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Functional rare males in diploid parthenogenetic *Artemia*

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Abstract

Functional males that are produced occasionally in some asexual taxa – called 'rare males' – raise considerable evolutionary interest, as they might be involved in the origin of new parthenogenetic lineages. Diploid parthenogenetic *Artemia* produce rare males, which may retain the ability to mate with females of related sexual lineages. Here, we (i) describe the frequency of male progeny in populations of diploid parthenogenetic *Artemia*, (ii) characterize rare males morphologically, (iii) assess their reproductive role, using cross-mating experiments with sexual females of related species from Central Asia and characterize the F1 hybrid offspring viability and (iv) confirm genetically both the identity and functionality of rare males using DNA barcoding and microsatellite loci. Our result suggests that these males may have an evolutionary role through genetic exchange with related sexual species and that diploid parthenogenetic *Artemia* is a good model system to investigate the evolutionary transitions between sexual species and parthenogenetic strains.

Introduction

Parthenogenetic reproduction occurs in one of 10 000 animal species (Lynch *et al.*, 2008). Populations in these species are made of females that reproduce through apomixis (strict asexuality where there is no meiotic division) or automixis, where some of the products of a single meiosis fuse in diverse ways to restore diploidy (Bell, 1982). However, the presence of occasional males in all-female populations is not an uncommon phenomenon (Schön *et al.*, 2009). Some of these species are cyclical parthenogens, where sexual and parthenogenetic phases are regulated environmentally and males and sexual females are part of the life cycle (Bell, 1982; De Meester *et al.*, 2004). Other species are androdioecious, where self-fertilizing hermaphrodites coexist with a small proportion of males, such as the branchiopods *Eulimnadia*, *Limnadia* and *Triops* and the nematode *Caenorhabditis elegans* (Weeks, 2006; Weeks *et al.*, 2008; Zierold *et al.*, 2009; Anderson *et al.*, 2010).

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Lineages of sperm-dependent apomictic flatworm *Schmidtea polychroa* have also been shown to present occasional male function (D'Souza & Michiels, 2010). Female-biased populations can also be due to infection with *Wolbachia* or other feminizing bacteria, rather than being genetically determined (Plantard *et al.*, 1998; Stouthamer *et al.*, 1999). Research, however, has confirmed the occurrence of rare males in various obligate parthenogens (Blackman, 1972; Butlin *et al.*, 1998; Martens, 1998; Rispe *et al.*, 1999; Simon *et al.*, 1999; Delmotte *et al.*, 2001; Snyder *et al.*, 2006; Engelstädter *et al.*, 2011). These observations of rare males raise important questions, such as their role in the origin and persistence of asexual lineages, the mechanisms involved in replenishing the diversity of such lineages, the avoidance of mutation accumulation and the occurrence of contagious parthenogenesis (Lynch, 1984; Butlin *et al.*, 1998). In addition, functional rare males may challenge assumptions of evolution of sex theory, such as the complete reproductive isolation between sexual and parthenogenetic lineages (Lynch, 1984), or the absence of a 'cost of males' in parthenogenetic lineages (Neiman *et al.*, 2012). Despite the importance of this topic, little research has been devoted to characterize their population frequency or to understand their mechanisms of origin. Most rare males found in parthenogenetic species appear to exhibit abnormal

spermatogenesis and sterility, although some are functional (Lynch, 1984). Rare males, purportedly, cannot fertilize conspecific females as these females are parthenogenetic, and, given the low frequency of males in these populations, they are often seen as 'atavisms' of little consequence with their potential evolutionary impact deemed unimportant (Schön *et al.*, 2009). However, if parthenogenetic lineages retain the ability to produce occasional males on a regular basis, and reproductive isolation between them and their sexual relatives is incomplete, such males may represent a vector for genetic exchange between parthenogenetic and sexual lineages when both coexist (Lynch, 1984; Rispe *et al.*, 1999; Simon *et al.*, 1999; Delmotte *et al.*, 2001; Engelstädter *et al.*, 2011). Indeed, males produced by parthenogenetic females, when mating with sexual females of related species, may transmit the genes conferring parthenogenesis to their offspring (Innes & Hebert, 1988; Lynch *et al.*, 2008; Engelstädter *et al.*, 2011; Eads *et al.*, 2012), a mechanism termed 'contagious parthenogenesis' (Simon *et al.*, 2003). This mechanism could (i) increase the fitness of parthenogenetic lineages producing rare males, (ii) boost the genetic diversity of such asexual lineages and (iii) potentially contribute to the ecological success and the evolutionary potential of such asexual lineages.

Brine shrimps of the genus *Artemia* (Crustacea, Branchiopoda, Anostraca) include gonochoric sexual species with separate males and females, and lineages of obligate parthenogenetic populations of different ploidy levels (Abatzopoulos *et al.*, 2002). Parthenogenetic populations occur only in the Old Word, from the Canary Islands in the west to China in the east, and they have been introduced in Australia (Gajardo *et al.*, 2002; McMaster *et al.*, 2007). These parthenogenetic lineages co-occur with diverse sexual species across their range, including *A. salina* (Linnaeus 1758) in the Mediterranean region and South Africa (Amat *et al.*, 1995), *A. urmiana* (Günther 1899) in and around lake Urmia (Iran) and Crimean salt lakes (Abatzopoulos *et al.*, 2009), *A. sinica* (Cai 1989) in Central and Northern China, *A. tibetiana* (Abatzopoulos *et al.*, 2002; Van Stappen *et al.*, 2007) in the Tibetan plateau and a yet undescribed sexual species in Kazakhstan (Pilla & Beardmore, 1994; Litvinenko & Boyko, 2008). In Australia, introduced populations of diploid parthenogenetic *Artemia* may coexist with endemic brine shrimps of the genus *Parartemia* (McMaster *et al.*, 2007). Parthenogenetic lineages are closely related genetically to Central Asian sexual species (in particular *A. urmiana*, *A. sinica* and the undescribed *Artemia* sp. from Kazakhstan), and they have originated independently several times (Baxevanis *et al.*, 2006; Muñoz *et al.*, 2010; Maniatsi *et al.*, 2011).

Parthenogenetic diploid *Artemia* populations, which reproduce through automictic parthenogenesis (Abreu-Grobois, 1987), produce males in low numbers, and these are usually referred to as rare males (Stefani, 1964; Bowen *et al.*, 1978; MacDonald & Browne, 1987; Amat *et al.*, 1991; Cai, 1993; Mura & Nagorskaya, 2005). Rare males are produced by a yet unknown cytogenetic mechanism, possibly involving crossing over between sex chromosomes (Stefani, 1964; Abreu-Grobois & Beardmore, 2001). These males have normal and functional reproductive organs and display normal sexual behaviour (MacDonald & Browne, 1987), their sperm being slightly larger than those of sexual males (Stefani, 1964). Rare males have not been shown to fertilize females from their own diploid parthenogenetic lineages (Stefani, 1964; MacDonald & Browne, 1987) or sexual females from *A. franciscana, A. persimilis* or *A. salina* (MacDonald & Browne, 1987; but see Bowen *et al.*, 1978). In contrast, rare males can fertilize sexual females of the closely related species *A. urmiana* (Bowen *et al.*, 1978) and *A. sinica* (Cai, 1993), thus potentially enabling gene flow among these lineages. The coexistence of parthenogenetic lineages with their close sexual relatives therefore may provide an opportunity for rare males to mate with sexual females and have an evolutionary impact.

The aims of this study were (i) to describe the frequency of male progeny in populations of diploid parthenogenetic *Artemia*, (ii) to characterize rare males morphologically in the context of the variation in closely related sexual Central Asian *Artemia* species, (iii) to assess the reproductive role of rare males in cross-mating experiments with sexual females of Central Asian sexual populations and estimate the viability of F1 hybrid offspring and (iv) to confirm genetically both the identity and functionality of rare males. The evolutionary role and functionality of rare males are discussed on the basis of the results obtained.

Materials and methods

Samples

Brine shrimp cyst samples were used to establish laboratory populations of diploid parthenogenetic *Artemia* (see Table 1). Samples covering most of the known geographical distribution of diploid parthenogenetic *Artemia* were obtained from the collection of the cyst bank kept in the Instituto de Acuicultura de Torre de la Sal (IATS-CSIC). Most cultured populations of diploid parthenogenetic individuals were obtained from cyst samples of pure parthenogenetic natural populations. In some cases, original cyst samples contained an additional species (see Table 1). Whenever cyst samples containing other *Artemia* species were obtained, as indicated by the presence of abundant males, diploid parthenogenetic females were carefully isolated from the cultures according to the morphological traits described by Amat (1980). Parthenogenetic females were then allowed to reproduce, and their naupliar or

Table 1 Rare male frequency in diploid parthenogenetic *Artemia* populations. Population name and location details, year of sample collection, additional co-occurring species found in the sample, total individuals sexed and number of males found and male ratio are given. In other species, the tetraploid parthenogenetic *Artemia* is denoted as 4n.

Population	Coordinates	Year	Other species	Individuals sexed	Number of males	Males/1000 individuals
Odiel, Huelva, Spain	37°15′26″N–06°58′53″W	1987	4n	14 188	14	0.99
Rocío, Cádiz, Spain*	36°51′19″N–06°20′14″W	2001	*A. salina*	12 202	12	0.98
Hortales, Cádiz, Spain	36°44′18″N–05°32′06″W	2009	–	2297	0	0.00
San Fernando, Cádiz, Spain	36°27′58″N–06°10′41″W	1990	*A. salina*	12 504	12	0.96
Calpe, Alicante, Spain	38°38′37″N–00°03′60″W	1986	–	12 000	12	1.00
La Mata, Alicante, Spain*	38°02′08″N–00°42′02″W	1989	–	19 690	51	2.59
Bonmatí, Alicante, Spain	38°10′20″N–00°37′16″W	1980	*A. salina*	7268	19	2.61
Bras de Port, Alicante, Spain	38°11′22″N–00°36′36″W	2004	*A. salina*	3283	1	0.30
Rasall, Murcia, Spain	37°38′03″N–00°43′23″W	2011	–	3250	9	2.77
Cabo de Gata, Almería, Spain	36°45′48″N–02°13′19″W	1998	–	4040	20	4.95
Gerri, Lleida, Spain	42°19′39″N–01°04′04″E	1990	–	12 320	1	0.08
Aveiro, Portugal	40°38′01″N–08°40′49″W	1992	–	18 105	36	1.99
Rio Maior, Santarem, Portugal	39°21′49″N–08°56′45″W	2004	–	7062	2	0.28
Giraud, Camargue, France	43°23′58″N–04°43′37″E	1990	–	348	1	2.87
Aigües Mortes, Camargue, France*	43°33′35″N–04°10′54″E	2003	*A. franciscana*	1272	5	3.93
Margherita Di Savoia, Puglia, Italy	41°22′50″N–16°05′24″E	2004	4n, *A. franciscana*	12 103	12	0.99
Torre Colimena, Puglia, Italy	40°18′13″N–17°44′03″E	2004	–	1993	5	2.51
Santa Gilla, Sardinia, Italy	39°13′33″N–09°02′53″E	1988	*A. salina*	4647	5	1.08
Molentargius, Sardinia, Italy	39°13′51″N–09°12′33″E	2004	*A. salina*	1631	8	4.90
Notteri, Sardinia, Italy*	39°07′04″N–09°30′55″E	2009	*A. salina*	5715	16	2.80
Atanosovsko, Bulgaria*	42°29′39″N–27°25′54″E	2006	–	8707	22	2.53
Narte, Albania	40°30′02″N–19°27′03″E	2006	–	5160	2	0.39
Koyashskoe, Ukraine*	45°02′57″N–36°11′02″E	2007	*A. urmiana*	2908	7	2.41
Kujalnik, Ukraine	46°38′00″N–30°43′21″E	1991	–	12 656	91	7.19
Maloje Jarove Lake, Russia	53°01′35″N–79°08′54″E	1993	–	8031	13	1.62
Janubio, Lanzarote, Spain	28°56′16″N–13°49′14″W	1988	–	13 092	0	0.00
Tenefé, Gran Canaria, Spain	27°48′51″N–15°25′19″W	2005	–	14 810	0	0.00
Guatiza, Lanzarote, Spain	29°03′29″N–13°27′41″W	2010	–	9374	20	2.13
El Río, Lanzarote, Spain	29°13′03″N–13°29′41″W	2010	–	2418	0	0.00
Larache, Morocco	35°11′52″N–06°07′24″W	2005	4n	5290	1	0.19
Relisane, Algeria	35°50′31″N–00°39′10″E	2009	4n, *A. salina*	9659	34	3.52
Bethioua, Algeria	35°42′31″N–00°16′53″W	2009	4n, *A. salina*	6308	12	1.90
Oran, Algeria	35°32′09″N–00°48′00″W	2009	*A. salina*	5951	3	0.50
Ezzemoul, Algeria	35°52′54″N–06°30′14″E	2008	*A. salina*	2065	11	5.33
Adrar, Algeria	27°46′30″N–00°14′17″E	2008	–	2891	9	3.11
Chergui, Algeria	35°13′02″N–03°34′55″E	2008	–	1231	6	4.87
Wadi Natron, Egypt	30°27′29″N–30°10′15″E	2003	*A. salina*	4947	5	1.01
El Max, Egypt	31°06′54″N–29°50′13″E	2010	–	3931	1	0.25
Walvis Bay, Namibia	23°00′17″S–14°25′37″E	1990	–	10 066	10	0.99
Bjurliu, Kazakhstan	51°49′00″N–78°00′00″E	1989	–	18 946	181	9.55
Aral Sea, Uzbekistan	44°43′41″N–59°34′22″E	2004	–	1497	3	2.00
Bagdad, Iraq*	33°17′06″N–44°15′13″E	2004	–	41 568	398	9.57
Urmia, Iran*	37°36′20″N–45°28′21″E	1988	*A. urmiana*	4619	78	16.89
Korangi Creek, Pakistan*	24°47′46″N–67°09′07″E	2005	–	8387	58	6.92
Madras, India	12°44′29″N–80°13′19″E	1993	–	3352	9	2.68
Aibi, Xinjiang, China*	44°53′05″N–82°53′55″E	1991	–	2207	19	8.61
Gahai, Qinghai, China	37°00′38″N–97°59′04″E	1991	–	1464	13	8.88
Dong Fang, Hainan, China	19°05′17″N–108°37′35″E	1992	–	8920	14	1.57
Tanggu, Tianjin, China	38°55′55″N–117°37′17″E	1989	*A. sinica*	1747	8	4.58
Luannan, Tianjin, China	39°06′03″N–118°25′55″E	2005	*A. sinica, A. franciscana*	3904	4	1.02
Dagang, Tianjin, China	38°48′50″N–117°32′44″E	2005	*A. sinica, A. franciscana*	8920	14	1.57
Xiaotan, Shandong, China*	36°07′38″N–120°04′36″E	1992	–	16 570	92	5.55
Yingkou, Liaoning, China	40°37′15″N–122°08′16″E	1989	–	5920	9	1.52
Lagkor Co, Tibet, China	32°01′30″N–84°10′46″E	2005	*A. tibetiana*	2232	8	3.58

*Males of these populations were used in the multivariate discriminant analysis.

encysted offspring used to obtain pure cultured laboratory parthenogenetic populations.

Culture conditions

Hatching was induced by incubating cyst samples under standard conditions, in 35 g L^{-1} sea water, at 28 °C, with continuous fluorescent lighting and gentle aeration (Vanhaecke & Sorgeloos, 1980). The resulting nauplii were mass-cultured in different volumes according to cyst availability and hatching efficiency. Mass cultures were usually kept in 60-L containers at 80 g L^{-1} brine salinity, at 20–24 °C, and fed *Dunaliella* sp. and *Tetraselmis* sp. (1 : 1) microalgae mixture every other day.

Sex ratio estimates and geographical patterns

Rare male frequencies were estimated for 54 laboratory populations of diploid parthenogenetic *Artemia* from a wide range of geographical locations (Table 1). Individuals were reared until maturity in mass cultures as detailed above and the sex ratio for each population (males per 1000 sexed individuals) were calculated as soon as most females showed signs of reproductive maturity (first ovulation or first offspring filling the ovisac), to minimize any possible effects of selective mortality. For sexing, animals were placed in Petri dishes with seawater and anaesthetized with a few drops of freshwater saturated with chloroform, and males carefully searched for with a binocular microscope.

To test whether there was a geographical pattern of distribution of the frequency of rare males, we carried out a spatial correlation of rare male frequencies using Moran's Index (Griffith, 1987). Given a set of locations and an associated variable, in this case rare male frequency, Moran's Index estimates whether the pattern is dispersed, random or clustered. For this purpose, we added the coordinates of each sampling site, confirmed in Google Earth, into spatial data using the ArcGIS package v. 10.0 (ESRI Inc., Redlands, CA, USA). In addition, to identify areas where the presence of rare males is highest, we looked for hot spots using the Gi* statistical test of Getis-Ord (Getis & Ord, 2010).

DNA barcoding

A 709-bp fragment of mitochondrial cytochrome *c* oxidase subunit I (COI) gene region was amplified and sequenced in 28 rare males from 14 diploid parthenogenetic *Artemia* populations across its distribution range. This same fragment was also sequenced in 12 females from 9 populations (Table 2) to confirm that these derived from parthenogenetic strains, instead of resulting from culture contamination by a sexual female. Total DNA was extracted from part of the antenna of ethanol-preserved adult males and from the first phyllopod for females, using the HotSHOT protocol optimized for zooplanktonic invertebrate organisms and their diapausing eggs (Montero-Pau *et al.*, 2008). We used the COI primers HCO2198 and LCOI490 (Folmer *et al.*, 1994). PCR was carried out in a total volume of 50 μL containing 5 μL of template DNA, 0.2 mM of each nucleotide, 0.2 μM of each primer, 0.05 U of *Taq* polymerase (Bioline) and 10 × Bioline buffer (with a $MgCl_2$ final concentration of 2 mM). The cycling profile consisted of one cycle of 3 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 20 s at 50 °C and 30 s at

Table 2 DNA barcoding of rare males of diploid parthenogenetic *Artemia*. Two males per population were sequenced for a fragment of COI. Individuals' codes as they appear in the phylogenetic tree and comparison of rare males sequences with the haplotypes of parthenogenetic females of the same population are presented.

Population	Rare male codes (GenBank Acc. Num)	Females codes (GenBank Acc. Num)	Comparison male-female haplotypes
Rocío, Cadiz, Spain	rmROC1,2 (KC193640-41)	Not done	–
La Mata, Alicante, Spain	rmMATA1,2 (KC193661-62)	MATA1 (KC193677)	5 bp difference
Notteri, Sardinia, Italy	rmNOT1,2 (KC193642-43)	Not done	–
Margherita di Savoia, Italy	rmMAR1,2 (KC193638-39)	APD02 (7)*	1 bp difference
Aigües Mortes, France	rmAIG1,2 (KC193646-47)	AIG1 (KC193670)	Same
Atanosovsko, Bulgaria	rmATA1,2 (KC193663-50)	APD02 (5)*, APD07 (1)*, ATA15 (KC193674)	Same
Koyashskoe, Ukraine	rmKOY1,2 (KC193648-49)	KOY1 (KC193667)	Same
Kujalnik, Ukraine	rmKUJ1,2 (KC193664-65)	APD04 (2)*	11 bp difference
Bagdad, Iraq	rmIRAQ1,2 (KC193651-52)	IRAQ2 (KC193666)	Same
Urmia Lake, Iran	rmURM1,2 (KC193653-54)	URM4 (KC193671)	Same
Korangi Creek, Pakistan	rmPAK1,2 (KC193659-60)	PAK2 (KC193669)	5 bp difference
Aibi Lake, Xinjiang, China	rmAIBI1,2 (KC193655-56)	AIBI 1,3,7 (KC193672-73-75)	2 bp difference with AIBI1
Xiaotan, Shandong, China	rmXIAO1,2 (KC193657-58)	Not done	–
Lagkor Co, Tibet, China	rmLAGK1,2 (KC193644-45)	LAGK1,4 (KC193668-76)	Same

*Sequences, haplotype names and number of individuals analysed from Muñoz *et al.* (2010).

72 °C, with a final step of 5 min at 72 °C. PCR products were purified and sequenced in both directions by Macrogen Inc. (Macrogen Europe, Amsterdam, the Netherlands, www.macrogen.com) using an ABI PRISM 3700 DNA analyser.

The electropherograms were checked with eye using CodonCode Aligner v. 3.5 (CodonCode Corporation, Dedham, MA, USA). Sequences obtained here were aligned with published sequences from the same COI fragment from diploid parthenogenetic *Artemia* populations (DQ426824–DQ426826, GU591380–GU591384) and Central Asian sexual species *A. urmiana* (DQ119651), *A. sinica* (DQ119650), *A. tibetiana* (EF615588) and *Artemia* sp. from Kazakhstan (DQ119653, GU591385-GU591389), from GenBank, using Clustal in MEGA5 (Tamura *et al.*, 2011). We used *A. franciscana* (DQ119645) and *A. sinica* (DQ119650) as outgroups. Phylogenetic reconstructions were carried out using MEGA5. The neighbour-joining (NJ) tree was reconstructed using evolutionary distances computed with the maximum composite likelihood method. The maximum likelihood (ML) tree was obtained using a GTR plus gamma model. The robustness of the branches was assessed with 1000 bootstrap pseudo-replicates. All sequences generated here were deposited in GenBank (Accession Numbers: KC193638–KC193677).

Morphometry

Reproductively mature males were characterized according to specific morphological traits following standard procedures (Hontoria & Amat, 1992) for a total of 11 parthenogenetic populations where 30 rare males were available (see Table 1). For this procedure, males were anaesthetized as described above and measured under a dissecting microscope. The following 12 morphometric characters were measured: total length, abdominal length, abdominal width, head width, distance between the compound eyes, eye diameter, length of the first antenna, furca length, number of setae on the left branch of the furca, number of setae on the right branch of the furca, ratio of abdominal length to total length (×100) and width of the genital segment. Morphometric data of males from the Asian sexual species were taken from the database of the Instituto de Acuicultura de Torre de la Sal (Amat *et al.*, 1994) including two *A. urmiana* (Urmia and Koyashskoe), one *Artemia* sp. from Kazakhstan, three *A. sinica* (Tanggu, Yuncheng and Tonkhil) (Abatzopoulos *et al.*, 2009) and four *A. tibetiana* (Lagkor Co, Hayan, Gaize, Jingyu) (Van Stappen *et al.*, 2003). The full data matrix was subjected to multivariate discriminant analysis (Hontoria & Amat, 1992) using SPSS v. 15.0 (SPSS Inc., Chicago, IL, USA). The morphological variables mentioned above were used to establish relationships among the populations (Anderson, 1984) setting the geographical origin of the cyst samples as the separation criterion.

Mating experiments

Mating experiments between rare males and females of Asian sexual populations were set up to obtain successful fertilization as evidenced by production of live viable or encysted offspring. The diploid parthenogenetic population from Bagdad (Iraq) was chosen as a source of males due to its high incidence of rare males and good cyst availability. Females used were chosen from sexual Asian populations, *A. urmiana* from Koyashskoe lake (Ukraine), *A. sinica* from Yuncheng lake (China), *A. tibetiana* from Lagkor Co lake (Tibet) and *Artemia* sp. from Kazakhstan (*Artemia* Reference Center code – ARC1039, unknown locality). Females used were either virgin (paired when still sexually immature) or kept isolated during the 2 weeks prior to the experiments to ensure that they had not been inseminated. Sperm storage does not occur in *Artemia,* and each copulation fertilizes the eggs present in the brood pouch (Bowen, 1962; M. Maccari & F. Amat, unpublished results). Isolated size-matched male–female single pairs were kept in small beakers (60 mL) under the culture conditions described above. Quantitative and qualitative reproductive outputs of each pair were monitored every other day during culture medium renewal. The total number of fertilized and unfertilized eggs produced per female in each mating experiment was recorded. Offspring quality was also characterized using the number of live and dead nauplii, as well as the number of abortive embryos (pale yellow-orange colour eggs) in ovoviviparous offspring. The number of normally shelled dormant cysts (pale grainy surface floating in 200 g L^{-1} brine), as opposed to abortive, abnormally shelled embryos (bright brown colour cyst not floating in 200 g L^{-1} brine), in oviparous offspring was also monitored. Mating experiments between sexual males and their conspecific females following the same procedure as above were used as controls.

We tested whether the means of the proportion of fertilized and unfertilized eggs and the means of the proportion of offspring quality variables per female were the same in the crosses involving rare males and in the corresponding controls. If the data were normal and homoscedastic, we used *t*-tests, otherwise Mann–Whitney tests were conducted. Statistical analyses were performed with SPSS v. 15.0.

Microsatellite analysis of hybrid F1 offspring

To obtain evidence of rare males' functionality regarding their ability to transmit genetic material to their offspring, we screened three microsatellite loci in the rare males, in the sexual females used in the crosses and in their F1 offspring. DNA extractions were obtained as described above. Each microsatellite locus (Apdq02-TAIL, Apdq03TAIL and Apd05TAIL) (Muñoz *et al.*, 2009) was amplified separately in PCRs performed in a total volume of 20 μL containing 2 μL of template

DNA, 10 μL of 2× QIAGEN® (Qiagen, Hilden, Germany) PCR Master Mix (including 3 mM $MgCl_2$, dNTP Mix and HotStarTaq® Polymerase; Qiagen), 2 μL of 10× Primer Mix (2 μM each primer) and 2 μL of Q solution (QIAGEN). The 5′ end of each reverse primer was labelled with a fluorescent dye (Apdq02TAIL, Apd05TAIL with Cy5 and Apdq03TAIL with Cy5.5, MWG Biotech, Eurofins MWG Operon, Ebersberg, Germany). The following PCR programme was used: 95 °C for 15 min, 35 cycles of 94 °C for 30 s, 53 °C for 90 s, 72 °C for 90 s, followed by 60 °C for 10 min. Diluted PCR products (1 : 20) were combined with a 400-bp size standard and separated on a Beckman-Coulter CEQ™ 8000 analysis system. Alleles were scored using the CEQ Fragment Analysis software (Beckman Coulter™, Fullerton, CA, USA) and checked manually.

Results

Rare male frequency and geographical patterns

In total, 415 666 diploid parthenogenetic *Artemia* specimens were sexed in this experiment (see Table 1 for male ratio and population details). The number of specimens sexed for each diploid parthenogenetic population varied depending on its cyst availability, cyst hatching efficiency and nauplii survival rate to maturity and ranged from 348 individuals for Salin de Giraud (France) to 41 568 individuals for Bagdad (Iraq). The presence of rare males was verified in 50 of the 54 populations sampled. Janubio and El Rio (Lanzarote) and Tenefé (Gran Canaria) in the Canary Islands and Hortales (Cádiz) in Spain were the only populations where the presence of rare males could not be confirmed.

The spatial autocorrelation analysis was not significant (Moran's Index, 0.10; *z*-score, 0.50; *P*-value: 0.61), indicating that the distribution of the male ratio does not appear to be significantly different than random. Despite that, we found the highest ratios – reaching or surpassing 1% of rare males – in the Central Asian populations: Bagdad saltern (Iraq), Urmia Lake (Iran), Bjurliu Lake (Kazakhstan) and Aibi and Gahai Lakes (Inner China); and the lower ratios in the western, eastern and southern populations (Iberian Peninsula, China, India and Africa). This was confirmed by the Gi* test, which indicated that there are three statistically

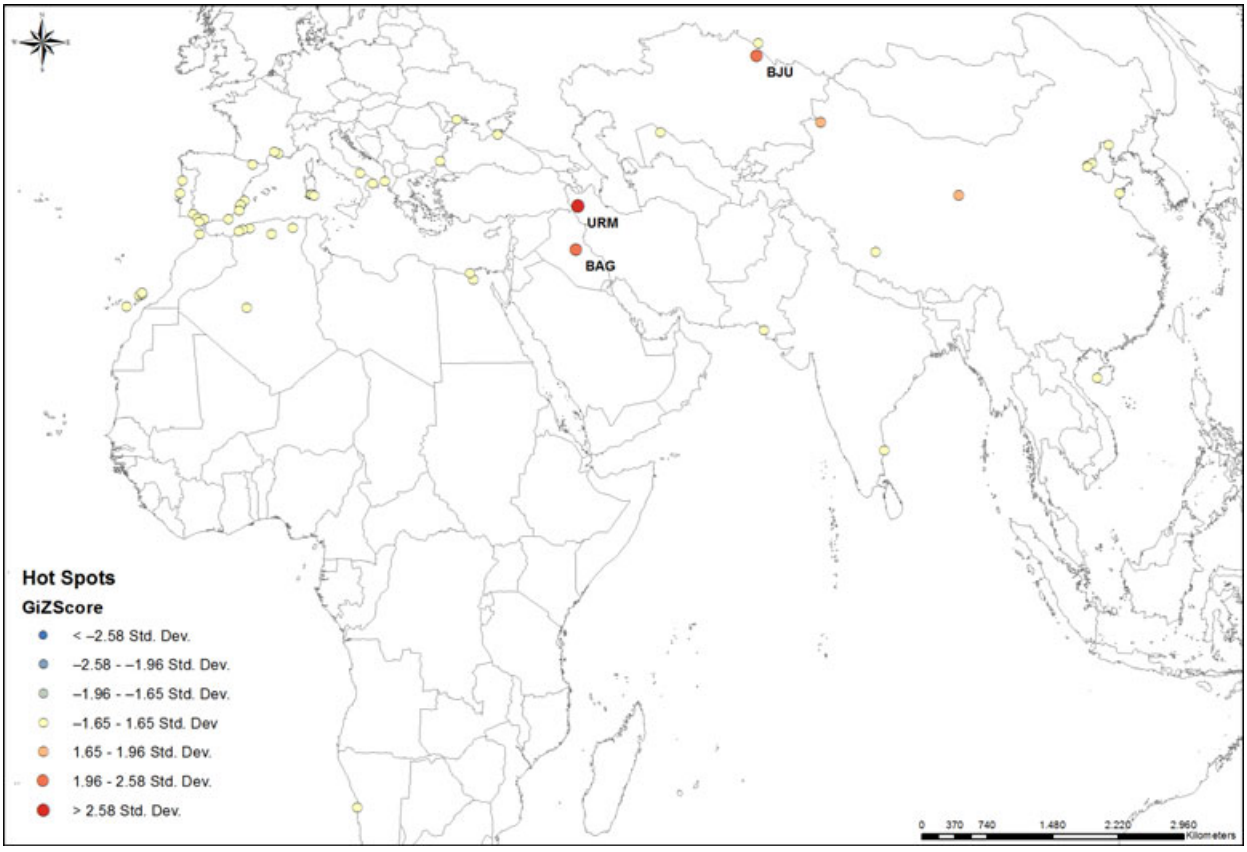

Fig. 1 Geographical distribution of diploid parthenogenetic *Artemia* sampling sites. The colour codes represent the GIZ score values obtained with the Gi* test, which are related with the frequency of rare males. GIZ score values >1.96 indicate high statistically significant clustering value of rare male ratio ($P \leq 0.05$).

significant male ratio hot spots, Urmia Lake, Bagdad Saltern and Bjurliu Lake (Fig. 1), where a hot spot is a population with a high male ratio surrounded by other populations with high male ratio.

DNA barcoding

Cytochrome *c* oxidase subunit I sequences from 28 rare males from 14 populations (two individuals for each one) and 12 parthenogenetic females from nine populations were obtained (Table 2). After trimming, collapsing identical haplotypes for each population, and adding sequences from GenBank, the alignment had a length of 617 bp and comprised 47 sequences including outgroups. No insertions, deletions or stop codons were present. There was a total of 161 variable sites, 63 of them parsimony informative. Rare male sequences collapsed into eight haplotypes. NJ and ML phylogenetic reconstructions had a virtually identical topology and branch support. The most widespread haplotype in rare males, found in 15 rare males from eight populations, was identical to APD02, the most common haplotype in Mediterranean diploid parthenogenetic *Artemia*, and was closely related to haplotypes in sexual *Artemia* sp. from Kazakhstan (Muñoz *et al.*, 2010; Fig. 2). The remaining seven haplotypes were found in single diploid parthenogenetic populations. Four of these haplotypes (rmMAR1-2, rmAIBI1-2, rmXIAO1-2 and rmPAK1-2) were closely related to APD02 and differed from it by 1, 2, 5 and 5 substitutions, respectively. Two haplotypes (rmATA1 and rmMATA1-2) were identical or closely related to haplotypes previously found in the diploid parthenogenetic population of Atanosovsko (APD07), which are closely related to the *A. urmiana* haplotype. The last haplotype, rmKUJ1-2, was very divergent, forming a sister branch to the remaining parthenogenetic sequences and differing in 10 and 8 substitutions from the APD02 haplotype and from the *A. urmiana* reference sequence, respectively.

Rare male mtDNA haplotypes in 6 of the 14 populations were identical to those found in parthenogenetic females from the same population (see Table 2 for details). In Margherita di Savoia and Aibi Lake, the rare male haplotype differed in 1 or 2 bp, respectively, from haplotypes in parthenogenetic females from the same population, whereas in Korangi Creek and La Mata, rare male haplotypes differed from the common haplotypes in females from these populations by 5 bp. Although female haplotypes from Rocio and Notteri were not available, rare males displayed the common APD02 haplotype. Sequences from females of Xiaotan were not available and the haplotypes obtained in the rare males from this population had never been reported before, although they differed in 5 bp from APD02. The rare males from Kujalnik differed from the two available sequences from the same population in 11 bp, and this haplotype has not been reported before.

Rare male morphometry

The morphometric multivariate analysis produced twelve discriminant functions. When they were included in the model, all except the last function significantly ($P \leq 0.05$) accounted for the variance with the first five discriminant functions accounting for 88.9% of the variation. The ratio of abdominal length to total length, and the length of the furca were highly correlated with the first discriminant function, and the length of the first antenna and the total length made the highest contributions to the second function. Data of the mean values of the morphological traits measured for each population are available upon request.

Discriminant analysis separated morphometrically the males belonging to sexual species *A. urmiana* and *A. tibetiana* from the rest (Fig. 3). The morphometry of the parthenogenetic males was very variable, and their population centroids were located within the limits of the sexual populations. However, most rare males were morphologically closer to the males from *A. sinica* and *Artemia* sp. from Kazakhstan. No obvious association between the haplotype group that the parthenogenetic rare male mtDNA belonged to and their morphological resemblance to either *A. urmiana* or *Artemia* sp. from Kazakhstan was found. For example, rare males from Atanosovsko or La Mata have haplotypes very similar to those of *A. urmiana* from Koyashskoe, but they do not appear morphologically closer to males of this sexual species.

Mating experiments

A total of 30 mating pairs were set up for each combination of sexual species with rare males, and between females of each sexual species with their conspecific males (controls). As some individuals died before mating, the final number of experimental pairs was between 8 and 25 per mating experiment (Table 3). Rare males were observed clasping and copulating with the sexual females of all species tested during the mating trials. Mating trials resulted in a total of 220 fertile hybrid broods and in 558 conspecific broods (controls). The proportion of fertilized eggs was always high (over 70%), and it was slightly higher in two of the four hybrid crosses (rare male × *A. urmiana* and rare male × *A. tibetiana*) than its corresponding controls, but in any case, there were no statistically significant differences between rare male crosses and controls (Table 3).

Crosses involving rare males resulted in viable ovoviviparous and oviparous hybrid offspring (Fig. 4). Remarkably, all interspecific crosses between Central Asian sexual females and rare males had a similar or higher F1 offspring quality than controls (intraspecific

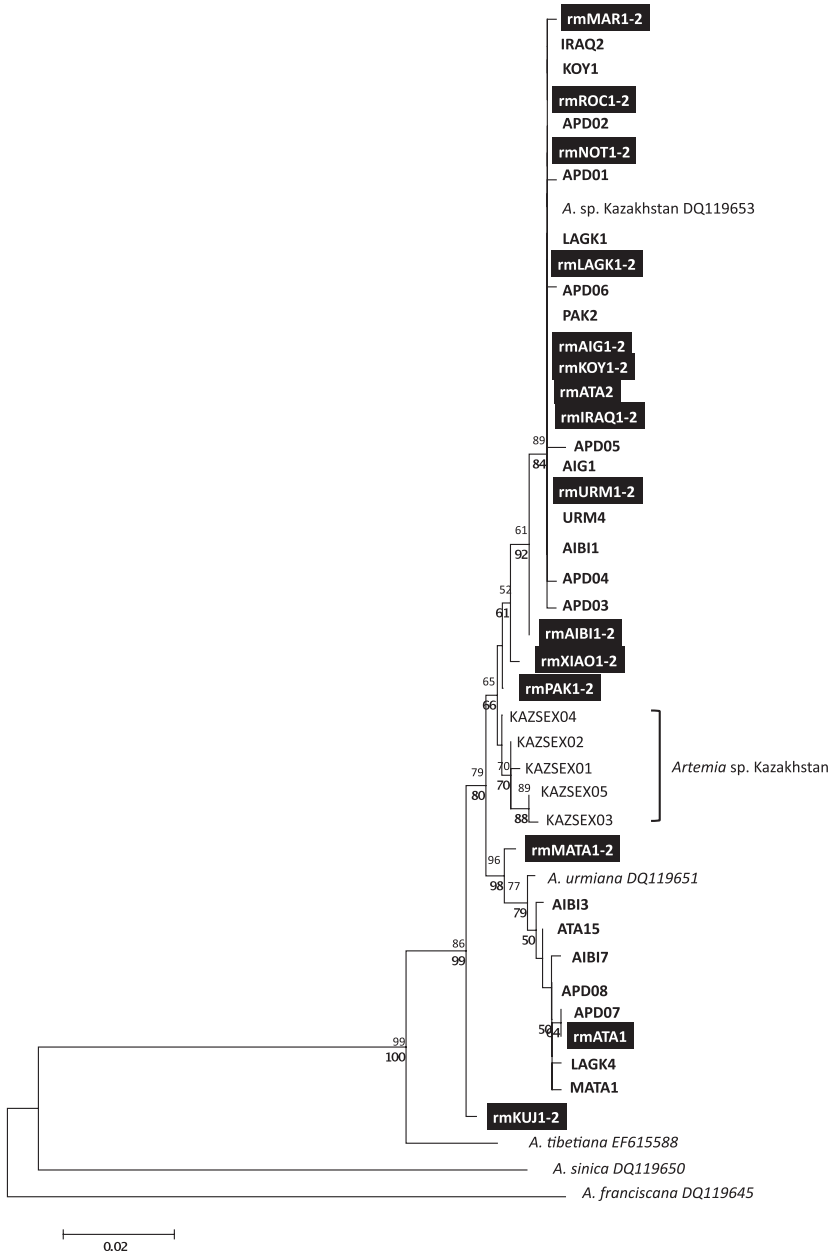

Fig. 2 Phylogenetic relationships of diploid parthenogenetic *Artemia* rare male mtDNA haplotypes (which are noted by the code "rm" followed by the population code), diploid parthenogenetic female haplotypes (in bold) and Central Asian species based on COI sequences. The neighbour-joining (NJ) topology is shown with NJ bootstrap values above the branches and maximum likelihood values under the branches.

sexual crosses). There were no statistically significant differences between rare male crosses and controls for most of the features analysed in both in ovoviviparous and oviparous quality traits. The only significant differences occurred in the proportion of dead nauplii obtained from ovoviviparous offspring from the crosses between rare males and *A. urmiana* or *A. sinica* females, which were higher in the controls (Fig. 4 and Table S1).

Microsatellite analysis

Microsatellite scoring showed that diploid parthenogenetic *Artemia* rare males underwent meiotic reduction and successfully fertilized sexual Central Asian *Artemia* females, transferring their alleles to the F1 progeny, and producing diploid hybrid offspring as a result (Table 4). Most males were heterozygotes for all loci (with the exception of male Iraq8 for locus Apd05). In those cases where the male was heterozygous, only one of the alleles was transmitted to each offspring, indicating that rare males produced haploid sperm through meiosis. No evidence for triploid offspring was found. In all the crosses performed, we found evidence of null alleles in the mother for one or more of the analysed loci. In these cases, the allele or alleles present in the father were found in the F1 offspring, demonstrating

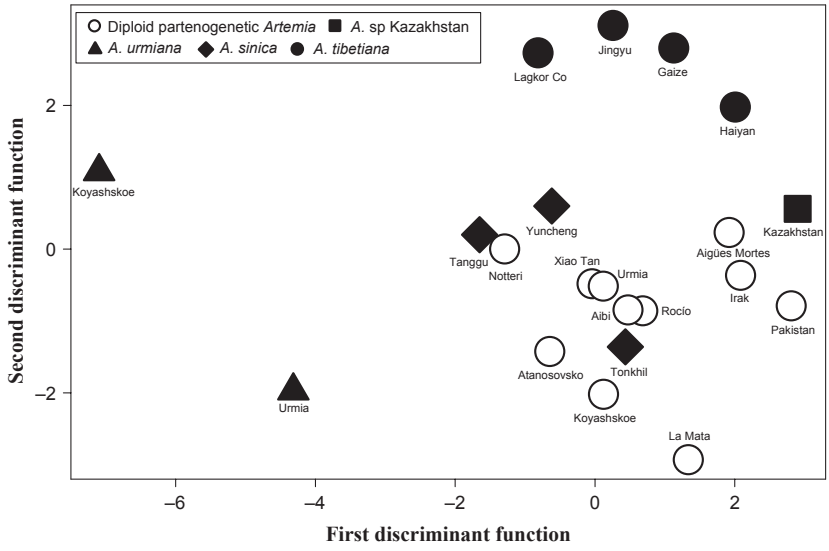

Fig. 3 Multivariate discriminant analysis of *Artemia* rare males morphometric traits. Mean values of each *Artemia* population solved for the two first discriminant functions (centroids).

Table 3 Egg fertilization in cross-mating experiments involving diploid parthenogenetic *Artemia* rare males and females of Central Asian sexual species and in conspecific matings used as controls (Mann–Whitney *U*-test because normality tests failed in all cases).

Cross	Pairs	Broods	Fertilized eggs (%)	*P*-value
rare male × *A. urmiana*	18	58	77.99	1.000
A. urmiana	13	72	76.93	
rare male × *Kazakhstan* sp.	15	61	90.39	0.472
Kazakhstan sp.	25	179	96.37	
rare male × *A. sinica*	25	102	89.54	0.436
A. sinica	25	246	90.99	
rare male × *A. tibetiana*	18	40	94.03	0.102
A. tibetiana	8	17	90.72	

that the father had transmitted the amplifiable copy to the offspring.

In the two crosses between a rare male and a female from *A. urmiana*, the mother amplified a single allele at Apd03 and Apd05, and for Apd02, the mother was heterozygous in the first cross and only amplified a single allele in the second, whereas the father was heterozygous at all three loci. All F1 hybrid offspring of both crosses amplified one paternal allele, whereas they either amplified one maternal allele or showed evidence of a null allele inherited from her.

In the cross between a rare male and a female from *Artemia* sp. from Kazakhstan, the mother was heterozygous at Apd03 and homozygous at Apd02 and failed to amplify, probably due to null alleles at loci Apd05. The male was heterozygous at Apd02 and Apd03 and homozygous at Apd05. All alleles present at the three loci in the father were detected in the five hybrid offspring screened.

In the crosses between rare males and *A. sinica* females, none of the three microsatellite loci tested amplified successfully in *A. sinica*. Despite this, in all hybrids, progeny produced one of the paternal alleles amplified. The lack of amplification of these three microsatellite loci in *A. sinica* was confirmed by checking additional individuals from this species. Microsatellite scoring in crosses between rare males and *A. tibetiana* females was problematic in both parents and the resulting hybrid offspring, and therefore, paternity analysis was not carried out.

Discussion

The presence of fertile males in otherwise parthenogenetic lineages raises questions about their potential role in genetic exchange with sexual species and in generating new parthenogenetic lineages. Here, we have described the presence, frequency, functionality and reproductive potential of parthenogenetically produced rare males in the genus *Artemia*.

Our results indicate that most diploid parthenogenetic *Artemia* populations produce males sporadically with a frequency up to 17 per 1000 individuals. Statistical analysis showed three statistically significant male ratio hot spots, Urmia Lake, Bagdad saltern and Bjurliu Lake. Populations showing a higher ability to produce rare males are therefore found in a geographical region around 40°N between the Mediterranean–Caspian basin and the salt lakes region in Kazakhstan, a region where the coexistence with closely related sexual species is more likely. Phylogenetic and phylogeographical

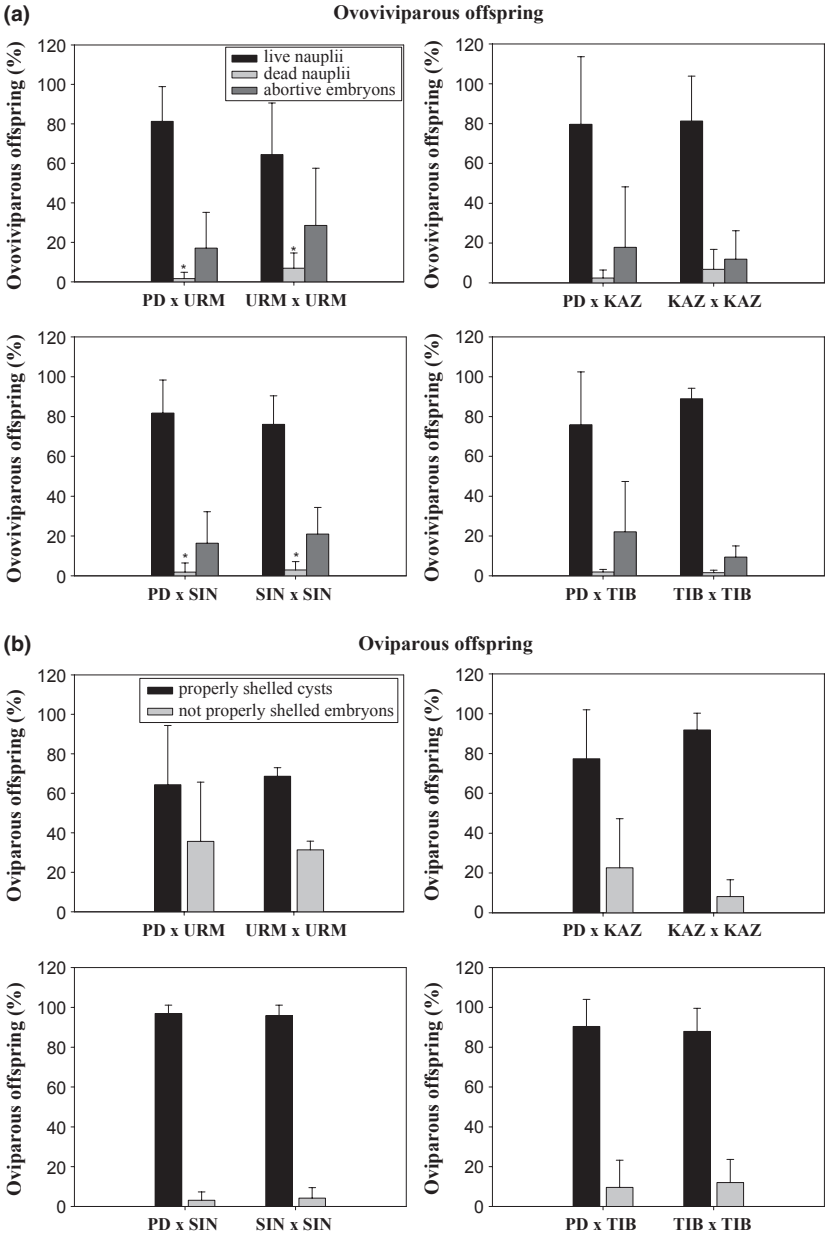

Fig. 4 Offspring quality in cross-breeding experiments in ovoviviparous (a) and oviparous broods (b) between *Artemia urmiana* (URM), *Artemia sinica* (SIN), *Artemia tibetiana* (TIB), *Kazakhstan* sp. (KAZ) and diploid parthenogenetic *Artemia* rare males (PD) (hybrid crosses) and in conspecific crosses (controls). Error bars are standard deviations. Asterisks ($P \leq 0.05$) indicate significant differences for each quality trait between hybrid and control offspring (*t*-test when normality and equal variance tests were not significant, otherwise Mann–Whitney test was employed).

analyses suggest that diploid parthenogenetic lineages may be evolutionarily recent (Holocene), having arisen in a region of Central Asia around Iran and Kazakhstan and subsequently expanded towards the Mediterranean and other regions (Muñoz *et al.*, 2010). Our results indicate that male production is a general feature in diploid parthenogenetic *Artemia* with the possible exception of the most western populations.

Similarly to the pattern found in the obligate parthenogenetic *Daphnia pulex* (Innes & Hebert, 1988) where some clones have the ability to produce males, whereas others have lost it, there is also intrapopulational

Table 4 Microsatellite paternity analysis for crosses between diploid parthenogenetic *Artemia* rare males and Central Asian sexual females. Results of screening females, males and F1 offspring for three microsatellite loci (allele sizes in base pairs are shown). Alleles present in the rare male father and not in the mother are shown in bold in the father and in the F1 offspring. The presence of presumably null alleles (no amplification could be obtained, or evidence of no amplification of maternal alleles in the offspring) is noted by Ø. Rare males belonged to the Iraq population. One individual F1-16-6, amplified weakly, and no amplification could be obtained for locus Apd03 (n.a.).

Cross	Individual code	Apd02	Apd03	Apd05
rare male ×	F0 (F-Koy 15)	233-281	207-Ø	170-Ø
A. urmiana	F0 (M-Iraq 15)	**254-**233	**216-231**	**115-185**
	F1-15-1	233-**254**	207-**216**	**185**-Ø
	F1-15-2	233-233	207-**231**	**115**-170
	F1-15-3	233-**254**	**231**-Ø	**185**-Ø
	F1-15-4	233-281	**216**-Ø	**115**-170
	F1-15-5	233-281	207-**231**	**115**-Ø
	F1-15-6	233-281	207-**216**	170-**185**
rare male ×	F0 (F-Koy 16)	248-Ø	208-Ø	90-90
A. urmiana	F0 (M-Iraq 16)	**233-251**	**216-230**	**117-189**
	F1-16-1	248-**251**	208-**216**	90-**189**
	F1-16-2	248-**251**	208-**230**	90-**189**
	F1-16-3	**233**-Ø	**216**-Ø	90-**189**
	F1-16-4	**233**-Ø	**216**-Ø	90-**189**
	F1-16-5	248-**251**	**230**-Ø	90-**189**
	F1-16-6	248-**251**	n.a	90-**117**
rare male ×	F0 (F-Kaz 8)	233-233	213-245	Ø-Ø.
Artemia sp.	F0 (M-Iraq 8)	233-**242**	**208-231**	**115**-Ø
Kazakhstan	F1-8-1	233-233	**208**-213	**115**-Ø
	F1-8-2	233-233	**208**-245	**115**-Ø
	F1-8-3	233-**242**	**231**-245	**115**-Ø
	F1-8-4	233-233	**208**-213	**115**-Ø
	F1-8-5	233-**242**	**208**-245	Ø-Ø
	F1-8-6	233-233	**231**-245	**115**-Ø
Rare male ×	F0 (F-sin 7)	Ø-Ø	Ø-Ø	Ø-Ø
A. sinica	F0 (M-Iraq 7)	**233-254**	**216-231**	**115-180**
	F1-7-1	**233**-Ø	**216**-Ø	**115**-Ø
	F1-7-2	**254**-Ø	**231**-Ø	**180**-Ø
	F1-7-3	**254**-Ø	**216**-Ø	**115**-Ø
	F1-7-4	**254**-Ø	**231**-Ø	**115**-Ø
	F1-7-5	**254**-Ø	**231**-Ø	**180**-Ø

variation in the tendency to generate rare males in diploid parthenogenetic *Artemia*, which differs between clonal lineages from 0.12% to 0.60% in a population in Salin de Giraud (France) (MacDonald & Browne, 1987), which could explain our results. However, the role of genetic vs. environmental effects in the ability of diploid parthenogenetic *Artemia* to produce rare males should be the focus of further studies.

DNA barcoding confirmed the identity of the rare males produced by diploid parthenogenetic *Artemia* populations. The haplotypes of most of the rare males analysed were identical to those of diploid parthenogenetic *Artemia* females. COI haplotypes of rare males form two main mtDNA clades, the more widespread one is closely related to the sexually reproducing *Artemia* sp. from Kazakhstan that is awaiting formal description, and the second one is found only in four diploid parthenogenetic populations and is more closely related to *A. urmiana*. These results agree with previous studies of phylogenetic relationships of diploid parthenogenetic populations, indicating close phylogenetic relationships between diploid parthenogenetic *Artemia* and both *A. urmiana* and *Artemia* sp. from Kazakhstan (Baxevanis *et al.*, 2006; Muñoz *et al.*, 2010; Maniatsi *et al.*, 2011). The haplotypes of some rare males, although related to haplotypes in rare males of other parthenogenetic populations, differed from the common haplotypes in females sequenced from their own population. The intrapopulation variability in the propensity to generate males reported in *Artemia* (MacDonald & Browne, 1987) mentioned above may explain this discrepancy between the haplotypes of rare males and the common haplotypes in the females of their populations, as this would be expected if, by chance, rarer lineages in the population (bearing rarer mtDNA haplotypes) had a higher propensity to produce males. In addition, as we had no available sequences from Xiaotan population females to compare with their divergent rare male haplotypes, further analyses are needed to understand the genetic diversity held by parthenogenetic *Artemia* populations, as these haplotypes had never been reported before. Overall however, it is clear that in most populations, rare males have the same haplotype as the parthenogenetic females from their populations, and these haplotypes were identical, or closely related, to haplotypes previously found in diploid parthenogenetic lineages.

Discriminant analysis proved to be a useful tool to separate *Artemia* rare males into different morphological clusters. Rare males differed morphologically from both *A. urmiana* and *A. tibetiana* males, whereas they were more similar to males from Kazakhstan *Artemia* sp. and from *A. sinica*. In a previous analysis (Triantaphyllidis *et al.*, 1997), the morphology in *Artemia* was studied through a discriminant analysis, but the sexual and the parthenogenetic populations were analysed separately and parthenogenetic males were not included in the analysis. In that work, the sexual population from Kazakhstan appears morphologically close to *A. sinica*, but it is considered a different species (Triantaphyllidis *et al.*, 1997). Possibly, rare males show higher morphological variability than the males from the Asian sexual species, because similar results are obtained when parthenogenetic females were compared with the sexual females (Mura *et al.*, 2006; Amat *et al.*, 2007). This could be explained by the heterogeneous geographical origin of parthenogenetic lineages (from Portugal to the Chinese coast) and the inability for them to interbreed.

The results of cross-mating experiments were used to evaluate the fertility and the reproductive potential of rare males. There are different kinds of isolating mecha-

nisms which determine the degree of divergence among populations: (i) inability of the two populations to live in the same medium (habitat isolation); (ii) failure of the male to clasp the female (ethological isolation); (iii) failure to produce a viable F1 (mechanical isolation, gametic or zygote mortality or hybrid inviability); and (iv) hybrid sterility (absence of an F2 or production of a deficient F2) (Mayr, 1963). Our findings show that rare males from obligate parthenogenetic diploid *A. parthenogenetica* populations (i) often coexist in the same habitat as sexual Asian species and (ii) show normal pairing behaviour with central Asia sexual females, excluding the first two isolating mechanisms described above. We also showed that (iii) rare males are fully functional and capable of fertilizing eggs from females of sexual Asian species, and hybrid crosses resulted in similar or higher offspring viability than the controls, in both ovoviviparous and oviparous broods. We (iv) obtained live nauplii from ovoviviparous F1 hybrid broods, which, upon culture, were morphologically normal and produced viable hybrid sexual populations (unpublished results).

The paternity analysis using microsatellite markers further shows that rare males from a parthenogenetic population undergo normal meiosis, produce viable haploid sperm and contribute to the genetic material of the hybrid offspring when mated with females from three of four sexual Asian *Artemia* species (*A. urmiana, Artemia* sp. from Kazakhstan and *A. sinica*). Given that this set of microsatellite loci were developed initially for diploid parthenogenetic *Artemia* (Muñoz *et al.*, 2008, 2009), it is not surprising that we found evidence of null alleles in some mothers for some loci, whereas the fathers (rare males of the diploid parthenogenetic lineage) amplified well and show a high degree of heterozygosis. Despite the fact that this set of microsatellites failed to amplify in *A. sinica* females, the cross gave informative results because the F1 offspring obtained when mating rare males with *A. sinica* inherited one paternal allele.

In an early pioneering work, Bowen *et al.* (1978) obtained four rare males – which they called exceptional males – from three diploid parthenogenetic *Artemia* populations. They documented a transfer of genes from a Yamaguchi (Japan) parthenogenetic population rare male to an *A. urmiana* female by polymorphism of three genetic markers (one haemoglobin and two esterase isozymes). They also obtained viable offspring mating a rare male from a Madras (India) parthenogenetic population with an *A. franciscana* female and documented transfer of genes from this male to the hybrid offspring. However, and in agreement with previous results (MacDonald & Browne, 1987), we have been unable to obtain viable offspring when mating *A. franciscana* females with rare males (unpublished results). Our study has considerably extended these early experiments, as we have produced more than 250 hybrid broods between rare males and Central Asian sexual females.

Artemia is one of the few known examples of parthenogenetic animal species that produce functional males. These rare males can successfully mate with congeneric sexual females, transmitting their genes to their diploid highly viable F1 offspring. Such ability makes the brine shrimp an exceptional model system to study the evolutionary process and to investigate the potential of these rare asexual males in generating new parthenogenetic lineages. In the absence of available coexisting sexual relatives, parthenogenetic lineages producing rare males or investing in male function incur a fitness cost compared with parthenogenetic lineages not producing such males (D'Souza & Michiels, 2010; Neiman *et al.*, 2012). Although the costs of producing rare males might be regarded as very low, the highly competitive conditions in *Artemia* populations, where rapid reproduction and resource limitation can be important, make it possible that this ability has persisted due to compensating direct or indirect benefits to the parthenogenetic lineage. An indirect benefit can be obtained if male production is linked to an advantageous trait, for example if males were the product of sex chromosome recombination during automixis, and parthenogenetic strains producing more males were benefiting from increased recombination rates generating more diverse offspring or purging deleterious alleles. As our results suggest, in the presence of potential partners such as sexual females of related species, rare male production could also obtain direct benefits as such rare males can produce fertile hybrid offspring as a result of mating with sexual females. In addition, these *Artemia* diploid parthenogenetic males might be able to transmit the parthenogenesis trait to their offspring (Lynch, 1984; Eads *et al.*, 2012), a topic that will be the subject of a future study. Alternatively, rare male production might persist in populations due to genetic drift, as genetic bottlenecks are likely to occur during colonization and migration between habitats, is likely to be constrained by habitat monopolization (De Meester *et al.*, 2002; Muñoz *et al.*, 2008, 2009). More research is needed into the cytological mechanisms behind rare male production, to understand the genetic basis of the variation in male production rates among and within populations and potential interactions between genetic and environmental effects into rare male production.

The occurrence and potential reproductive role of parthenogenetic *Artemia* rare males led MacDonald & Browne (1987) and Browne & Bowen (1991) to suggest that cross-fertilizations of sexual females by parthenogenetic males could provide a source of gene flow between the different genotypes. Further, Abreu-Grobois & Beardmore (1982) suggested that fertilization by rare males might result in the generation of polyploid parthenogenetic *Artemia* lineages. Recent mitochondrial DNA and microsatellite analysis of polyploid

parthenogenetic *Artemia* strains (Maniatsi *et al.*, 2011) suggests that triploid strains might have originated by fertilization of an unreduced ovum by a parthenogenetic rare male. Further research is needed to fully understand the evolutionary role of rare males into the origin of polyploid parthenogenetic *Artemia*.

Our work demonstrates the functionality of rare males and, given that co-occurrence between these rare males and sexual species is common in Central Asia, suggests an evolutionary role for males of parthenogenetic origin through hybridization and genetic exchange between parthenogenetic and sexual *Artemia* lineages through hybridization via rare males.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Results of statistical tests on proportions of offspring quality in cross-breeding experiments in ovoviviparous and oviparous broods between *Artemia urmiana* (URM), *Artemia sinica* (SIN), *Artemia tibetiana* (TIB), *Kazakhstan* sp. (KAZ) and diploid parthenogenetic *Artemia* rare males (PD) (hybrid crosses) and in conspecific crosses (controls).

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