts W. 1994 Sperm competition in horseshoe crabs. *Behav. Ecol. Sociobiol.* **35**, 153–160.
2. Jones A.G., Avise J.C. 1997 Microsatellite analysis of maternity and the mating system in the Gulf pipefish *Syngnathus scovelli*, a species with male pregnancy and sex-role reversal. *Mol. Ecol.* **6**, 203–213. (doi:10.1046/j.1365-294x.1997.00173.x).
3. Griffith S.C., Owens I.P., Thuman K.A. 2002 Extra pair paternity in birds: a review of interspecific variation and adaptive function. *Mol. Ecol.* **11**, 2195–2212. (doi:10.1046/j.1365-294x.2002.01613.x).
4. Birkhead T.R., Pizzari T. 2002 Postcopulatory sexual selection. *Nat. Rev. Genet.* **3**, 262–273. (doi:10.1038/nrg774).
5. Parker G.A., Birkhead T.R. 2013 Polyandry: the history of a revolution. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **368**, 20120335. (doi:10.1098/rstb.2012.0335).
6. Fisher D.O., Double M.C., Blomberg S.P., Jennions M.D., Cockburn A. 2006 Post-mating

- sexual selection increases lifetime fitness of polyandrous females in the wild. *Nature* **444**, 89-92. (doi:10.1038/nature05206).
7. Boulton R.A., Shuker D.M. 2015 The costs and benefits of multiple mating in a mostly monandrous wasp. *Evolution* **69**, 939-949. (doi:10.1111/evo.12636).
8. Magnhagen C. 1991 Predation risk as a cost of reproduction. *Trends. Ecol. Evol.* **6**, 183-186. (doi:10.1016/0169-5347(91)90210-O).
9. Shuker D.M., Day T.H. 2001 The repeatability of a sexual conflict over mating. *Anim. Behav.* **61**, 755-762.
10. Roberts K.E., Evison S.E., Baer B., Hughes W.O. 2015 The cost of promiscuity: sexual transmission of *Nosema* microsporidian parasites in polyandrous honey bees. *Sci. Rep.* **5**, 10982. (doi:10.1038/srep10982).
11. Laubu C., Dechaume-Moncharmont F.X., Motreuil S., Schweitzer C. 2016 Mismatched partners that achieve postpairing behavioral similarity improve their reproductive success. *Sci. Adv.* **2**, e1501013. (doi:10.1126/sciadv.1501013).
12. Snekser J.L., Itzkowitz M. 2019 Serial monogamy benefits both sexes in the biparental convict cichlid. *Peer J.* **7**, e6535. (doi:10.7717/peerj.6535).
13. Komdeur J. 2001 Mate guarding in the Seychelles warbler is energetically costly and adjusted to paternity risk. *Proc. Biol. Sci.* **268**, 2103-2111. (doi:10.1098/rspb.2001.1750).
14. Hosken D.J., Stockley P., Tregenza T., Wedell N. 2009 Monogamy and the battle of the sexes. *Annu. Rev. Entomol.* **54**, 361-378. (doi:10.1146/annurev.ento.54.110807.090608).
15. Emlen S.T., Oring L.W. 1977 Ecology, sexual selection, and the evolution of mating systems. *Science* **197**, 215-223. (doi:10.1126/science.327542).
16. Wittenberger J.F., Tilson R.L. 1980 The evolution of monogamy: hypotheses and evidence. *Annu. Rev. Ecol. Syst.* **11**, 197-232.
17. Young R.L., Ferkin M.H., Ockendon-Powell N.F., Orr V.N., Phelps S.M., Pogany A., Richards-Zawacki C.L., Summers K., Szekely T., Trainor B.C., et al. 2019 Conserved transcriptomic profiles underpin monogamy across vertebrates. *Proc. Natl. Acad. Sci. U S A* **116**, 1331-1336. (doi:10.1073/pnas.1813775116).
18. Whiteman E.A., Cote I.M. 2004 Monogamy in marine fishes. *Biol. Rev. Camb. Philos. Soc.* **79**, 351-375. (doi:10.1017/s1464793103006304).
19. Kaiser S.A., Risk B.B., Sillett T.S., Webster M.S. 2017 Ecological and Social Factors Constrain Spatial and Temporal Opportunities for Mating in a Migratory Songbird. *Am. Nat.* **189**, 283-296. (doi:10.1086/690203).

20. Komers P.E., Brotherton P.N. 1997 Female space use is the best predictor of monogamy in mammals. *Proc. Biol. Sci.* **264**, 1261-1270. (doi:10.1098/rspb.1997.0174).
21. Kvarnemo C. 2018 Why do some animals mate with one partner rather than many? A review of causes and consequences of monogamy. *Biol. Rev. Camb. Philos. Soc.* **93**, 1795-1812. (doi:10.1111/brv.12421).
22. Iwata Y., Shaw P., Fujiwara E., Shiba K., Kakiuchi Y., Hirohashi N. 2011 Why small males have big sperm: dimorphic squid sperm linked to alternative mating behaviours. *BMC Evol. Biol.* **11**, 236. (doi:10.1186/1471-2148-11-236).
23. Squires Z.E., Wong B.B., Norman M.D., Stuart-Fox D. 2012 Multiple fitness benefits of polyandry in a cephalopod. *PLoS One* **7**, e37074. (doi:10.1371/journal.pone.0037074).
24. Marian J., Apostolico L.H., Chiao C.C., Hanlon R.T., Hirohashi N., Iwata Y., Mather J., Sato N., Shaw P.W. 2019 Male Alternative Reproductive Tactics and Associated Evolution of Anatomical Characteristics in Loliginid Squid. *Front. Physiol.* **10**, 1281. (doi:10.3389/fphys.2019.01281).
25. Morse P., Huffard C.L. 2019 Tactical Tentacles: New Insights on the Processes of Sexual Selection Among the Cephalopoda. *Front. Physiol.* **10**, 1035. (doi:10.3389/fphys.2019.01035).
26. Hanlon R.T., Smale M.J., Sauer W.H.H. 2002 The mating system of the squid *Loligo vulgaris reynaudii* (Cephalopoda, Mollusca) off South Africa: fighting, guarding, sneaking, mating and egg laying behavior. *Bull. Mar. Sci.* **71**, 331–345.
27. Hirohashi N., Alvarez L., Shiba K., Fujiwara E., Iwata Y., Mohri T., Inaba K., Chiba K., Ochi H., Supuran C.T., et al. 2013 Sperm from sneaker male squids exhibit chemotactic swarming to CO₂. *Curr. Biol.* **23**, 775-781. (doi:10.1016/j.cub.2013.03.040).
28. Hirohashi N., Tamura-Nakano M., Nakaya F., Iida T., Iwata Y. 2016 Sneaker Male Squid Produce Long-lived Spermatozoa by Modulating Their Energy Metabolism. *J. Biol. Chem.* **291**, 19324-19334. (doi:10.1074/jbc.M116.737494).
29. Apostolico L.H., Marian J. 2017 Dimorphic ejaculates and sperm release strategies associated with alternative mating behaviors in the squid. *J. Morphol.* **278**, 1490-1505. (doi:10.1002/jmor.20726).
30. Hanlon R.T., Ament S.A., Gabr H. 1999 Behavioural aspects of sperm competition in cuttlefish, *Sepia officinalis* (Sepioidea: Cephalopoda). *Mar. Biol.* **134**, 719–728.
31. Hoving H.J., Bush S.L., Robison B.H. 2012 A shot in the dark: same-sex sexual behaviour in a deep-sea squid. *Biol. Lett.* **8**, 287-290. (doi:10.1098/rsbl.2011.0680).

32. Franklin A.M., Squires Z.E., Stuart-Fox D. 2012 The energetic cost of mating in a promiscuous cephalopod. *Biol. Lett.* **8**, 754-756. (doi:10.1098/rsbl.2012.0556).
33. Hanlon R.T., Messenger J.B. 2018 Cephalopod behaviour 2nd ed. Cambridge University Press, Cambridge.
34. Nigmatullin C.M., Arkhipkin A.I., Ssabirov M.S. 1995 Age, growth and reproductive biology of diamond-shaped squid *Thysanoteuthis rhombus* (Oegopsida: Thysanoteuthidae). *Mar. Ecol. Prog. Ser.* **124**, 73-87.
35. Lenz T.L., Hafer N., Samonte I.E., Yeates S.E., Milinski M. 2018 Cryptic haplotype-specific gamete selection yields offspring with optimal MHC immune genes. *Evolution* **72**, 2478-2490. (doi:10.1111/evo.13591).
36. Firman R.C., Gasparini C., Manier M.K., Pizzari T. 2017 Postmating Female Control: 20 Years of Cryptic Female Choice. *Trends. Ecol. Evol.* **32**, 368-382. (doi:10.1016/j.tree.2017.02.010).
37. Sato N., Yoshida M.A., Kasugai T. 2017 Impact of cryptic female choice on insemination success: Larger sized and longer copulating male squid ejaculate more, but females influence insemination success by removing spermatangia. *Evolution* **71**, 111-120. (doi:10.1111/evo.13108).
38. Iwata Y., Sato N., Hirohashi N., Kasugai T., Watanabe Y., Fujiwara E. 2019 How female squid inseminate their eggs with stored sperm. *Curr. Biol.* **29**, R48-R49. (doi:10.1016/j.cub.2018.12.010).
39. Hooper R.E., Siva-Jothy M.T. 1996 Last male sperm precedence in a damselfly demonstrated by RAPD profiling. *Mol. Ecol.* **5**, 449-452. (doi:10.1111/j.1365-294x.1996.tb00335.x).
40. Wada T., Takegaki T., Mori T., Natsukari Y. 2010 Sperm removal, ejaculation and their behavioural interaction in male cuttlefish in response to female mating history. *Anim. Behav.* **79**, 613-619.
41. Squires Z.E., Wong B.B.M., Norman M.D., Stuart-Fox D. 2015 Last male sperm precedence in a polygamous squid. *Biol. J. Linn. Soc.* **116**, 277-287.
42. Neff B.D., Svensson E.I. 2013 Polyandry and alternative mating tactics. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **368**, 20120045. (doi:10.1098/rstb.2012.0045).
43. Hanlon R.T. 1998 Mating systems and sexual selection in the squid *Loligo*: How might commercial fishing on spawning squids affect them? *Calif. Coop. Oceanic Fish. Invest. Rep.* **39**, 92-100.

44. Wada T., Takegaki T., Natsukari Y. 2005 Alternative male mating behaviors dependent on relative body size in captive oval squid *Sepioteuthis lessoniana* (Cephalopoda, Loliginidae). *Zool. Sci.* **22**, 645-651.
45. Naud M.J., Shaw P.W., Hanlon R.T., Havenhand J.N. 2005 Evidence for biased use of sperm sources in wild female giant cuttlefish (*Sepia apama*). *Proc. Biol. Sci.* **272**, 1047-1051. (doi:10.1098/rspb.2004.3031).
46. Naud M.J., Sauer W.H., McKeown N.J., Shaw P.W. 2016 Multiple Mating, Paternity and Complex Fertilisation Patterns in the Chokka Squid *Loligo reynaudii*. *PLoS One* **11**, e0146995. (doi:10.1371/journal.pone.0146995).
47. Mather J. 2016 Mating games squid play: reproductive behaviour and sexual skin displays in Caribbean reef squid *Sepioteuthis sepioidea*. *Mar. freshw. behav. physiol.* **49**, 359-373.
48. Herring P.J. 2000 Species abundance, sexual encounter and bioluminescent signalling in the deep sea. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **355**, 1273-1276. (doi:10.1098/rstb.2000.0682).
49. Hayashi S. 1995 Fishery biological studies of firefly squid, *Watasenia scintillans*, (Berry) in Toyama Bay. *Bull. Toyama Pref. Fish. Res. Inst.* **7**, 1-128.
50. Arkhipkin A.I.e.a. 2015 World Squid Fisheries. *Rev. Fish. Sci. Aqua.* **23**, 92-252.
51. R. L.M., G. U.L. 1995 Sexual maturation in squid: quantum or continuum? *S. Afr. J. mar. Sci.* **15**, 207-223.
52. Iwata Y., Munehara H., Sakurai Y. 2005 Dependence of paternity rates on alternative reproductive behaviors in the squid *Loligo bleekeri*. *Mar. Ecol. Prog. Ser.* **298**, 219-228.
53. Apostólico L.H., Marian J.E.A.R. 2018 Dimorphic male squid show differential gonadal and ejaculate expenditure. *Hydrobiologia* **808**, 5-22.
54. Thiel T., Michalek W., Varshney R.K., Graner A. 2003 Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). *Theor. Appl. Genet.* **106**, 411-422. (doi:10.1007/s00122-002-1031-0).
55. Untergasser A., Cutcutache I., Koressaar T., Ye J., Faircloth B.C., Remm M., Rozen S.G. 2012 Primer3--new capabilities and interfaces. *Nucleic Acids Res.* **40**, e115. (doi:10.1093/nar/gks596).
56. Jones O.R., Wang J. 2010 COLONY: a program for parentage and sibship inference from multilocus genotype data. *Mol. Ecol. Resour.* **10**, 551-555. (doi:10.1111/j.1755-0998.2009.02787.x).

57. Kalinowski S.T., Taper M.L., Marshall T.C. 2007 Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Mol. Ecol.* **16**, 1099-1106. (doi:10.1111/j.1365-294X.2007.03089.x).
58. Sherman G.B., Kachman S.D., Hungerford L.L., Rupp G.P., Fox C.P., Brown M.D., Feuz B.M., Holm T.R. 2004 Impact of candidate sire number and sire relatedness on DNA polymorphism-based measures of exclusion probability and probability of unambiguous parentage. *Anim. Genet.* **35**, 220-226. (doi:10.1111/j.1365-2052.2004.01143.x).
59. Yuuki Y. 1985 Spawning and growth of *Watasenia scintillans* in the southwestern, Japan Sea. *Bull. Jpn. Soc. Fish. Oceanogr.* **49**, 1–6.
60. Fromhage L., Elgar M.A., Schneider J.M. 2005 Faithful without care: the evolution of monogyny. *Evolution* **59**, 1400-1405.
61. Gomes B.V., Guimaraes D.M., Szczupak D., Neves K. 2018 Female dispersion and sex ratios interact in the evolution of mating behavior: a computational model. *Sci. Rep.* **8**, 2467. (doi:10.1038/s41598-018-20790-7).
62. Simmons L.W., Garcia-Gonzalez F. 2008 Evolutionary reduction in testes size and competitive fertilization success in response to the experimental removal of sexual selection in dung beetles. *Evolution* **62**, 2580-2591. (doi:10.1111/j.1558-5646.2008.00479.x).
63. Baker J., Humphries S., Ferguson-Gow H., Meade A., Venditti C. 2019 Rapid decreases in relative testes mass among monogamous birds but not in other vertebrates. *Ecol. Lett.* (doi:10.1111/ele.13431).
64. Bonduriansky R., Maklakov A., Zajitschek F., Brooks R. 2008 Sexual selection, sexual conflict and the evolution of ageing and life span. *Funct. Ecol.* **22**, 443-453. (doi:10.1111/j.1365-2435.2008.01417.x).
65. Reichard U.H. 2003 Monogamy: past and present In: Monogamy: mating strategies and partnerships in birds, humans and other mammals. Eds. Reichard, U.H. & Boesch, C. Cambridge Cambridge University Press p. 3–25.
66. Yamamura O., Inada T. 2001 Importance of micronekton as food of demersal fish assemblages. *Bull. Mar. Sci.* **68**, 13-25.
67. Rodriguez-Munoz R., Bretman A., Tregenza T. 2011 Guarding males protect females from predation in a wild insect. *Curr. Biol.* **21**, 1716-1719. (doi:10.1016/j.cub.2011.08.053).
68. Franklin A.M., Squires Z.E., Stuart-Fox D. 2014 Does predation risk affect mating behavior? An experimental test in dumpling squid (*Euprymna tasmanica*). *PLoS One* **9**,

e115027. (doi:10.1371/journal.pone.0115027).

69. Hou Z., Luo C., Roberts J.D., Wei C. 2017 Sexual pair-formation in a cicada mediated by acoustic behaviour of females and positive phonotaxis of males. *Sci. Rep.* **7**, 6453. (doi:10.1038/s41598-017-06825-5).
70. Wedell N. 2005 Female receptivity in butterflies and moths. *J. Exp. Biol.* **208**, 3433-3440. (doi:10.1242/jeb.01774).
71. Ruthera J., Thal K., Blaul B., Steiner S. 2010 Behavioural switch in the sex pheromone response of *Nasonia vitripennis* females is linked to receptivity signalling. *Anim. behav.* **80**, 1035-1040.
72. Guevara-Fiore P., Skinner A., Watt P.J. 2009 Do male guppies distinguish virgin females from recently mated ones? *Anim. behav.* **77** 425-431.
73. Kubodera T., Koyama Y., Mori K. 2007 Observations of wild hunting behaviour and bioluminescence of a large deep-sea, eight-armed squid, *Taningia danae*. *Proc. Biol. Sci.* **274**, 1029-1034. (doi:10.1098/rspb.2006.0236).
74. Seidou M., Sugahara M., Uchiyama H., Hiraki K., Hamanaka T., Michinomae M., Yoshihara K., Kito Y. 1990 On the three visual pigments in the retina of the firefly squid, *Watasenia scintillans*. *J. Comp. Physiol. A* **166**, 769-773.

Fig. 1

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587

588 **Fig. 2**

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Fig. 3

593 Supplemental data

594 Table S1

Table S1 References for testicularsomatic index of various squid species used in Fig. 3F

ref#	
S ³	Markaida U. and Sosa-Nishizaki O. (2001) Reproductive biology of jumbo squid <i>Dosidicus gigas</i> in the Gulf of California, 1995–1997. <i>Fish. Res.</i> 54, 63–82.
S ⁴	Murata M., Ishii M. and Osako S. Some Information on Copulation of the Oceanic Squid <i>Onychoteuthis borealijaponica</i> Okada. (1982) <i>Bull. Jap. Soc. Sci. Fish.</i> 48,351–354.
S ⁵	Apostólico L.H. and Marian J.E.A.R. (2018) Dimorphic male squid show differential gonadal and ejaculate expenditure. <i>Hydrobiologia</i> 808, 5–22.
S ⁶	Iwata Y., Munehara H., Sakurai Y. (2005) Dependence of paternity rates on alternative reproductive behaviors in the squid <i>Loligo bleekeri</i> , <i>Mar. Ecol. Prog. Ser.</i> 298, 219–228.
S ⁷	Salman A. and Onsoy, B. (2004) Analysis of fecundity of some bobtail squid of the genus <i>Sepioida</i> (Cephalopoda: Sepioida) in the Aegean Sea (eastern Mediterranean). <i>J. Mar. Biol. Ass. UK</i> 84, 781–782.
S ⁸	Thulasitha W.S., Charles G.A., and Sivashanthini K. (2010) Reproductive Characteristics of Squid <i>Sepioteuthis lessoniana</i> (Lesson, 1830) from the Northern Coast of Sri Lanka. <i>J. Fish. Aqu. Sci.</i> 5, 12–22.
S ⁹	Cuccu D., Mereu M., Masala P., Cau A. and Jere P. (2011) Male reproductive system in <i>Neorossia caroli</i> (Joubin 1902) (Cephalopoda: Sepioidae) from Sardinian waters (western Mediterranean Sea) with particular reference to sexual products. <i>Invert. Reprod. Develop.</i> 55, 16–21.
S ¹⁰	Costa P.A.S., Fernandes F.C. (1993) Reproductive cycle of <i>Loligo sanpa ulensis</i> (Cephalopoda: Loliginidae) in the Cabo Frio region, Brazil. <i>Mar. Eco. Pro. Ser.</i> 101, 91–97.
S ¹¹	Gabr H.R., Hanlon R.T., Hanafy M.H. and El-Etreby S.G. (1998) Maturation, fecundity and seasonality of reproduction of two commercially valuable cuttlefish, <i>Sepia pharaonis</i> and <i>Sepia dollfus</i> in the Suez Canal. <i>Fish. Res.</i> 36, 99–115.
S ¹²	Jackson G.D., Semmens J.M., Phillips K. L., Jackson C.H. (2004) Reproduction in the deepwater squid <i>Moroteuthis ingens</i> , what does it cost? <i>Mar. Biol.</i> 145, 905–916.
S ¹³	MARCELAL B., ANÍBALAUBONE I. and PASCUAL L. (2006) REPRODUCTIVE BIOLOGY OF RED SQUID (<i>Ommastrephes bartramii</i>) IN THE SOUTHWEST ATLANTIC NORMAE. <i>Rev. Invest. Desarr. Pesq.</i> N° 18, 5–19.
S ¹⁴	Cerola L. and Milone N. (2017) Growth and reproduction of the squid <i>Illex coindetii</i> Verany, 1839 in the southern Adriatic Sea, central Mediterranean. <i>Medit. Mar. Sci.</i> , 18, 107–120.
S ¹⁵	Onsoy B., Ceylan B. and Salman A. (2013) Reproductive biology of the lentil bobtail squid, <i>Rondeletiola minor</i> (Cephalopoda: Sepioidae) from the eastern Mediterranean. 93, 851–854.
S ¹⁶	Ikeda Y., Sakurai Y. and Shimazaki K. (1991) Development of Male Reproductive Organs During Sexual Maturation in the Japanese Common Squid <i>Todarodes pacificus</i> . <i>Nippon Suisan Gakkaishi</i> 57, 2237–2241.
S ¹⁷	Hoving H.J.T. and Laptikhovskiy V. (2008) Reproduction in <i>Heteroteuthis dispar</i> (Rüppell, 1844) (Mollusca: Cephalopoda): A sepioid reproductive adaptation to an oceanic lifestyle. <i>Mar. Biol.</i> 154, 219–230.
S ¹⁸	Jackson G.D. and Moltschaniwskyj N.A. (2001) Temporal variation in growth rates and reproductive parameters in the small near-shore tropical squid <i>Loliolus noctiluca</i> ; is cooler better? <i>Mar. Ecol. Prog. Ser.</i> 218,167–177.
S ¹⁹	Sato N., Yoshida MA. and Kasugai T. (2017) Impact of cryptic female choice on insemination success: Larger sized and longer copulating male squid ejaculate more, but females influence insemination success by removing spermatangia. <i>Evolution</i> 71, 111–120.
S ²⁰	Giordano D. et al. (2009) Distribution and biology of <i>Sepietta oweniana</i> (Pfeffer, 1908) (Cephalopoda: Sepioidae) in the Southern Tyrrhenian Sea (Central Mediterranean Sea), <i>Cah. Biol. Mar.</i> 50, 1–10.

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Table S2 Information of microsatellite makers for *Watasenia scintillans*

Locus	(motif) _n *	primer sequence	T _m	k	N	Ho	He	PIC	F(Null)
Wsci5311	(GATA) ₁₈ / N ₁₈ (TAGA) ₂₀	Fw: GGGGTAGGTGATTCATCGC Rv: TCGGACTGATGTTGCTGTCT	58.4 59.0	15	31	0.935	0.921	0.899	-0.0160
Wsci4372	(TAT) ₃₀	Fw: GCGACAGAACCTGCAGTTTG Rv: ACGTCAAGGTGTCGCAATG	60.0 59.4	26	31	0.903	0.964	0.945	0.0242
Wsci2424	(ATT) ₃₀	Fw: ACTCCTGGTATTTCCGCACC Rv: ACACCGCTGCTTATGTCACA	59.7 60.0	20	31	0.774	0.939	0.919	0.0886
Wsci7910	(AAT) ₂₉	Fw: GGCAACAAAAGATCGGAAGCT Rv: AGGTTTTTGCCGGTTTCGTG	59.5 59.9	39	31	0.935	0.977	0.960	0.0140
Average				25	31	0.887	0.950	0.931	0.0277

*repeat number of a specimen used in next-generation sequencing

k: number of alleles observed, N: number of individuals examined, Ho: observed heterozygosity, He: expected heterozygosity, PIC: polymorphic information content, F(Null): Null allele frequency.

The percentage of PCR amplification success with newly developed microsatellite markers was 99.0% (1172/1184) and resulting overall success of paternity identification was 98.9% (270/273). By using Cervus 3.0, the combined non-exclusion probability for all microsatellite loci was calculated to be < 0.0001, which is sufficiently low value for correctly identifying the real sire.

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598 **Datasheet S1**

Experiment 1	Wsci2424		Wsci4372		Wsci7910	
female mantle	211	215	135	135	204	216
sperm mass	197	208	135	156	114	180
embryo #2	197	215	135	156	114	216
embryo #3	208	215	135	135	180	216
embryo #4	197	215	135	135	114	216
embryo #5	208	212	135	135	180	204
embryo #6	197	215	135	156	114	216
embryo #7	197	215	135	156	114	216
embryo #8	197	212	135	135	114	204
embryo #9	208	212	135	156	180	204
embryo #11	208	212	135	156	180	204
embryo #13	197	212	135	156	114	204
embryo #15	197	215	135	156	114	216
embryo #16	208	212	135	156	180	204
embryo #17	208	215	135	135	180	216
embryo #18	197	212	135	156	114	204
embryo #19	197	215	135	156	114	216

Experiment 2	Wsci2424		Wsci7910	
female mantle	173	185	171	318
sperm mass	191	216	105	180
embryo #1	185	215	105	318
embryo #2	173	215	105	171
embryo #3	185	215	105	318
embryo #4	185	191	180	318
embryo #5	185	191	180	318
embryo #7	185	191	180	318
embryo #8	173	215	105	171
embryo #9	173	215	105	171

embryo #10	185	191	180	318
embryo #11	185	215	105	171
embryo #12	173	215	105	171
embryo #13	173	215	105	171
embryo #14	173	191	171	180
embryo #15	173	191	171	180
embryo #16	185	191	180	318
embryo #17	185	215	180	318
embryo #18	185	215	105	318
embryo #19	185	191	180	318
embryo #20	185	191	180	318

Experiment 3	Wsci2424		Wsci5311		Wsci4372	
female mantle	200	204	146	208	116	166
sperm mass	182	200	170	203	107	123
embryo #1	182	200	170	208	107	116
embryo #3	200	204	170	208	122	166
embryo #4	200	200	146	203	107	116
embryo #5	200	204	146	170	107	116
embryo #7	200	204	170	208	107	116
embryo #8	182	204	146	203	122	166
embryo #9	182	204	203	207	107	116
embryo #10	200	204	170	208	107	116
embryo #11	182	200	146	203	107	166
embryo #12	200	200	146	170	107	166
embryo #13	200	200	203	208	116	122
embryo #14	182	200	146	203	107	116
embryo #15	182	204	203	208	122	166
embryo #16	182	204	170	208	166	122
embryo #17	200	204	203	208	116	122
embryo #18	200	200	203	208	107	116

embryo #19	182	204	146	170	107	166
embryo #20	200	204	203	208	116	122

Experiment 4	Wsci2424		Wsci5311		Wsci4372		Wsci7910	
female mantle	150	267	221	225	203	241	266	280
sperm mass	165	279	204	241	225	251	272	298
embryo #2	150	165	225	242	225	241	280	298
embryo #3	267	279	204	225	203	251	266	272
embryo #4	165	267	221	242	225	241	266	272
embryo #5	150	279	204	225	203	225	272	280
embryo #6	150	279	205	225	203	225	272	280
embryo #7	150	279	205	225	241	250	272	280
embryo #8	150	165	205	221	203	251	280	298
embryo #9	150	165	204	221	203	251	280	298
embryo #10	165	267	221	242	225	241	266	298
embryo #11	150	279	221	241	241	251	272	280
embryo #12	150	279	225	242	203	251	272	280
embryo #13	150	279	225	241	203	225	272	280
embryo #14	267	279	205	225	203	251	272	280
embryo #15	150	279	221	242	241	251	272	280
embryo #16	150	165	204	225	203	225	280	298
embryo #17	165	267	221	242	203	225	266	298
embryo #18	165	267	221	242	225	241	266	298
embryo #19	267	279	221	242	203	225	272	280
embryo #20	150	279	204	225	203	251	272	280
embryo #21	165	267	221	241	225	241	280	298
embryo #22	267	279	221	242	225	241	266	272
embryo #23	150	165	221	242	203	251	280	298
embryo #24	150	279	225	242	241	251	272	280
embryo #25	267	278	205	221	204	251	266	272
embryo #26	165	267	225	242	204	251	266	298

embryo #27	150	279	221	242	225	241	272	280
embryo #28	150	279	225	241	241	251	280	298
embryo #29	165	267	204	221	225	241	266	298
embryo #30	150	165	204	221	241	251	280	298
embryo #31	150	279	204	225	225	241	272	280
embryo #32	267	279	204	221	241	251	266	272
embryo #33	150	165	221	242	241	251	280	298
embryo #34	165	267	204	225	225	241	266	298
embryo #35	165	268	204	225	241	251	266	298
embryo #37	150	279	221	242	241	251	272	280
embryo #38	165	267	221	242	203	251	266	298
embryo #39	165	267	221	242	203	251	280	298
embryo #40	165	267	225	242	203	222	266	298
embryo #41	267	279	204	225	225	241	266	272
embryo #43	150	165	225	242	225	241	280	298
embryo #44	150	279	204	221	241	251	272	280
embryo #45	267	279	204	221	203	225	266	272
embryo #46	150	165	225	241	225	241	280	298
embryo #47	267	279	204	221	203	225	266	298
embryo #48	150	279	204	221	203	225	272	280

Datasheet S1 Parentage analysis.

Genotyping was carried out with the mother, her stored spermatangia and spawned eggs using four SSR markers ($n = 4$). All hatched paralarvae, every single spermatangium, i.e., six on left side, six on right side, stored by the female and her mantle tissue were subjected to DNA extraction followed by PCR and then fragment analysis. Microsatellite null alleles were eliminated from the analysis. The estimated size (bp) of each amplicon was shown. The data clearly show that in all microsatellite loci amplified from paralarval samples, one of two alleles was derived from spermatozoa stored in the spermatangia, and the other from the mother.