



# A nudibranch removes rival sperm with a disposable spiny penis

Ayami Sekizawa<sup>1,2</sup> · Shin G. Goto<sup>1</sup> · Yasuhiro Nakashima<sup>3</sup>

Received: 23 January 2018 / Accepted: 21 September 2018 / Published online: 27 October 2018  
© The Author(s) 2019, corrected publication 2019

## Abstract

Simultaneous hermaphroditism is, at least initially, favoured by selection under low density — and therefore it can be assumed that sperm competition has little importance in this sexual system. However, many simultaneously hermaphroditic nudibranchs have both an allo-sperm storage organ (the seminal receptacle) and an allo-sperm digesting organ (the copulatory bursa), suggesting the possibility of the occurrence of sperm competition. A nudibranch, *Chromodoris reticulata*, autotomizes its penis after every copulation and replenishes it within about 24 h to perform another copulation. We observed that the surface of the autotomized penis was covered with many backward-pointing spines and that a sperm mass was often entangled on the spines. This suggests that the nudibranch removes sperm that is already stored in a mating partner's sperm storage organ(s) with its thorny penis. Using six microsatellite markers, we determined that the sperm mass attached to the penis were allo-sperm originating from individual(s) that had participated in prior copulations. We revealed that *C. reticulata* performed sperm removal using the thorny penis. These results suggest that competition in fertilization is quite intense and mating frequency in the wild is relatively high in this species.

**Keywords** Sexual selection · Sperm removal · Sperm competition · Nudibranch · Simultaneous hermaphrodite

## Introduction

It was originally thought that simultaneous hermaphroditism was favoured by selection under low density, such as in deep-sea fishes, or low mobility, such as snails, and that sperm competition had little importance in simultaneous hermaphrodites (Ghiselin 1969). However, some simultaneous hermaphrodites are known to live in high density populations and show complicated mating behaviour (Fischer 1980, 1981: egg trading in hamlets; Leonard et al. 2007: phally polymorphism in banana slugs). Moreover, multiple paternity was confirmed in some simultaneously hermaphroditic molluscs (Baur 1994; Angeloni et al. 2003; Evanno

et al. 2005; Kupfernagel et al. 2010), suggesting that sperm competition occurred in these hermaphroditic animals. These findings indicate that sexual selection functions in simultaneous hermaphrodites as strongly as in gonochorists, if not more strongly. Furthermore, mating behaviours, such as penis fencing in flatworms (Michiels and Newman 1998), cephalo-traumatic secretion transfer in head-shielded slugs (Lange et al. 2013, 2014), and disposable penises in nudibranchs (Sekizawa et al. 2013), show that some simultaneous hermaphrodites undergo severe competition to acquire the chance to inseminate, and suggest that they expose themselves to sperm competition. Additionally, some simultaneously hermaphroditic invertebrates have an organ(s) to digest and absorb sperm received from mating partners (Baur 1998; Michiels 1998). Together, these characteristics indicate the existence of intense sperm competition among simultaneous hermaphrodites as well as in gonochorists.

All nudibranchs are simultaneous hermaphrodites. During mating, they simultaneously play “male roles” as sperm donors and “female roles” as sperm recipients, in principle. The morphology of their female reproductive system is complicated, with two sperm storage organs, the copulatory bursa and the seminal receptacle. Hermaphroditic gastropods are generally thought to be able to digest allo-sperm

✉ Ayami Sekizawa  
aym-ca15@hotmail.co.jp

<sup>1</sup> Department of Biology and Geosciences, Graduate School of Science, Osaka City University, 3-3-138 Sugimoto Sumiyoshi-ku, Osaka-shi, Osaka 558-8585, Japan

<sup>2</sup> Present Address: Graduate School of Agricultural Science, Tohoku University, 468-1 Aramaki Aza Aoba, Aoba-ku, Sendai 980-0845, Japan

<sup>3</sup> Nihon University, College of Economics, 3-2-1 Kanda-Misakicho, Chiyoda-ku, Tokyo 101-8360, Japan

stored in the copulatory bursae (Brandriff and Beemen 1973; Dillen et al. 2009; Lind 1973). The morphology, arrangement, and connectivity of the copulatory bursa and seminal receptacle are highly variable among nudibranch species (Rudman 1984; Valdés et al. 2010). This rich diversity in female reproductive systems implies that mating strategies vary among species and intense sexual selection operates, for example, in the form of sperm competition. Although reproductive system morphology is relatively well studied in nudibranchs, the function of each reproductive organ is not entirely clear. Additionally, direct evidence of sperm competition has not yet been obtained in nudibranchs (Baur 1998).

*Chromodoris reticulata* is reported to autotomize its penis after every copulation and replenish it within about 24 h in order to perform another copulation (Sekizawa et al. 2013). The fact that this nudibranch has a mechanism to repeat copulation within a short period of time with disposable penises indicates their high mating frequency. The autotomized penis is covered with many backward-pointing spines on its surface. It is known that simultaneously hermaphroditic nudibranchs have a wide variety of penis morphologies, including hooks at the tip and spines on the surface (Valdés et al. 2010). These hooks and spines are supposed to provide anchorage to prevent the penis from being unplugged from the mating partner during copulation (Valdés et al. 2010). However, the exact function of these projections on the penis has not been understood. Since a sperm mass was often entangled in the spines on the surface of an autotomized penis after copulation in *C. reticulata* (Sekizawa et al. 2013), this nudibranch has been proposed to scrape out allo-sperm already stored in the allo-sperm storage organ(s) of the mating partner with its thorny penis.

The genetic diversity of stored sperm in female sperm storage organs (Siva-Jothy and Hooper 1995), multiple paternity of a clutch of offspring, and last male precedence in reproductive success (Cooper et al. 1996) have been studied in insects using DNA analysis. These results suggest many insects perform sperm displacement by sperm removal (Córdoba-Aguilar et al. 2003; Kamimura 2000, 2003). Furthermore, Takami (2007) showed that spermatophore displacement was highly probable by DNA analysis of a spermatophore placed in the female genitalia of a ground beetle. However, verification of sperm removal by DNA analysis of scraped out sperm has never been carried out in any animal to our best knowledge, including gastropods and the well-studied damselflies.

In this study, we clarified, by DNA analysis using microsatellite markers, that the sperm mass attached to the autotomized penis in *C. reticulata* originated, at least in part, from allo-sperm removed from the copulatory pouch(es) of the sperm recipient, and certified that the nudibranch performed sperm removal. We compared the traits and mechanisms of sperm displacement in this nudibranch to those of previous

studies in insects. Subsequently, this showed direct evidence of sperm competition by sperm removal. We propose that sexual selection in this simultaneously hermaphroditic nudibranch functions as strongly as that in gonochorists.

## Materials and methods

### Study animals

The study animal, *C. reticulata* (Quoy and Gaimard 1832) (Opisthobranchia, Nudibranchia, Chromodoridae), is distributed in the Indo-West Pacific. In Japan, *C. reticulata* occurs on the rocky coast from central to southern Honshu and around the Ryukyu Islands. This nudibranch has a reticulated network of red lines over the surface of its mantle, reaches a length of 60 mm, and feeds on sponges (Gosliner et al. 2008). The reproductive season of this species ranges from spring to early summer, and individuals spawn ribbon-shaped, yellow egg masses. As is typical of most opisthobranchs (Baur 1998), *C. reticulata* is a non-selfing, simultaneously hermaphroditic nudibranch. The allo-sperm received from the mating partner is stored in the copulatory bursa and seminal receptacle (Rudman 1984). *C. reticulata* exchanges sperm reciprocally during copulation in principle.

### Collection and maintenance

The animals used in this study were collected using SCUBA equipment during their reproductive season, between April and May of 2012, in the shallow coral reefs (2–7 m depth) in the vicinity of the Sesoko Station of the University of the Ryukyus, Okinawa, Japan (26°38'N, 127°52'E). Collected animals were kept individually in a perforated small plastic case sunk in a seawater tank. The tank was submerged in running seawater and aerated. We did not feed the nudibranchs, as their food consumption decreases substantially during their reproductive season (Thompson 1966).

### Repeated copulation experiment

We designated five copulatory groups, each consisting of four individuals, and put two individuals into a clear, acrylic frame experimental tank measuring 27 × 9 × 6 cm (length, breadth, height: 1458 ml) for copulation. The other pair of each group was also permitted to copulate. The mating trials were performed 3 times per animal with each of three different partners within the mating group, with an interval of 2–3 days between each mating trial. We observed a total of 30 copulations. After each copulation, animals were kept individually in small plastic cases until the next trial, and the seawater in the experimental tank was replaced to prevent sperm contamination. We succeeded in collecting 36 out

of the 40 sperm mass samples after the second and third copulations. We graded the amount of sperm attached to the autotomized penis into three ranks (Fig. 1a), collected each sperm mass with tweezers, and preserved them in 90% ethanol. We also cut off a part of the mantle (approximately 20 mg) of each individual and preserved it in 90% ethanol, after all of the copulation trials.

## DNA analysis

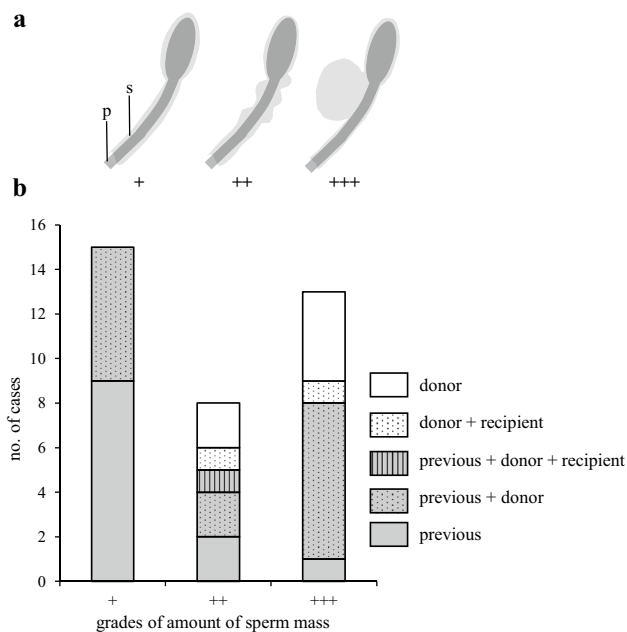
We compared the genotypes of sperm donors and recipients and alleles of sperm samples with six microsatellite markers: *sara 10, 18, 20, 44, 45, and 59* (Sato et al. 2011). We extracted DNA from the mantle of each adult animal and from each sperm mass following the Sato et al. (2011) method. We identified the alleles of the six microsatellite markers in the DNA samples (Sato et al. 2011) using a 3130 Genetic Analyzer (Applied Biosystems) and Gene Mapper 4.0 (Applied Biosystems), and used CERVUS 3.0 (Kalinowski et al. 2007) to calculate the observed and expected heterozygosities and null allele frequencies. When all the donor's alleles were found in a sperm mass in all of the six markers, these alleles were judged to be derived from the donor (D). When all the recipient's alleles were found in a sperm mass, these alleles were judged to be derived from the recipient (R). If the alleles do not correspond to the alleles of the recipient or donor, they are possibly derived from the sperms of the recipient's previous

mating partner(s). Among them, we classified the same alleles as those detected from experimentally copulated individuals (recipient's ex-partner or ex-ex-partner) as E alleles, and those alleles not detected from all four individuals within each experimental group as W alleles. W alleles have a possibility of being derived from individuals with which the study animals copulated in the wild before capture. In case only some but not all the alleles of the donor/recipient were detected, these were not assigned to either D, R, E, or W alleles.

## Results

We identified the alleles of the sperm mass attached to the backward-pointing spines on the surface of autotomized penises after the second and third copulations of the repeated copulation experiments. We also identified the alleles of the sperm donor and recipient, respectively. The number of alleles, observed and expected heterozygosities, and null allele frequencies of the donors and recipients are shown in Table 1. Negative values for the null allele frequency estimates imply that the observed heterozygote genotype exceeded the statistical expectation (Marshall et al. 1998). Six microsatellite markers successfully distinguished each individual from the others.

It is noteworthy that the number of alleles detected in the sperm samples was generally larger than that detected in the donors and recipients (Table 1). Moreover, we detected three or more alleles in each locus from each of the sperm samples (Table 2). That is, the sperm mass was a mixture of sperm from multiple individuals. In 24 samples, the same alleles as the donor were detected at all of six loci used in the experiments, and thus, the alleles were possibly derived from the donor (Table 2). Similarly, the same alleles as the recipient were detected in three samples, and thus, the alleles were possibly derived from the recipient (Table 2). In 21 samples, the same alleles as the ex- or ex-ex-partner were detected at some, but not all of the six loci (allele<sup>E</sup>, Table 2). For example, the sperm mass sample of *Fsp2* was collected from the penis of individual F after the second copulation trial. In this second copulation trial, F's mating partner was B that had copulated with A in the first copulation trial. Therefore, there is some possibility that B received and stored sperm from A in its allo-sperm storage organs in the first copulation trial, and then F removed the sperm originally from A from B's allo-sperm storage organs in the second copulation trial. We assumed that allele 149 in locus *sara 20* of 'Fsp2' originated from A and thus indicated it as allele<sup>E</sup> (previously copulated individual). However, it is also possible that the allele is derived from an unknown wild individual. Therefore, we could not specify the origin of the sperm. In 23 samples, some alleles were not identical with any alleles of individuals of the experimental group. We thought that those



**Fig. 1** Amount and origin of sperm mass attached to an autotomized penis after copulation. **a** Schematic presentation of three grades of the amount of sperm mass. *p* penis, *s* sperm mass. **b** Detected allele patterns in each sperm mass. "Previous" indicates the sperm mass contained allele<sup>E</sup> or allele<sup>W</sup> (see Table 2)

**Table 1** Characterization of six microsatellite loci from *Chromodoris reticulata*

Locus	Individuals ( $n=20$ ) Sperm masses ( $n=36$ )	No. of alleles	$H_O$	$H_E$	Null allele frequency
<i>sara10</i>	Individuals	9	0.850	0.860	-0.008
	Sperm masses	11			
<i>sara18</i>	Individuals	19	0.650	0.958	0.179
	Sperm masses	21			
<i>sara20</i>	Individuals	18	0.750	0.947	0.102
	Sperm masses	20			
<i>sara44</i>	Individuals	22	0.450	0.972	0.355
	Sperm masses	23			
<i>sara45</i>	Individuals ( $n=19$ ) <sup>a</sup>	17	0.474	0.954	0.327
	Sperm masses	18			
<i>sara59</i>	Individuals	5	0.350	0.579	0.249
	Sperm masses	5			

CERVUS 3.0 (Kalinowski et al. 2007) was used to calculate the observed and expected heterozygosities and null allele frequencies. Negative values for null allele frequency estimates imply an observed heterozygote genotype in excess of statistical expectation (Marshall et al. 1998)

$H_O$  observed heterozygosity,  $H_E$  expected heterozygosity of individuals

<sup>a</sup>In the locus of *sara45*, we could analyse only 19 samples

samples with alleles different from the donor or the recipient had a possibility of originating from the recipient's previous partner prior to collection (allele<sup>W</sup>, Table 2).

In the results of the genotype and allele comparison of sperm masses, donors and recipients, alleles from neither donors nor recipients were detected in 28 sperm masses [sperm masses including alleles of the recipient's previous partner(s) only (previous),  $n=12$ ; alleles of recipient's previous partner(s) and alleles of donor,  $n=15$  (donor + previous); alleles of the recipient's previous partner(s) and alleles of donor and recipient (donor + recipient + previous),  $n=1$ ; Table 2]. By contrast, the alleles of the recipient's previous partner(s) were not detected in eight sperm masses [sperm mass including alleles of donor only (donor),  $n=6$ , donor and recipient (donor + recipient),  $n=2$ ; Table 2].

Some samples contained sperm of the recipient's previous partner(s), irrespective of the grade of the amount of sperm attached to the autotomized penis ( $n=28$ , Fig. 1b). Sperm from the recipient's previous partner(s) was detected from all the sperm mass samples of grade '+' ( $n=15$ , Fig. 1b). Some sperm mass samples of grade '++' and '+++' did not contain sperm of the recipient's previous partner(s) ( $n=8$ , Fig. 1b). All of the samples that lacked the sperm of the recipient's previous partner(s) contained the sperm of the donor.

## Discussion

In this study, we obtained the first proof of sperm removal from the reproductive or copulatory organ by DNA analysis of scraped-out sperm. In 28 out of the 36 samples of sperm

mass attached to the autotomized penis, we detected alleles that originated from the recipient's previous partner(s) (Fig. 1) This result demonstrates sperm removal in *C. reticulata*, in which allo-sperm already stored in the reproductive pouch(s) of a mating partner was scraped out using the thorny penis. It is well known that some damselflies remove sperm from the copulatory pouch of mating partners (Córdoba-Aguilar et al. 2003). However, sperm displacement in damselflies was evaluated based on increases and decreases in the amount of stored sperm (Córdoba-Aguilar et al. 2003). Direct evidence of sperm removal by DNA analysis of scraped-out sperm has not yet been shown in animals reported to perform sperm displacement (Kamimura 2005).

The manner of sperm removal in *C. reticulata* differed from that in damselflies. Some damselflies such as *Calopteryx maculata* are known to use a hook-shaped structure at the tip of the appendage of male copulatory organ to remove allo-sperm already stored in the copulatory pouch (Waage 1979; Cordero and Miller 1992; Miller 1987). Damselflies removed allo-sperm with a voluntary movement by the tergo-sternal muscle that is associated with a hook-shaped appendage (Córdoba-Aguilar et al. 2003). Unlike the hook-shaped appendage in damselflies, the disposable penis in *C. reticulata* does not have a retractor muscle (Sekizawa et al. 2013), so it appears impossible to move each spine in a controlled manner. It is plausible that they insert their penis deeply into the copulatory pouch of the mating partner, and ejaculate their own sperm at the innermost part, then entangle allo-sperm with backward-pointing spines on the swollen tip of the penis, pull out the penis entangled with allo-sperm, and finally autotomize the penis after copulation.

**Table 2** Alleles of individuals and sperm masses

Pairing of each group	Sample name	Locus										Origin of sperm mass			
		<i>sara10</i>	<i>sara18</i>	<i>sara20</i>	<i>sara44</i>	<i>sara45</i>	<i>sara59</i>								
<b>Group 1</b>	A	196	212	297	325	143	149	223	244	280	119	123	sperm mass		
	Asp2	196 <sup>D</sup>	212 <sup>D</sup>	297 <sup>D</sup>	325 <sup>D</sup>	143 <sup>D</sup>	149 <sup>D</sup>	223 <sup>D</sup>	244 <sup>D</sup>	280 <sup>D</sup>	119 <sup>D</sup>	123 <sup>D</sup>		Donor	
	Asp3	194 <sup>W</sup>	196 <sup>D</sup>	286 <sup>W</sup>	297 <sup>D</sup>	143 <sup>D</sup>	149 <sup>D</sup>	201 <sup>W</sup>	223 <sup>D</sup>	237 <sup>W</sup>	244 <sup>D</sup>	280 <sup>D</sup>		Donor + previous	
	<b>B</b>	190	208	276	317	143	153	231	231	-	-	123		Donor + previous	
	Bsp2	190 <sup>D</sup>	208 <sup>D</sup>	276 <sup>D</sup>	317 <sup>D</sup>	143 <sup>D</sup>	153 <sup>D</sup>	231 <sup>D</sup>	240	262	123 <sup>D</sup>	Donor			
	Bsp3	190 <sup>D</sup>	194 <sup>W</sup>	276 <sup>D</sup>	317 <sup>D</sup>	136 <sup>W</sup>	143 <sup>D</sup>	231 <sup>D</sup>	-	-	123 <sup>D</sup>	Donor + previous			
	<b>D</b>	208	304	320	320	161	167	202	219	232	242	123		Previous	
	Dsp2	192 <sup>W</sup>	194 <sup>W</sup>	208	212	143	149	202	219	242	280	119			123
	Dsp3	188 <sup>W</sup>	192 <sup>W</sup>	200 <sup>W</sup>	208	143	161	187 <sup>E</sup>	219	232	121 <sup>W</sup>	123			Previous
	<b>F</b>	204	208	320	320	153	167	187	187	242	242	101		123	Donor + previous
Fsp2	194 <sup>W</sup>	200 <sup>W</sup>	204 <sup>D</sup>	208 <sup>D</sup>	149 <sup>E</sup>	153 <sup>D</sup>	187 <sup>D</sup>	167 <sup>D</sup>	242 <sup>D</sup>	280 <sup>E</sup>	101 <sup>D</sup>	123 <sup>D</sup>			
Fsp3	188 <sup>W</sup>	208	297	320	149	153	187	206 <sup>W</sup>	223	252 <sup>W</sup>	238 <sup>W</sup>	280	119	123	
<b>Group 2</b>	<b>C</b>	190	208	265	276	149	163	227	262	262	119	121	Previous		
Csp2	192 <sup>E</sup>	194 <sup>E</sup>	200 <sup>W</sup>	212	136	149	196 <sup>W</sup>	227	-	-	101 <sup>W</sup>	123		Previous	
Csp3	192 <sup>E</sup>	194	265	276	149	163	194 <sup>E</sup>	196 <sup>W</sup>	212	227	262	276 <sup>W</sup>		121	123
<b>I</b>	192	194	320	345	156	161	201	241	246	302	123	Donor			
Isp2	192 <sup>D</sup>	194 <sup>D</sup>	320 <sup>D</sup>	345 <sup>D</sup>	156 <sup>D</sup>	161	201 <sup>D</sup>	241 <sup>D</sup>	246 <sup>D</sup>	302 <sup>D</sup>	123 <sup>D</sup>		Donor		
Isp3	192 <sup>D</sup>	194 <sup>D</sup>	320 <sup>D</sup>	345 <sup>D</sup>	156 <sup>D</sup>	161	201 <sup>D</sup>	241 <sup>D</sup>	246 <sup>D</sup>	302 <sup>D</sup>	123 <sup>D</sup>		Donor		
<b>J</b>	212	335	136	149	194	219	234	246	119	123	Previous				
Jsp3	192	194	198 <sup>W</sup>	200 <sup>W</sup>	282 <sup>W</sup>	301 <sup>W</sup>	201	219	246	123		123	Previous		
<b>L</b>	194	212	308	308	153	161	212	254	254	123		Previous			
Lsp2	192	194	212	320	140 <sup>W</sup>	153	196 <sup>W</sup>	227 <sup>E</sup>	246	254	276 <sup>W</sup>		302	123	Previous
Lsp3	182 <sup>W</sup>	192 <sup>E</sup>	194	212	136 <sup>E</sup>	153	194 <sup>E</sup>	209 <sup>W</sup>	248 <sup>W</sup>	123	123		Previous		

Table 2 (continued)

Pairing of each group	Sample name		Locus										Origin of sperm mass					
	sara10		sara18	sara20	sara44	sara45	sara49	sara59										
<b>Group 3</b>	<b>G</b>	<b>190 192</b>	<b>276 297</b>	<b>141</b>	<b>206 227</b>	<b>266</b>	<b>123</b>											
1	G×N	K×M	190 <sup>D</sup> 192 <sup>D</sup> 200 <sup>E</sup>	276 <sup>D</sup> 297 <sup>D</sup>	126 <sup>W</sup> 141 <sup>D</sup>	206 <sup>D</sup> 227 <sup>D</sup>	266 <sup>D</sup>	123 <sup>D</sup>										Donor + previous
2	G×K	N×M	190 <sup>D</sup> 192 <sup>D</sup> 200	276 <sup>D</sup> 297 <sup>D</sup> 325	141 <sup>D</sup> 163 <sup>W</sup>	196 206 <sup>D</sup> 227 <sup>D</sup>	266 <sup>D</sup> 280 <sup>E</sup>	123 <sup>D</sup>										Donor + previous
3	G×M	N×K	<b>188 196</b>	<b>280 325</b>	<b>147 167</b>	<b>266</b>	<b>256 280</b>	<b>123</b>										Donor + previous
			188 <sup>D</sup> 196 <sup>D</sup>	280 <sup>D</sup> 325 <sup>D</sup>	147 <sup>D</sup> 167 <sup>D</sup>	217 <sup>E</sup> 266 <sup>D</sup>	256 <sup>D</sup> 280 <sup>D</sup>	101 <sup>E</sup> 123 <sup>D</sup>										Donor + previous
			192 <sup>E</sup> 194 <sup>W</sup> 200 <sup>E</sup>	280 294 325	147	202 <sup>W</sup>	- -	123										Previous
<b>Group 4</b>	<b>M</b>	<b>190 200</b>	<b>325</b>	<b>138 161</b>	<b>196 229</b>	<b>256 266</b>	<b>123 125</b>											Previous
			192 <sup>E</sup> 194 <sup>W</sup> 208	286 <sup>W</sup>	147 <sup>E</sup>	196	256 266	101 123										Previous
			190 <sup>D</sup> 200 <sup>D</sup>	325 <sup>D</sup>	138 <sup>D</sup> 161 <sup>D</sup>	196 <sup>D</sup> 229 <sup>D</sup>	256 <sup>D</sup> 266 <sup>D</sup>	123 <sup>D</sup> 125 <sup>D</sup>										Donor
	<b>N</b>	<b>190 208</b>	<b>282 294</b>	<b>145</b>	<b>201 217</b>	<b>284 292</b>	<b>101</b>											Previous
			190 192 <sup>E</sup> 194 <sup>W</sup> 200	282 294	145	201 217	254 <sup>W</sup> 292	101 123										Previous
			188 194 <sup>W</sup> 196	200 <sup>E</sup> 208 282 294	145	201 217	284 292	123										Previous
<b>Group 4</b>	<b>O</b>	<b>192 208</b>	<b>276 317</b>	<b>130 147</b>	<b>225</b>	<b>234</b>	<b>123</b>											Donor + previous
1	O×R	P×Q	192 <sup>D</sup> 208 <sup>D</sup>	276 <sup>D</sup> 317 <sup>D</sup>	130 <sup>D</sup> 147 <sup>D</sup>	225 <sup>D</sup> 289 <sup>W</sup>	123 <sup>D</sup>											Donor + previous
2	O×P	R×Q	<b>192 194</b>	<b>302 335</b>	<b>128 165</b>	<b>198</b>	<b>121</b>											Donor + previous
			192 <sup>D</sup> 194 <sup>D</sup>	276 302 <sup>D</sup> 317	335 <sup>D</sup> 128 <sup>D</sup> 130	147 165 <sup>D</sup> 198 <sup>D</sup> 235 <sup>E</sup>	240 <sup>D</sup>	121 <sup>D</sup> 123										Donor + previous
3	O×Q	R×P	<b>192<sup>D</sup> 194<sup>D</sup></b>	<b>302<sup>D</sup> 335<sup>D</sup></b>	<b>128<sup>D</sup> 136</b>	<b>147<sup>E</sup> 165<sup>D</sup> 198<sup>D</sup> 209<sup>W</sup> 235</b>	<b>234<sup>E</sup> 240<sup>D</sup></b>	<b>121<sup>D</sup> 123</b>										Donor + previous
			192 <sup>D</sup> 194 <sup>D</sup>	302 <sup>D</sup> 335 <sup>D</sup>	128 <sup>D</sup> 136	147 <sup>E</sup> 165 <sup>D</sup> 198 <sup>D</sup> 209 <sup>W</sup> 235	234 <sup>E</sup> 240 <sup>D</sup>	121 <sup>D</sup> 123										Donor + previous
<b>Group 4</b>	<b>Q</b>	<b>192 208</b>	<b>286</b>	<b>140 147</b>	<b>215</b>	<b>254 304</b>	<b>123</b>											Donor + previous
			188 192 <sup>D</sup> 194 <sup>E</sup> 200 <sup>W</sup> 208 <sup>D</sup>	286 <sup>D</sup> 317 <sup>E</sup>	136 140 <sup>D</sup> 147 <sup>D</sup>	215 <sup>D</sup> 231 <sup>W</sup> 289 <sup>W</sup> 254 <sup>D</sup> 304 <sup>D</sup>	119 123 <sup>D</sup>											Donor + previous
			192 <sup>D</sup> 194 <sup>E</sup> 200 <sup>W</sup> 208 <sup>D</sup>	286 <sup>D</sup> 302 <sup>E</sup>	136 <sup>E</sup> 140 <sup>D</sup>	215 <sup>D</sup> 225	254 <sup>D</sup> 304 <sup>D</sup>	123 <sup>D</sup>										Donor + previous
	<b>R</b>	<b>188 208</b>	<b>333 345</b>	<b>136</b>	<b>235</b>	<b>241 254</b>	<b>119 123</b>											Donor + previous
			188 <sup>D</sup> 192 194 200 <sup>W</sup> 208 <sup>D</sup>	317 <sup>E</sup> 333 <sup>D</sup> 345 <sup>D</sup>	136 <sup>D</sup> 147 <sup>E</sup>	198 235 <sup>D</sup> 289 <sup>W</sup> 234 <sup>E</sup> 241 <sup>D</sup> 254 <sup>D</sup> 266 <sup>W</sup> 119 <sup>D</sup> 121 123 <sup>D</sup>												Donor + previous

Table 2 (continued)

Pairing of each group	Sample name	Locus										Origin of sperm mass							
		<i>sarzi10</i>	<i>sarzi18</i>	<i>sarzi20</i>	<i>sarzi44</i>	<i>sarzi45</i>	<i>sarzi59</i>	sperm mass											
<b>Group 5</b>	<b>S</b>	<b>190</b>	<b>323</b>	<b>143</b>	<b>202</b>	<b>248</b>	<b>101</b>	<b>123</b>											
1 S×W	T×U	190 <sup>D</sup>	208 <sup>R</sup>	212 <sup>R</sup>	294 <sup>R</sup>	323 <sup>D</sup>	331 <sup>D</sup>	143 <sup>D</sup>	147 <sup>OR</sup>	202 <sup>D</sup>	219 <sup>E</sup>	252 <sup>R</sup>	240 <sup>R</sup>	248 <sup>D</sup>	101 <sup>OR</sup>	119 <sup>R</sup>	123 <sup>D</sup>	Donor + recipient + previous	
2 S×T	W×U	190 <sup>D</sup>	208 <sup>R</sup>	212 <sup>R</sup>	294 <sup>R</sup>	323 <sup>OR</sup>	331 <sup>D</sup>	143 <sup>D</sup>	147 <sup>D</sup>	156 <sup>R</sup>	161 <sup>R</sup>	202 <sup>D</sup>	217 <sup>R</sup>	248 <sup>D</sup>	304 <sup>R</sup>	101 <sup>D</sup>	123 <sup>OR</sup>	Donor + recipient	
3 S×U	T×W	<b>208</b>	<b>212</b>	<b>147</b>	<b>252</b>	<b>240</b>	<b>101</b>	<b>119</b>											
	Tsp2	208 <sup>D</sup>	212 <sup>D</sup>	147 <sup>D</sup>	202	217 <sup>E</sup>	252 <sup>D</sup>	240 <sup>D</sup>											
	Tsp3	208 <sup>D</sup>	212 <sup>D</sup>	147 <sup>D</sup>	202 <sup>E</sup>	217 <sup>E</sup>	252 <sup>D</sup>	240 <sup>D</sup>											
	U	<b>200</b>	<b>212</b>	<b>156</b>	<b>161</b>	<b>304</b>	<b>123</b>												
	Usp2	196 <sup>R</sup>	200 <sup>D</sup>	208 <sup>R</sup>	212 <sup>D</sup>	290 <sup>R</sup>	304 <sup>R</sup>	323 <sup>D</sup>	132 <sup>R</sup>	151 <sup>R</sup>	156 <sup>D</sup>	161 <sup>D</sup>	217 <sup>D</sup>	219 <sup>R</sup>	241 <sup>R</sup>	244 <sup>R</sup>	304 <sup>D</sup>	123 <sup>OR</sup>	Donor + recipient
	Usp3	200 <sup>D</sup>	212 <sup>D</sup>	156 <sup>D</sup>	161 <sup>D</sup>	323 <sup>D</sup>	217 <sup>D</sup>												
	W	<b>196</b>	<b>208</b>	<b>132</b>	<b>151</b>	<b>244</b>	<b>123</b>												
	Wsp2	192 <sup>W</sup>	194 <sup>W</sup>	196 <sup>D</sup>	200	208 <sup>D</sup>	290 <sup>D</sup>	304 <sup>D</sup>	132 <sup>D</sup>	151 <sup>D</sup>	219 <sup>D</sup>	241 <sup>D</sup>	244 <sup>D</sup>	304	123 <sup>D</sup>			Donor + previous	

Alphabetic characters on each sample name indicate 20 individuals used for the experiments. Pairings of each group is surrounded with a square. -*sp*<sup>2</sup> represents a sperm mass attached to the autotomized penis after the second copulation of successive copulation experiment, -*sp*<sup>3</sup> represents a sperm mass after the third copulation. Numbers indicate the base pairs of amplified DNA; – indicates that the sample was not amplified. Allele(s)<sup>D</sup> represents the same allele as the donor in the sperm mass in which the same allele pattern as the donor was detected in all six loci. Allele(s)<sup>R</sup> represents the same allele as the recipient in the sperm mass in which the same allele pattern as the recipient was detected in all six loci. Allele(s)<sup>E</sup> represent the same alleles as the recipient's ex-partner or ex-ex-partner. Allele(s)<sup>W</sup> represents alleles seen in none of the four individuals in a group of successive copulation experiments. "Previous" indicates the sperm mass contained allele<sup>F</sup> or allele<sup>W</sup>

No alleles of the recipient's previous partner(s) detected in the removed sperm mass was coincident with that of the recipient's ex- or ex-ex-partner used in the mating experiments in all six loci. Alleles that did not match with any of the four individuals in each group were detected from 23 sperm masses (allele<sup>W</sup>, Table 2). This suggests that *C. reticulata* removed sperm originating from the mating partner(s) of preceding copulations that occurred in the wild prior to collection. There are a few possible reasons why the same alleles as the recipient's ex- and ex-ex-partner were not detected from the removed sperm mass. The primary possibility is that the amount of sperm originating from the ex- and ex-ex-partner was too little to detect the same alleles at all of the six loci necessary to determine genotype, because the detection sensitivity of alleles varied among markers (loci). The other possibility is that the amount of the sperm of the recipient's ex- and ex-ex-partner was greatly reduced in (the removable area of) the copulatory pouches of the sperm recipient at the time of fixation, owing to sperm movement and transport, or sperm digestion in the copulatory pouches. The sperm of the recipient's previous partner(s) was detected from some sperm mass samples of each grade of the amount of sperm. This shows that sperm displacement succeeded irrespective of the amount of sperm attached to the autotomized penis. Some sperm mass samples of grade '++' and '+++' did not contain sperm of the recipient's previous partner(s) but sperm of the donor itself. In these cases, the sperm donor may ejaculate in an inappropriate place (for example the diverticulum connecting the copulatory bursa and seminal receptacle) before inserting its penis into the correct position. This may cause the attachment of auto-sperm to the surface of the penis to prevent it from scraping out allo-sperm. There are possible causes for the detection of the DNA of the recipient from the sample of sperm mass: (1) cells of the recipient's female reproductive organ were scraped and attached to the penis of the sperm donor at copulation, (2) sperm that leaked out from the penis of the recipient was attached to the penis of the donor when both partners pulled out their respective penises at the end of copulation.

*C. reticulata* autotomizes its penis after every copulation (Sekizawa et al. 2013) and it is thought that the autotomy of the penis evolved to remove allo-sperm from the mating partner efficiently. We clarified in this study that sperm donors removed allo-sperm already stored in the copulatory pouch(es) of sperm recipients with backward-pointing spines on the penis as the final process of their copulation. Though a long and thorny penis is advantageous in scraping out allo-sperm at copulation, such a penis is difficult to pull back into the body again after copulation. And the backward-pointing spines on the penis covered with sperm at copulation will not remove allo-sperm efficiently at the next copulation, like a Velcro<sup>TM</sup> tape. Such morphological

and functional inconveniency may have made *C. reticulata* develop a cheap and fragile penis and dispose of it, rather than a robust but expensive one and reuse it.

The mating behaviour of *C. reticulata* suggests that sexual selection functions intensely in simultaneous hermaphrodites. In *C. reticulata*, we clarified that sperm donors removed allo-sperm stored from a third or more preceding copulation, and that sperm originating from 2 or more individuals was stored in the seminal receptacle of recipients. The ability to displace allo-sperm with a thorny penis and the ability to copulate repeatedly at intervals of 24 h by rapid replenishment of the disposed penis (Sekizawa et al. 2013) in *C. reticulata* suggest that competition to fertilize is quite intense and mating frequency in the wild is not so low in this species. As the disposable penis in *C. reticulata* has a similar function as the accessory structure of the copulatory organ of damselflies, we suppose that sexual selection may work as intensely in simultaneously hermaphroditic animals as it does in gonochorists. Additionally, we propose that the low-density model, which states that simultaneous hermaphroditism evolved to fully utilize scarce mating opportunities caused by low density or low mobility (Ghiselin 1969), should be re-evaluated, at least in the case of *C. reticulata*.

**Acknowledgements** We are grateful to K. Sakai of the Sesoko Station of the University of Ryukyus, for his help during the field survey in Okinawa. We are also thankful to A. Shimizu and M. Okubo of the National Research Institute of Fisheries Science, Fisheries Research; N. Sato of Shimane University; and S. Nanami of Osaka City University, for their help performing DNA analysis. We thank S. Shiga for her help and encouragement. This work was financially supported by a Grant-in-Aid (#22570029) for Scientific Research to Y. N. from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants performed by any of the authors. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

**Informed consent** Irrelevant.

**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made.

The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.



## References

- Angeloni L, Bradbury JW, Burton RS (2003) Multiple mating, paternity, and body size in a simultaneous hermaphrodite, *Aplysia californica*. *Behav Ecol* 14(4):554–560
- Baur B (1994) Multiple paternity and individual variation in sperm precedence in the simultaneously hermaphroditic land snail *Arianta arbustorum*. *Behav Ecol Sociobiol* 35(6):413–421
- Baur B (1998) Sperm competition in molluscs. In: Birkhead TR, Møller AP (eds) Sperm competition and sexual selection. Academic Press, London, pp 255–305
- Brandriff B, Beemen RD (1973) Observations on the gametolytic gland in the anaspidean opisthobranchs, *Phyllaplysia taylori* and *Aplysia californica*. *J Morphol* 141:395–409
- Cooper G, Miller P, Holland PWH (1996) Molecular genetic analysis of sperm competition in the damselfly *Ischnura elegans* (Vader Linden). *Proc R Soc Lond B* 263:1343–1349
- Cordero A, Miller PL (1992) Sperm transfer, displacement and precedence in *Ischnura graellsii* (Odonata: Coenagrionidae). *Behav Ecol Sociobiol* 30:261–267
- Córdoba-Aguilar A, Uhía E, Rivera AC (2003) Sperm competition in Odonata (Insecta): the evolution of female sperm storage and rivals' sperm displacement. *J Zool* 261:381–398
- Dillen L, Jordaens K, Backeljau T (2009) Sperm transfer, sperm storage, and sperm digestion in the hermaphroditic land snail *Succinea putris* (Gastropoda, Pulmonata). *Invertebr Biol* 128:97–106
- Evanno G, Madec L, Arnaud J (2005) Multiple paternity and post-copulatory sexual selection in a hermaphrodite: what influences sperm precedence in the garden snail *Helix aspersa*? *Mol Ecol* 14(3):805–812
- Fischer EA (1980) The relationship between mating system and simultaneous hermaphroditism in the coral reef fish *Hypoplectrus nigricans* (Serranidae). *J Anim Behav* 28:620–633
- Fischer EA (1981) Sexual allocation in a simultaneously hermaphroditic coral reef fish. *Am Nat* 117:64–82
- Ghiselin MT (1969) The evolution of hermaphroditism among animals. *Q Rev Biol* 44:189–208
- Gosliner TM, Behrens DW, Valdés Á (2008) Indo-Pacific nudibranchs and sea slugs: a field guide to the world's most diverse fauna. Sea Challengers Natural History Books, Washington, p 426
- Kalinowski ST, Taper ML, Marshall TC (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Mol Ecol* 16:1099–1106
- Kamimura Y (2000) Possible removal of rival sperm by the elongated genitalia of the earwig, *Euborella plebeja*. *Zool Sci* 17:667–672
- Kamimura Y (2003) Effects of broken male intromittent organ on the sperm storage capacity of female earwig, *Euborella plebeja*. *J Ethol* 21:29–35
- Kamimura Y (2005) Last-male paternity of *Euborella plebeja*, an earwig with elongated genitalia and sperm-removal behavior. *J Ethol* 23:35–41
- Kupfernagel S, Rusterholz H, Baur B (2010) Variation in multiple paternity and sperm utilization patterns in natural populations of a simultaneous hermaphroditic land snail. *Biol J Linn Soc* 99(2):350–361
- Lange R, Werminghausen J, Anthes N (2013) Does traumatic secretion transfer manipulate mating roles or reproductive output in hermaphroditic sea slug? *Behav Ecol Sociobiol* 67:1239–1247
- Lange R, Werminghausen J, Anthes N (2014) Cephalo-traumatic secretion transfer in a hermaphroditic sea slug. *Proc R Soc B* 281:20132424. <https://doi.org/10.1098/rspb.2013.2424>
- Leonard J, Westfall J, Pearse J (2007) Phally polymorphism and reproductive biology in *Ariolimax (Ariolimax) buttoni* (Pilsbry and Vanatta, 1896) (Stylommatophora: Arionidae). *Am Malacol Bull* 23:121–135
- Lind H (1973) The functional significance of the spermatophore and the fate of spermatozoa in the genital tract of *Helix pomatia* (Gastropoda: Stylommatophora). *J Zool* 169:39–64
- Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. *Mol Ecol* 7:639–655
- Michiels NK (1998) Mating conflicts and sperm competition in simultaneous hermaphrodites. In: Birkhead TR, Møller AP (eds) Sperm competition and sexual selection. Academic Press, London, pp 219–254
- Michiels NK, Newman LJ (1998) Sex and violence in hermaphrodites. *Nature* 391:647
- Miller PL (1987) Sperm competition in *Ischnura elegans* (Vander Linden) (Zygoptera: Coenagrionidae). *Odonatologica* 16:201–207
- Rudman WB (1984) The *Chromodorididae* (Opisthobranchia: Mollusca) of the Indo-West Pacific: a review of the genera. *Zool J Linn Soc Lond* 81:115–273
- Sato N, Sekizawa A, Awata S, Munehara H, Nakashima Y (2011) Isolation and characterization of microsatellite markers in the nudibranch *Chromodoris tinctoria*. *Venus* 69:214–217
- Sekizawa A, Seki S, Tokuzato M, Shiga S, Nakashima Y (2013) Disposable penis and its replenishment in a simultaneous hermaphrodite. *Biol Lett* 9:20121150. <https://doi.org/10.1098/rsbl.2012.1150>
- Siva-Jothy MT, Hooper R (1995) The disposition and genetic diversity of stored sperm in the damselfly *Calopteryx splendens xanthostoma* (Charpentier). *Proc R Soc Lond B* 259:313–318
- Takami Y (2007) Spermatophore displacement and male fertilization success in the ground beetle *Carabus insulicola*. *Behav Ecol* 18(3):628–634
- Thompson TE (1966) Studies on the reproduction of *Archidoris pseudoargus* (Rapp) (Gastropoda Opisthobranchia). *Phil Trans R Soc Lond B* 343:343–374
- Valdés Á, Gosliner TM, Ghiselin MT (2010) Opisthobranchs. In: Leonard J, Córdoba-aguilar A (eds) The evolution of primary sexual characters in animals. Oxford University, UK, pp 148–172
- Waage JK (1979) Dual function of the damselfly penis: sperm removal and transfer. *Science* 203:916–918