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First evidence of self-fertilization in a marine microturbellarian (*Platyhelminthes*)

Marco Casu *, Piero Cossu, Tiziana Lai, Fabio Scarpa, Daria Sanna, Gian Luca Dedola, Marco Curini-Galletti

Dipartimento di Scienze della Natura e del Territorio, Università di Sassari, Via F. Muroni 25, 07100 Sassari, Italy

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ABSTRACT

The reproductive strategy of the simultaneous hermaphrodite *Pseudomonocelis paupercula* (Platyhelminthes: Proseriata) was investigated using multiple approaches including breeding experiments, anatomical reconstruction, and parentage analysis of offspring. The 18 allozyme loci tested were monomorphic. Conversely, the ISSR markers showed differences among the populations, and allowed us to ascertain whether the offspring were derived from selfing or cross-breeding. The results suggest that selfing is the most common mode of reproduction in this species, with only 8% of the offspring resulting from cross-reproduction. Age at first reproduction of selfers does not differ from that of paired, potentially cross-breeding, specimens. The presence of sperm in the female ducts of individuals that have been isolated since birth suggests the existence of a connection between the male and female reproductive systems that allows self-fertilization. Habitat is suggested to be the key factor shaping the reproductive strategy of the species. *P. paupercula* is found in highly fragmented brackish-water microhabitats, and selfing may allow for colonization of new habitats that can start from single, unfertilized specimens.

1. Introduction

Hermaphroditism occurs in the majority of the animal phyla and is prevalent in major taxa including *Platyhelminthes*, *Gastrotricha*, *Pulmonata*, *Clitellata* and *Ascidiacea*. However, relatively few cases of selfing have been reported in animals (Jarne and Auld, 2006; Lamy et al., 2012). In fact, the negative genetic and evolutionary consequences of selfing (see Jarne and Charlesworth, 1993) are so significant that many hermaphroditic species display differential timing in the maturation of gonads, and simultaneous hermaphrodites may delay the onset of reproduction in the absence of mates (Escobar et al., 2011).

Yet, in marine habitats selfing may confer a significant advantage to poorly vagile or sessile species when the densities of conspecifics are low and mate availability is problematic. In the case of hermaphroditic, free-spawning species, self-fertilization may be determinant for assuring reproductive success in cases of sperm limitation and/or dilution (Brazeau et al., 1998). Furthermore, isolated, self-fertile individuals can rapidly establish populations in new habitat spaces (Ryland and Bishop, 1990), and high rates of selfing combined with phylopatry may allow for local adaptation (Parker, 1991).

A potentially viable alternative could be a mixed-mating system, where individuals shift between selfing and cross-breeding, according to the situation. The rarity of this reproductive strategy in animals has been explained by genetic models, which predict evolution towards pure selfing or pure outcrossing as a result of the disruptive selection against intermediate selfing rates (Lande and Schemske, 1985). However, in ecological models, forces external to the genetic costs and the benefits of selfing may lead to mixed-mating (Goodwillie et al., 2005), and the extent to which selfing occurs may reflect the diversity of the selective forces operating in the particular ecological setting where the species is present (Husband and Schemske, 1996).

The predominantly simultaneous hermaphroditic *Platyhelminthes* are among the taxa where alternative strategies to obligate cross-breeding are present. However, although selfing has been reported in parasitic taxa and in *Tricladida* (Jarne and Auld, 2006; Sluys, 1989), it has not been previously documented in marine microturbellarians, which include minute, predominantly interstitial, free-living marine *Platyhelminthes*. These organisms lack dispersal stages and occurring at low densities in the sediment (Curini-Galletti, 2001), set a plausible ecological scenario for the evolution of selfing.

Preliminary observations of isolated specimens of a recently described species, *Pseudomonocelis paupercula* Curini-Galletti, Casu and Lai, 2011 (Platyhelminthes: Proseriata), laying fertile cocoons suggested that the species may be capable of self-fertilization. However, on the basis of chance observations alone, the possibility of the long-term viability of allosperm in the female reproductive tract could not be ruled out. In this paper, the presence and relevance of selfing in this species was studied using multiple approaches including breeding experiments, anatomical reconstructions and parentage analysis of offspring.

* Corresponding author. Tel.: +39 079 228924; fax: +39 079 228665.
E-mail address: marcasu@uniss.it (M. Casu).

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2. Materials and methods

2.1. Sampling

The specimens of *P. paupercula* used in this study were collected from the following locations:

- Porto Pozzo (PP), Sardinia, Italy (Western Mediterranean) (41°11′ 20.22″N; 9°17′11.03″E);
- Maliakós (MA), Greece (Aegean Sea, Eastern Mediterranean) (38°54′26.05″N; 22°37′22.25″E);
- Alexandria (AL), Egypt (Levantine Sea, Eastern Mediterranean) (31°17′16.68″ N; 30°0′35.16″E);
- Akko (AK), Israel (Levantine Sea, Eastern Mediterranean) (32°55′ 29.94″N; 35°4′5.55″E).

At each station, 2 l of sediment was collected from the water line to approximately 20 cm deep by scraping the upper surface of the sand. The specimens were isolated from the sediment using the $MgCl_2$ technique (see Casu et al., 2011a for details).

Cultures of the specimens were maintained in the laboratory at 18 ± 0.5 °C and 37‰ salinity and fed weekly with crushed *Sphaeroma* sp. (Isopoda).

2.2. Experiments on reproductive biology

The main questions addressed were as follows: i) is selfing present in the species and is it relevant compared to cross-breeding and ii) what is the pathway through which selfing is achieved?

1) Testing selfing and its relevance. To quantify the number of offspring produced by isolated specimens and by pairs, a set of experiments was performed using newly hatched individuals (NHI) from the populations from Akko and Porto Pozzo:
   - Isolated specimens: 20 NHI per population were isolated into 20 ml containers filled with seawater, and maintained under the culture conditions described for the stock populations;
   - Paired specimens: 20 pairs, each pair consisting of two NHI from the same population, maintained as above.

   The specimens who died before reaching maturity were replaced with others maintained in individual containers as backups, to obtain the necessary number of replicates.

   The offspring were produced by specimens kept in pairs and in isolation. The offspring were counted and relocated weekly. To avoid bias due to senescence, only the 10 weeks following the hatching of the first offspring were considered in the calculation of the reproductive output.

   ANOVAs were used to analyze differences in a) the age of first reproduction by singles and pairs of the same population (i.e., the age when the first cocoons were laid – because it was impossible to assign the cocoon to one individual in the paired specimens, the laying of the first cocoon was taken as the onset of reproduction for both individuals) and b) the reproductive output (i.e., the number of offspring produced per individual per week) of individuals and pairs of specimens from the same population – the offspring were equally subdivided between parents for the paired specimens. To test the quantitative relevance of selfing, both the hypotheses, H_1: the average number of offspring produced by the isolated individuals = the average number of offspring produced by the pairs; and H_2: the average number of offspring produced by the isolated individuals × 2 = the average number of offspring produced by the pairs, were tested.

   One-way ANOVA was run for the variable "the age of first reproduction" separately for each population where the culture condition (isolation vs. pairs) was treated as a fixed factor. The variable "reproductive output" was analyzed by means of a one-way ANOVA, with population (PP and AK) and culture condition as random and fixed factor respectively. The Cochran's C-test was used to test the assumption of the homogeneity of variance, and the log-normal transformation was used to remove heteroscedasticity. The differences were deemed to be significant when $P \leq 0.05$. The Student–Newman–Keuls (SNK) test was used to compare the means following significant effects found in the ANOVA (Underwood, 1997).

2) Morphological study. Twenty NHI from Porto Pozzo were kept in individual containers until the birth of the first offspring. The specimens were then fixed in Bouin's fluid, embedded in 60 °C Paraplast and cut into 4-μm serial sagittal sections and stained with Hansen's haematoxylin and eosin-orange, and then mounted in Eukitt. The voucher slides were deposited in the collection of the Zoological Museum of the University of Sassari (Italy) (CZM).

2.3. Molecular analysis

Molecular analyses were performed to test the i) within and among population variability and ii) to ascertain whether the offspring produced by the specimens kept in pairs were derived from selfing or cross-breeding.

The genetic structure of *P. paupercula* was evaluated using both allozyme electrophoresis and ISSR (Inter-Simple Sequence Repeat) techniques (Zietckiewicz et al., 1994). Forty wild-caught individuals (ten per each population) were analyzed using both molecular markers.

To assess the origin of the offspring (i.e., whether they were derived from cross-breeding or from selfing, and in the latter case, from which parent) we performed a series of experiments pairing specimens from populations, which, based on results of the genetic population studies, showed adequate differences in the ISSR banding patterns. Thus, five pairs of NHI consisting of the combination AL × MA or AK × PP were formed and maintained in the culture conditions described above. Additionally, as a control of the uniformity of the ISSR banding pattern in offspring obtained by selfing, two NHI specimens per population (AL, MA, AK, and PP) were isolated and reared individually in the same culture conditions. The offspring produced by the pairs and isolated individuals were removed and fixed in 95% ethanol for molecular analysis. The parents were then fixed. All of the specimens were stored at 4 °C until the DNA was extracted. The ISSRs were used for the parentage analysis.

2.3.1. Allozyme electrophoresis

A total of 22 enzyme systems were assayed in Tris EDTA maleate electrode buffer [Tris (0.1 M), EDTA (0.01 M), $MgCl_2$ (0.001 M), corrected to pH 7.8 with maleic acid] (Table 1). Each specimen was placed in a 25 μl microwell containing 5 μl of 0.05 M Tris pH 8.0 grinding buffer, where it was manually homogenized. The supernatant fractions were applied to cellulose acetate membranes for electrophoresis (25–30 min at 350 V). The samples were maintained at less than 5 °C at all stages. The enzyme staining was performed according to the procedures described by Pasteur et al. (1987), with slight modifications. Enzymes with low activity and resolution were discarded, resulting in a focus on 12 enzymes (Table 1). Before estimating within and among-population genetic variability (see Casu and Curini-Galletti, 2006 for the routine analyses performed on allozymes), the arbitrary value of 100 was assigned to the most common allele.

2.3.2. ISSRs

The genomic DNA was extracted using the QIAGEN DNeasy Tissue kit (QIAGEN Inc.) according to the supplier's instructions. After extraction, the DNA was stored in solution at 4 °C. When amplification was poor, we used the GenomiPhi DNA Amplification Kit (GE Healthcare), a whole genome amplification kit that can perform unlimited DNA tests from small samples. A set of 18 primers provided by Proligo Primers and Probes (Proligo France SAS) was used as a preliminary screening to achieve wide genome coverage (Table 2). We chose to use only primers anchored at the 3′ end, to obtain greater reaction specificity and to minimize problems due to homoplasy and/or homology of bands.