Origin and evolution of *Artemia* reproductive and genetic diversity

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Origin and evolution of *Artemia* reproductive and genetic diversity

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Part I

GENERAL INTRODUCTION

1.- REPRODUCTIVE SYSTEMS. SEX V.S. ASEX

The majority of eukaryotic species have adopted two main reproductive strategies: sexual reproduction and asexuality. Sexual reproduction, which predominates in most living organisms (Bell, 1982; De Meeûs et al., 2007; Schurko et al., 2009), is a process involving the fusion (fertilization) of two specialized reproductive cells called gametes, one from a male source and one from a female source. Both male and female gametes are produced by a special cell division process known as meiosis, which halves the number of chromosomes in each resulting sex cell. Fertilization may occur between gametes produced by a single hermaphrodite individual (selfing) or, in most cases, between gametes formed by different female and male individuals. So, in a sexual life cycle different stages alternate: diploid cellular life, meiosis, haploid cellular life, and fertilization. Meiosis and fertilization occur regularly in life cycles (Normarck et al., 2003).

Asexuality is a less widespread strategy but it encompasses a variety of reproductive mechanisms (Schön et al., 2009). The term asexual reproduction *sensu strictu* implies the abolishment of sexes. In this case, it is considered synonymous to clonal reproduction or agametic reproduction. It occurs when an individual produces new individuals that are genetically identical to the parent at all loci in the genome, except at those sites that have experienced somatic mutations. This is, for example, the case of the *fragmentation* in colonial organism as reef-building corals and sponges and the case of the *fission* in unisexual organisms as echinoderms, turbellarian flatworms, and some polychaete and oligochaete annelid worms. Another case of clonal reproduction is

represented by *budding*, which concerns the production of new individuals from small parts of the parent without the division of the parent individual. It is common in cnidarian (jellyfishes, hydras, corals and sea anemones), phoronids (horseshoe worms), entoprocts (goblet worms), urochordates (sea squirts) and trematodes (flukes) (De Meeûs et al., 2007). But in animal biology, we refer to asexual organisms as all those that have all dropped out of the regular meiotic (sexual) cycle (Schön et al., 2009). So, asexual reproduction regroups other types of reproduction that are not all cases of clonal reproduction and in which gametes cell are involved. This is the case of parthenogenesis, gynogenesis and hybridogenesis (Simon et al., 2003; De Meeûs et al., 2007).

1.1.- Parthenogenesis, hybridogenesis, gynogenesis

Parthenogenesis refers to the development of eggs without fertilization. An unfertilized female gamete develops into a new organism (typically female, thelytokous parthenogenesis) without the need of male gamete. Modes of parthenogenetic reproduction fall into two main categories: apomixis or automixis, based on the presence or absence of meiosis. It will be explained it in more details later (Simon et al., 2003; Schlupp, 2005).

Instead, gynogenesis (also called pseudogamy) is a form of reproduction in which fertilized eggs are replaced by diploid cells from the mother. Offspring are produced from diploid oocytes that do not undergo meiosis and male haploid sperm of a related bisexual species is needed only to trigger embryo development (Simon et al., 2003; Schlupp, 2005).

In hybridogenesis, fertilization takes place and the offspring shows characters of both parents. It is a hemiclonal mode of reproduction because half genome (paternal) is transmitted sexually and the other half (maternal genome) is transmitted clonally. Sperm and egg fuse and paternal genes are expressed in the offspring but only the maternal genome is inherited. Hybrid condition is restored at each generation by mating with males of the parental species whose genome has been discarded from the egg (Simon et al., 2003; Schlupp, 2005).

1.2.- The paradox of sex

Despite sex being the predominant mode of reproduction among eukaryotes, it has been described as a paradox because it faces substantial and immediate costs compared to asexual alternatives (Maynard Smith, 1971, 1978; Williams, 1975). First of all, diploid anisogamous species with an even sex ratio pay the cost of males. Sexual females have a reduced reproductive potential because half of their eggs develop into male offspring. Two sexes are needed to restore the parental diploid state. So sexual females have to produce males, find or attract males and mate with them, what entails additional time and energy resources and all the risks associated with mating. Secondly, sexuality has a less efficient mode of transmitting genes to the offspring (cost of meiosis). Indeed, each individual transmits only 50% of its genes to the next generation. Finally, the re-assortment of parental genotypes to each generation may break-up favourable gene combinations of alleles at many loci, a process known as recombination load (Case and Taper, 1986).

In contrast, in asexual populations every individual in the population produces offspring and the whole genome is passed on to its progeny. Asexual females can potentially produce twice as many daughters as sexual females, so that the ratio of asexual to sexual females should initially double each generation. Thus, asexual populations are expected to have major demographic advantages. Everything else equal, they will grow much faster than any competing sexual species and they might be able to invade and displace them over the short term (Engelstädter, 2008). In addition, in an asexual population the lack of genetic recombination increases the possibility for amplification of coadapted genes, what can be an immediate advantage in some environments (Butlin, 2002).

1.3.- Advantages of sex

Given the costs of sex and the reproductive advantages of asexual reproduction, we would expect that many more unisexual taxa should exist. On the contrary, only one out of every 1000 eukaryotic taxa is unisexual (Vrijenhoek, 1998; Simon et al., 2003). How is it possible that asexual clones do not invade and displace sexual populations? Why is sex so common? The widespread occurrence of sex has been the focus of many studies but it is still one of unsolved enigmas in evolutionary biology and it is termed the "queen of problems" (Bell, 1982).

Many theories have been proposed to understand the advantages of sexual reproduction, which should counterbalance its costs. These theories can be broadly classified into ecological (or environmental) and mutation-based models.

On one hand, ecological theories affirm that recombination produces a more genetically diverse offspring compared with offspring from asexual females. This genetic diversity makes sexual populations less vulnerable to changing environments, parasites or diseases. In fact, sex may accelerate adaptation to a changing environment by creating new gene combinations (Bell, 1982) and may provide an advantage in antagonistic coevolutionary interactions (Hamilton et al., 1990; Lively et al, 1990; Ladle, 1992; Morran et al., 2011). On the other hand, mutational theories assert that sex and meiotic crossovers allow individuals to eliminate deleterious mutations more efficiently. Asexual lineages would accumulate in their genome deleterious mutations that cannot be purged without genetic recombination (Muller, 1964; Kondrashov, 1988; Lynch et al., 1993; Arkhipova and Meselson, 2004). However, there is no a single explanation which can account for the predominance of sex. The different mechanisms may act simultaneously and interact synergistically in many ways in different species (West et al., 1999; Gouyon, 1999; Normarck et al., 2003).

1.4.- Are asexual lineages evolutionary dead ends?

The mode of reproduction of a species determines its genetic diversity and, in turn, its ecological and evolutionary success (Normarck et al., 2003; Simon et al., 2003; De Meeûs et al, 2007). In a sexual interbreeding population new combinations of genes are constantly formed and destroyed. Offspring from sexual parents are generally more genetically diverse compared with offspring from asexual females. The genealogical relationship defining the genetic structure of sexual populations is

usually represented by vast and complex networks (Normark et al., 2003; Simon et al., 2003). On the contrary, in a strictly asexual lineage, where mutation is supposed to be the only source of genetic diversity, clonal diversity in the population is reduced every generation. The phylogenetic reconstruction of asexual populations is generally represented by strictly branching tree, where most asexuals occupy tip positions (Normark et al., 2003; Simon et al., 2003). In fact, a brief evolutionary life span is expected for asexual organisms which are generally regarded as evolutionary dead ends and supposed to go extinct within a short time (10^4 - 10^5 generations) (Lynch and Gabriel, 1990).

The first direct challenge of the assumption that asexual lineages are evolutionary dead-ends came from molecular studies which have identified a variety of "ancient asexual" lineages. There are asexual organisms which have persisted for millions of years without sex which are considered "evolutionary scandals" (Judson and Normark, 1996). Examples are bdelloid rotifers (80 Myr) or darwinulid ostracods (100 Myr) (Mark Welch and Melson, 2000; Martens et al., 2003; Butlin et al., 1998).

1.5.- Genotypic diversity in parthenogens

The mode and frequency of origin of asexual clones in natural populations plays a key role in determining the balance between cost and benefits of asexuality (Butlin et al., 1998, 1999). Different studies have shown that the genetic diversity of asexual populations may have levels comparable to those of sexual populations if they are produced at

high rate or through various mechanisms (Schwander et al., 2011; Delmotte et al., 2001, 2002, 2003). In these cases, asexual populations will emerge repeatedly generating a pool of diverse, polyphyletic asexual lineages. This will therefore influence their ecological adaptability and the outcome competitiveness with their sexual relatives in the short term and it will also determine their long term evolutionary potential (Bell, 1982; Simon et al. 2003).

High genotypic diversity among parthenogenetic lineages is often associated to multiple lineages origin, but it may be also related to different reproductive strategies. Many ancient asexual lineages of vertebrates engage in some form of gene exchange with closely related sexual taxa, so to incorporate a "bit of sex" and compensate the disadvantages caused by the lack of recombination or accumulation of deleterious mutations (Lampert and Schartl, 2010). For example, the asexual fish *Poecilia formosa* (Amazon Molly) reproduces by gynogenesis. Typically the sperm DNA is degraded and the offspring are clones of their mothers. But, sometimes, genomic fragments of (microchromosomes) or the paternal genome are included in the oocyte. That implies an occasional addition of fresh genetic material that slows down the degeneration process of Muller's ratchet and gives rise to new clones (Stöck et al., 2010). Also the unisexual salamander of the genus *Ambystoma* has adopted the reproductive strategy of kleptogenesis in which part of or even the whole of the maternal genome is frequently exchanged for paternal genetic material from sympatric sexual species. That has made possible the existence of nearly 30 genomic biotypes with ploidy ranging from diploid to pentaployd (kleptogenesis and

polyploidization) (Bogart et al., 2007; Bi and Bogart, 2010).

In addition, asexual and sexual reproduction may be not exclusive alternatives. Around 15000 animal species have evolved independently a mixed strategy called cyclical parthenogenesis. Sexual and parthenogenetic generations may alternate throughout the life cycle as in cladocerans and rotifers or exist simultaneously as in hymenopterans (Bell, 1982; De Meester et al., 2004). Cyclical parthenogenesis seems to combine the advantages of sexuality (such as the generation of genetically diverse offspring and a process of genome purging) with the high demographic potential of asexuality (Simon et al., 2002). *Daphnia*, for example, reproduce by amictic parthenogenesis, forming clonal lineages as long as environmental conditions remain favourable. This can be continued for several generations, resulting in an exponential growth of clonal lineages. When unfavourable conditions arise (e.g., food shortage, overcrowding, presence of predators), the population turns to sexual reproduction. Males are produced parthenogenetically, and females produce sexual eggs that need to be fertilized, which are long-lived dormant eggs able to hatch once environmental conditions become favourable again. The genetic structure of cyclically parthenogenetic *Daphnia* populations is so determined by the consequences of combining sexual and asexual reproduction. Populations are expected to be characterized by a high clonal diversity at the start of the growing season (in populations that re-establish from the dormant egg bank, clonal diversity at the beginning of the growing season equals the number of hatchlings), but during parthenogenetic reproduction, chance extinctions of clones and selection are expected to

erode clonal diversity within the population (Ortells et al., 2006).

Finally, there are intermediate strategies including obligate parthenogenesis that retain the capacity for male production (Blackman, 1972; Martens, 1998; Pongratz et al., 1998; Plantard et al., 1998). Fertile matings of these males and females from sexual lineages may generate repeatedly new asexual clones. The gene exchange will result in the introgression of genes of asexuality into sexual population, but it will also increase the genetic diversity of asexuals, producing new asexual genotypes purged from deleterious mutations. That is named contagious parthenogenesis (Simon et al., 2003; Schön et al., 2009)(for details see later).

Thus, studying the origin and evolution of asexual lineages, and understanding how genetic diversity is generated and preserved in such lineages is very important when assessing costs and benefits of asexual reproduction vs. sexual reproduction.

2.- PARTHENOGENESIS AND ITS ORIGIN

Different asexual modes of reproduction are found among animals (Schön et al., 2009). Thelytokous parthenogenesis consists in the development of unfertilized eggs that give rise to all female offspring.

Parthenogenetic reproduction fall into two main categories: apomixis or automixis, based on the presence or absence of meiosis (Simon et al., 2003). In apomictic parthenogenesis, meiosis is totally lacking: the divisions in the oocyte are mitotic. There is no recombination of alleles and the offspring are true clones of the mother. In automictic

parthenogenesis, meiosis is preserved but fusion occurs between two nuclei originating from the same individual. Gene recombination can occur. Various cytological mechanisms are known to restore the ploidy level, which represent different modifications of meiosis. Each mechanism has a different impact on the genetic diversity of the population since they may either maintain or eliminate genetic variation across generations, with very different evolutionary consequences (Pearcy et al., 2006; Noughé et al., 2015b).

The two simplest cytological mechanisms leading to automictic parthenogenesis are central fusion and terminal fusion, in which two products of the same meiosis, one oocyte and one haploid polar body, fuse to restore diploidy. In automictic parthenogenesis with terminal fusion, the oocyte fuses with the second polar body. So, it consists in the fusion between two haploid meiotic products that separated at meiosis II. Considering a given heterozygous locus in the parent, the offspring will become entirely homozygous, but heterozygosity might be maintained further away on the chromosome if recombination exchanged chromatids between homologous chromosomes during meiosis I. Each heterozygous locus has a probability ranging from 1/3 (far from centromere) to 1 (close to centromere) of becoming homozygous. In automictic parthenogenesis with central fusion, the oocyte fuses with a haploid product of the first polar body. It means that the fusion occurs between two haploid meiotic products separated at meiosis I. In this situation, the offspring is genetically similar to the mother (it will always remain heterozygous), except when there is recombination. Each heterozygous locus has a probability ranging from

0 (close to centromere) to 1/3 (far from centromere) of becoming homozygous.

Thus, automixis through central fusion combined with very low recombination rates leaves a genetic signature very similar to that of apomixis (with maintenance of high heterozygosity levels). In contrast, terminal fusions and central fusions combined with very high recombination rates leave a genetic signature very similar to self-fertilization (loss of heterozygosity) (Pearcy et al., 2006; Noughé et al., 2015b).

There are other cytological mechanisms leading to automictic parthenogenesis, which are characterized by modified meiotic steps. Among these, automictic parthenogenesis with 'random fusion' occurs when all four chromatids segregate independently and each heterozygous locus has a probability of 1/3 of becoming homozygous, independent of its position on the chromosome; instead, automictic parthenogenesis with 'gamete duplication' involves the duplication of the chromosomes after meiosis and the offspring will be homozygous for all loci (Pearcy et al., 2006; Noughé et al., 2015b).

Parthenogenesis in animals has evolved through different mechanisms: 1) spontaneous origin, 2) hybrid origin, and 3) infectious origin. Depending on the mechanisms involved in the loss of sex, parthenogenetic lineages may acquire different genotypic profiles compared to bisexual ancestors, which determines their initial genetic variability and therefore their evolutionary success and persistence (Simon et al., 2003).

2.1.- Spontaneous origin

Spontaneous transition to asexuality may occur when mutations involve the genes that suppress meiosis or the genes underlying the production of sexual forms (Simon et al., 2003). Such mutations could directly result in obligate asexual population, or they could be initially maintained as genetic variation for facultative parthenogenesis in a sexual population. In any case, it will result in the production of an all-female lineage reproductively isolated from its sexual ancestors (Schwander and Crespi, 2009).

Apomictic parthenogens could evolve directly from rare sexual females that produce their eggs mitotically or, secondarily, by a stepwise transition via automictic parthenogenesis. In the last case there will be an intermediate cytological process involving recombination suppression and an increase of the relative proportion of oocytes produced by central fusion (Schwander and Crespi, 2009).

Spontaneous origin is expected to occur in environments in which finding a mate is difficult or impossible, such as in marginal habitats with such low densities that stochastic fluctuations in the sex ratio may eliminate males by chance (Kramer and Templeton 2001). Spontaneous origin of diploid parthenogenetic lineages has been documented in different groups of invertebrates, as ostracods belonging to the genus *Eucypris* (Schön et al., 2000) or molluscs of the genus *Campeloma* (Johnson and Bragg, 1999) and *Potamopyrgus* (Neiman and Lively 2004). In the stick insect of the genus *Timema,* Schwander and Crespi (2009) have found that four of the five *Timema* parthenogens (*T. douglasi*, *T. monikensis*, *T. tahoe*, and *T. genevievae*) evolved through a spontaneous

loss of sex from four different sexual ancestors (respectively *T. poppensis*, *T. cristinae*, *T. bartmani*, and *T. tahoe*).

2.2.- Hybrid origin

Parthenogenetic lineages can result from hybridization between two co-occurring sexual species. Hybridization events occur when genetically differentiated populations come into contact after a previous allopatric condition. If reproductive isolation breaks down, a new hybrid population may arise, which acquires a novel genotype combining alleles from their parents, being transmitted to the next generation (Bullini, 1994). The frequency at which hybrid species are formed varies among groups and with the degree of similarity between parental species (Morgan-Richards and Trewick, 2005).

Hybridization is frequently associated to a switch from sexual to asexual reproduction (parthenogenesis, gynogenesis or hybridogenesis). In this regard, there are two theories that try to explain this linkage. On one hand, hybridization can disrupt normal gametogenesis and thus favour asexual reproduction (hybrid theory); on the other hand, asexual reproduction might already exist, as spontaneous or facultative reproductive strategy, in the sexual parental species and then be inherited by hybrids (spontaneous theory) (Bullini, 1994; Kearny et al., 2009).

Occasionally, individuals of a hybrid taxa can backcross with a sexual relative to generate asexual lineages of increased ploidy. Secondary hybridization events with repeated origin of asexual forms might thus generate complex patterns of relationships between the parthenogenetic

lineages (reticulate evolution pattern) (Bullini, 1994; Morgan-Richards and Trewick, 2005).

In a hybridization event, cytological processes disrupting meiosis as the pairing of divergent homologues might be difficult to accomplish. This can explain why some interspecific hybrids are sterile, or why they show lower offspring viability compared to parental species (Schwenk et al. 2001). But, at the other extreme, parthenogenetic lineages can benefit from heterosis (hybrid vigour) and generate offspring with higher viability and fecundity rates (Lynch, 1984).

In general, hybrid taxa are morphologically well differentiated from their parental species, showing intermediate phenotypes compared with parental species (Schwenk et al. 2001; Hobæk et al., 2004).

Hybrid lineages enjoy the advantages of sexual reproduction (recombination and increased genetic variability) and those of asexual reproduction (high rates of demographic growth, capacity of colonization), what might explain their evolutionary success (Bullini, 1994).

Hybridization appears to be the main route by which unisexual vertebrates arise. It is well documented in amphibians, fishes and reptiles (Neaves and Baumann, 2011). For example, hybridization combined with parthenogenesis has given rise to almost all unisexual lizards. Molecular data have shown that diploid parthenogenetic *Aspidoscelis* species arose from hybridization events between sexual progenitors (*A. inornata* and *A. exsanguis*); further secondary hybridization between these hybrid females and males of sympatric sexual species produces triploid unisexuals which, in turn, may produce

tetraploid hybrids (Lutes et al., 2011).

Most vertebrates of hybrid origin are gynogenetic or hybridogenetic, and still require insemination from bisexual relatives. It is the case of the gynogenetic Amazon molly, *Poecilia formosa* which arose by hybridization between *Poecilia mexicana* as maternal and *Poecilia latipinna* as paternal ancestors (Avise et al., 1991; Lampert and Schartl, 2008) or the hemiclonal frog *Rana esculenta* arisen from sexuals *Rana ridibunda and Rana lessone* (Avise et al., 1992).

In invertebrates, hybridization is common in crustaceans, insects and molluscs. Several interspecific hybrids have been found within the cladoceran genus *Daphnia*, which are capable of parthenogenetic reproduction (Hobæk et al., 2004).

In North America, among stick insects of the genus *Timema*, one parthenogenetic lineage *T. shepardi* likely derives from a hybrid between *T. poppensis* females and *T. californicum* males, which are the two sexual species with the same number of chromosomes (the other four have a spontaneous origin, see above) (Schwander and Crespi, 2009). In Europe, repeated interspecific hybridization of the sexual stick insect of the genus *Bacillus* has resulted in lineages that reproduce asexually (Scali et al., 2003)

Parthenogenetic triploids of the genus *Campeloma* (freshwater snail) also have a hybrid origin arisen through fertilization of diploid parthenogens by haploid sperm of sexual related species (Johnson and Bragg, 1999).

2.3.- Infectious origin

The loss of sex may occur through infection by vertically inherited

microorganisms able to alter the reproduction of their host to favour their persistence in populations. These microorganisms can be classified into three groups: 1) *Wolbachia pipientis* group, 2) the *Cytophaga-Flexibacter-Bacteroides* (CFB) group of bacteria, 3) *Xiphinematobacter* species (Koivisto and Braig, 2003). The best known example is *Wolbachia*, an intracellular alpha-proteobacteria. There are different ways by which *Wolbachia* can manipulate host reproductive processes, for example, by converting genetic males in functional females (feminizing), by killing males, by inducing parthenogenesis, or causing male sterility (Maniatsi et al., 2010).Parthenogenesis-inducing *Wolbachia* is known in several hymenopteran parasitoids, where the presence of *Wolbachia* causes diploidization of the unfertilized haploid eggs, which develop as females and not as haploid males (Plantard et al., 1998). A case of male killing has been reported in the genus *Ostrinia* (European corn worm) where *Wolbachia* kills genetic males ZZ during the larval stage, while genetic females WZ do not survive in absence of the bacterium (Sugimoto and Ishikawa, 2012).

2.4.- Contagious parthenogenesis

A secondary origin for the generation of new parthenogenetic lineages is contagious parthenogenesis (Simon et al., 2003; Schön et al., 2009). This mechanism involves a pre-existing parthenogenetic lineage able to produce functional males, which has arisen by any of the mechanisms described above. When the reproductive isolation between such males and their sexual relatives is incomplete, they may mate with coexisting sexual females producing fertile parthenogenetic hybrid offspring. The

new parthenogenetic lineages will combine genetic diversity from the maternal sexual species and from their paternal parthenogenetic ancestor, including the genetic fragments linked to the parthenogenesis (Simon et al., 2003; Tucker et al., 2013).

Many asexual lineages retain the ability to produce functional males as in aphids (Blackman, 1972; Rispe et al., 1999; Simon et al., 1999; Delmotte et al., 2001), ostracods (Butlin et al., 1998; Martens, 1998), freshwater flatworms (Pongratz et al. 1998) and wasps (Plantard et al. 1998), what indicates that the loss of sexual reproduction may not start with the complete loss of males, or that the mechanisms suppressing sexual reproduction fails occasionally.

In such systems, rare males may represent a vector for genetic exchange between asexual and sexual lineages when both coexist (Lynch, 1984; Rispe et al., 1999; Simon et al., 1999; Delmotte et al., 2001; Engelstädter et al., 2011). This occasional gene flow between sexual and asexual lineages, resulting in a regular emergence of asexual lineages, may be sufficient to significantly reduce the costs of the asexuality, contributing to the ecological success and to the evolutionary potential of such asexual lineages. Indeed, male-transmitted asexuality may create a genetically diverse assemblage of asexual lineages. Newly produced asexuals may continuously replace the oldest lineages suffering from the accumulation of deleterious mutations, allowing the persistence of the asexual populations in both short and long time.

This mechanism has been deeply studied in the water flea *Daphnia pulex* (Innes and Hebert, 1988; Paland et al., 2005). In the North American *D. pulex* parthenogenetic lineages, at least two distinct unrecombined

haplotypes on chromosome VIII and IX are implied in the sex-limited meiosis suppression (Lynch et al., 2008; Eads et al., 2012; Tucker et al., 2013). These haplotypes, leading to obligate parthenogenesis in *D. pulex*, stem from a single recent event of hybridization with its sister taxon *D. pulicaria* (Xu et al., 2013; Tucker et al., 2013). Multiple new parthenogenetic lineages have arisen since this event, as males produced by asexual lineages spread these parthenogenesis-inducing haplotypes by mating with sexual females.

The mechanism of contagious parthenogenesis has been also studied in the bee *Apis mellifera capensis* and in the parasitoid wasp *Lisyphlebus fabarum* (Schneider et al., 2002; Sandrock and Vorburger, 2011; Delmotte et al., 2013) in which the meiosis suppressor genes are recessive and not dominant as in *D. pulex*.

The retention of functional males in parthenogenetic lineages may involve a fitness cost compared to the asexual populations producing only females. For example, a recent study suggests that a 5-10% decrease in daughter production due to male production may influence the outcome of competition amongst asexual lineages (Neiman et al., 2012). On the other side, occasional sexual reproduction in predominantly asexual organisms reaps the benefits of sexual reproduction without paying its cost. Low levels of sex are sufficient to increase genotypic diversity and the fitness of a population (D'Souza and Michiels, 2010).

2.5.- Geographic parthenogenesis. Marginal habitats

Geographic parthenogenesis is the geographically distinct distribution of closely related sexual and asexual organisms (Vandel, 1928).

Many studies reveal that asexual populations are more frequently distributed in environments classified as marginal: extreme or disturbed areas, xeric habitats, islands or island–like habitats, high altitude and latitude biotypes (Vandel, 1928).

Different hypotheses have been postulated to explain this pattern, and they are not mutually exclusive. At first, asexuals are considered better colonizers than sexual species, since a single dispersing female or egg can establish a new population, whereas sexual individuals would have more difficulties to find mates in marginal biotopes where the demographic density is low (Peck et al., 1998). Moreover, the biotic pressure of parasites, competitors and predators is lower in extreme environments, so asexual populations would be better able to compete against sexual species (Glesener and Tilman, 1978; Jaenike, 1978; Hamilton, 1980). In marginal habitats populations are subdivided in metapopulations, which suffer of frequent events of extinction and recolonization. Due to repeated genetic bottlenecks, sexual populations can suffer increased homozygosity and inbreeding depression (Haag and Ebert, 2004). Finally, many asexual populations have hybrid origin and enjoy the heterosis enabling them to invade extreme environments (Kearney, 2005).

2.6.- Parthenogenesis and geographic distribution

Many parthenogenetic species are geographically and ecologically more widely distributed than their sexual relatives. Two major hypotheses describe how asexuals will use niches in relation to their sexual ancestors: the General Purpose Genotype (GPG) and the Frozen Niche

(FNV) hypotheses.

FNV affirms that asexual populations arising from sexual species will "freeze" the ecological niche of the latter: it means that asexuals will generally inherit the same range of tolerance to different environmental conditions (e.g. temperature, salinity, oxygen, etc.) of their sexual relatives. However, since a large number of different clones may arise from sexual ancestors, the total ecological tolerance of a set of clones might still cover a wide range of environmental conditions (Vrijenhoek, 1978, 1979).

Different way, the GPG considers that asexuals may occupy a broader range of environments because they are generalist clones. The selection in a temporally varying environment promotes the evolution of generalist clones, characterized by wide ecological tolerance ranges and low fitness variance in a wide range of ecological conditions (Lynch, 1984; Van Doninck et al., 2002).

3.- MODEL ORGANISM: *Artemia* GENUS

Artemia is a genus of anostracan crustaceans widely known as brine shrimps. It was first described by Schlosser in 1755 on material collected from the solar saltworks near Lymington, England, which do not currently exist (Kuenen and Baas–Becking, 1938, in Sorgeloos, 1980a). Later, in 1758 Linneaus classified it as *Cancer salinus* and only in 1818 Leach renamed it as *Artemia salina,* term with which is usually known in the scientific literature.

Phylum *Artropoda* (Siebold y Stannius, 1848)
Subphylum *Crustacea* (Pennant, 1777)
Class *Branchiopoda* (Latreille, 1817)
Order *Anostraca* (Sars, 1867)
Family *Artemiidae* (Grochowski, 1896)
Genus *Artemia* Leach, 1819

Artemia salina has been for a long time the only species belonging to the genus. The earliest genetic studies on chromosomes led to recognize first two different reproductive modes, parthenogenesis and bisexuality (Artom, 1906, 1911), and then to distinguish into several sexual sibling species and a number of parthenogenetic forms, so that the systematic of the genus has been reviewed during all the second half of the previous century (Halfer Cervini et al., 1968; Clark and Bowen, 1976; Bowen et al., 1980; Abreu-Grobois and Beardmore, 1982; Barigozzi, 1972, 1974, 1980).
Nowadays the denomination *Artemia salina* is maintained only for the original material upon which the first description was made and for the European sexual brine shrimp (Mura 1990) and multidisciplinary approaches have been used to characterize *Artemia* populations (Gajardo et al,. 2002, Mura et al., 2006; Maniatsi et al., 2011).
Artemia has been used as a model organism in many studies concerning physiology, ecotoxicology, genetics, phylogeography (Saez et al., 2000; Barahona and Sanchez-Fortún, 1999; Papeschi et al., 2008; Baxevanis et al., 2006; Muñoz et al., 2008) so much that it is considered a sort of "aquatic *Drosophila*"(Abreu-Grobois and Beardmore 1982; Gajardo and Beardmore 2001). That is due to the convenience with which *Artemia*

cysts may be stored, the ease with which an active population may be generated in the laboratory within a few days and the handiness with which the environmental parameters may be quantified to design an experiment.

In addition, *Artemia* is widely known for its beneficial effect in salt production as a filtrating and purifying organism in the brine, and for its extensive use in aquaculture as live food for fish and crustacean larvae (Lavens and Sorgeloos, 2000; Dhont and Sorgeloos, 2002; Dhont and Van Stappen, 2003; Kolkovski et al., 2004).

3.1.- Morphology

The crustacean class Branchiopoda is a morphologically diverse group of ecologically important freshwater organisms including the orders Anostraca, Notostraca, Concostraca and Cladocera. Branchiopod fossil record extends back to the upper Cambrian (Walosseck, 1993).

Artemia is a typical anostracan branchiopod with a segmented, elongated body, in which it is easy to distinguish a head, a thorax and an abdomen (Figure 1).

All the body is covered with a thin flexible exoskeleton of chitin, which sheds periodically to allow the growth of the animal. The total length is about 8 – 10 mm for adult males and 10 – 12 mm for adult females, depending on the species. Within the same species, size may also vary depending on the environmental parameters as temperature, salinity and pH (Amat, 1985; Ben Naceur et al., 2012).

The head is composed of six fused segments and bears a median eye and a pair of large, pedunculated compound eyes, first antennae, second

antennae, mandibles, first and second maxillae. The thorax is constituted by eleven segments, each provided whit a pair of appendices (thoracopods) with respiratory, locomotory, and filter feeding functions. The abdomen extends behind the thorax and is composed of eight annular segments. It lacks appendices (phyllopods) and ends with a telson or furca. The first two abdominal segments correspond to the genital segments and they bear the gonopods (Amat, 1985).

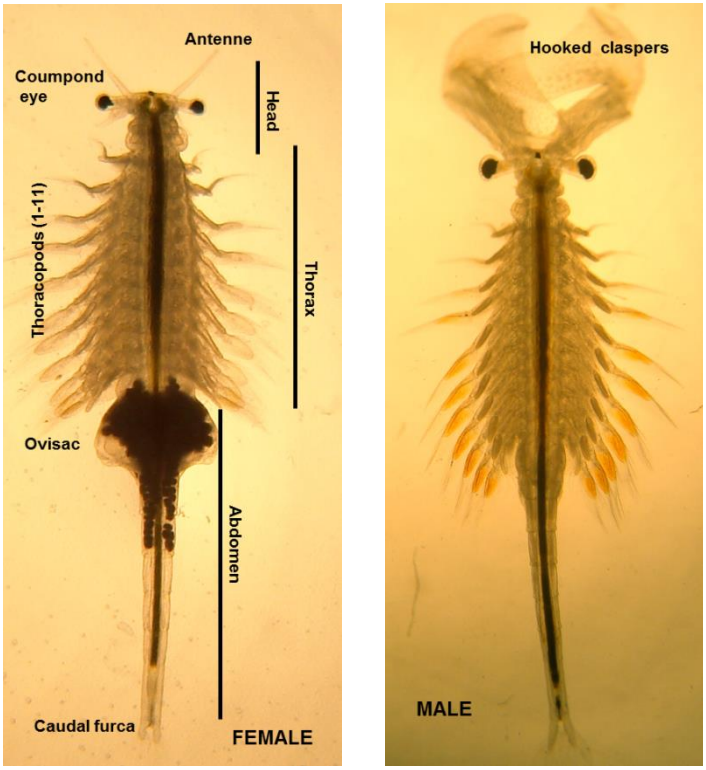

Figure 1. External morphological *Artemia* features
(female and male)
Photo credit: "own work"

As is typical in anostracans, *Artemia* displays external sexual dimorphism. The males show the second antennae enlarged and modified into hooked claspers used during mating to hold the female, and a pair of retractile penises on the genital segments, which include two separate reproductive systems, each consisting of testis, seminal vesicle and vas deferens. In the females, the second antennae are small and simple and act as sensorial appendages, and the reproductive system consists of two tubular ovaries, two pouch-like oviducts and a median uterus, which lies within a single ovisac situated just behind the 11th pair of thoracopods. Attached to the uterus are four clusters of shell glands (Amat, 1985; Criel and MacRae, 2002).

3.2.- Ecology and life cycle

Artemia is the most common invertebrate in hypersaline ecosystems such as inland salt lakes, coastal lagoons, ponds and solar saltworks (Triantaphyllidis et al., 1998). These aquatic biotopes have a markedly variable chemistry and seasonality, and they are commonly characterized by their high productivity and low species diversity (Lenz and Browne, 1991).

Salinity is certainly the predominant abiotic factor determining the presence or absence of *Artemia* since it conditions primarily the presence of potential predators, against which brine shrimp do not have any anatomical nor behavioural defence mechanisms. The other variables as temperature, light intensity, primary food production, may have an influence on the dynamic of the *Artemia* population, or may cause only a temporary absence of brine shrimp (Persoone and Sorgeloos, 1980; Van

Stappen, 2009).

The species of the genus *Artemia* display an exemplary series of biochemical and physiological adaptations to face the strong seasonal fluctuations of environmental parameters (mainly salinity and temperature) of these biotopes (Clegg and Trotman, 2002). First of all, brine shrimps are considered extremely osmotolerant organisms (Van Stappen, 2002). They live in environments with salinities ranging from 45 g/L to up to 370g/L and different anionic compositions (chloride, sulphate or carbonate waters) (Bowen et al., 1985, 1988; Triantaphyllidis et al., 1995; Abatzopoulos et al., 2003; Van Stappen, 2002). This is due to its efficient osmoregulatory capacity that consists of an active excretion of salt by phyllopods. Actually, the animal is able to exist and reproduce at normal sea water salinities but, because often predators will also be present, brine shrimp is generally found in nature only in waters of high salinity (> 70 g/L) (Bowen et al., 1978; Clegg and Trotman, 2002).

Artemia is also a eurythermal crustacean. It inhabits waters with different temperature regime, which are exposed to diverse climatic conditions from humid to arid climate types, and situated at different altitudes from sea level up to 4500 m (Persoone and Sorgeloos, 1980; Bowen et al., 1985, 1988; Vanhaecke et al., 1987; Campos et al., 1996; Gajardo et al., 1999; Van Stappen et al., 2003, 2008). The effect of temperature on the distribution of brine shrimp has been the subject of many studies, showing interspecific range of tolerance (Vanhaecke et al., 1984; Lenz, 1987; Browne et al., 1988; Vanhaecke and Sorgeloos, 1989; Abatzopoulos et al., 2003). Generally *Artemia* populations survive at temperatures ranging from 5°C to 35°C, with the species *Artemia*

franciscana also occurring at even higher temperature (Clegg et al., 2000; Kappas et al., 2004).

Saline waters are often characterized by low concentration of dissolved oxygen (< 2 ml O_2/L, hypoxic condition). At this regard, brine shrimp is able to regulate the concentration of respiratory pigment to increase the oxygen-carrying capacity of the blood; moreover, *Artemia* can synthesize different types of hemoglobin, specifically HbIII type which has a higher oxygen affinity (Bowen et al., 1978; Clegg and Trotman, 2002).

An additional adaptive strategy of *Artemia* to the variability and unpredictability of these habitats is a flexible life cycle. *Artemia* can reproduce both by ovoviviparity (producing free swimming nauplii) and by oviparity (producing diapausing cysts) and switch these modes of reproduction depending on the environmental conditions (Criel and MacRae, 2002; Clegg and Trotman, 2002). Under adverse conditions, they produce resistant, diapausing cysts (encysted embryos enveloped in a shell or chorion) which float and strand along the banks of the saltpans or lakes, where they dehydrate. When the environment becomes appropriate again, these cysts resume embryonic development, do hatch and a living population starts anew (Lavens and Sorgeloos, 1987).

If, on one side, these resistant eggs allow the continuity and the persistence of the population, on the other side, they are also very important for the dispersal of populations. As *Artemia* is incapable of active dispersion, waterfowl, wind and human activities are the most important dispersion vectors to spread the cysts to other water bodies (Gajardo et al., 2002; Figuerola et al., 2002, 2005; Sanchez et al., 2007).

Reproduction in *Artemia* is one of the most fascinating aspects of their biology. The genus includes both gonochoric sexual species, with separate males and females, and numerous parthenogenetic (asexual) lineages (Gajardo et al., 2002). The two modes of reproduction, sexual reproduction and thelytokous parthenogenesis, are alternative and exclusive modes.

As mentioned above, *Artemia* species and strains can reproduce both by ovoviviparity and oviparity and females can switch in-between two reproduction cycles from one mode of reproduction to the other. Mature eggs (fertilized or not) normally develop into free-swimming nauplii which are released by the mother. In adverse conditions, the embryos only develop up to the gastrula stage, then they get surrounded by a thick shell (secreted by the brown shell glands located in the uterus) and enter a state of metabolic standstill or dormancy (diapause) to be released by the female as cysts (or "resting eggs" or "diapausing eggs"). Diapausing cysts can withstand a wide variety of extraordinary environmental stresses, including long-term anoxia, temperature extremes, desiccation, g-irradiation (Persoone and Sorgeloos, 1980). They usually float in the high salinity brines and are blown ashore, where they accumulate and dry. Dormancy is terminated by a dehydration-rehydration cycle. The rehydrated cysts exist in a quiescent state termed anhydrobiosis (Browne and Bowen, 1991) and they can resume their further embryonic development when hydrated in optimal hatching conditions.

In the first larval stage, nauplii do not feed as their digestive system is not functional yet; they thrive completely on their yolk reserves. After

about 8 h, the animal is able to filter out small food particles (1 to 50 µm) by the second antennae, being ingested into the functional digestive tract. They take two weeks to reach to adult stage, surviving then several months depending on the species and on the environmental conditions (Amat, 1985).

3.3.- Biodiversity and biogeography

Artemia has a cosmopolitan distribution, since it is distributed over all continents, except Antarctica. Although it has been recorded in nearly 600 locations, the distribution of the genus has yet to be considered provisional, since it reflects exploration activities carried out so far, with all their limitations (natural, socio-political and linguistic barriers) (Vanhaecke et al., 1987; Triantaphyllidis et al., 1998; Van Stappen, 2002; Muñoz and Pacios, 2010)(Figure 2).

The *Artemia* genus includes both gonochoric sexual species with separate males and females, and a large number of obligate parthenogenetic lineages (Gajardo et al., 2002; Baxevanis et al., 2006). Currently seven sexual species have been documented in the scientific literature, with six of them described. Some of them have a vast area of distribution, whereas others are known from a single site. In the Old World, *A. salina* (Linnaeus 1758) occurs in the Mediterranean region and South Africa (Amat et al., 1995 a,b; Kaiser et al., 2006); *A. sinica* (Cai 1989) is broadly distributed in China and Inner Mongolia; *A. urmiana* (Günther 1890) is endemic to lake Urmia and surrounding area (Iran) and Crimean salt lakes (Abatzopoulos et al., 2009); *A. tibetiana* (Abatzopoulos et al., 2002a; Van Stappen et al., 2007) is only found in the

Tibetan plateau. Different studies (Pilla, 1992; Pilla and Beardmore, 1994; Litvinenko and Boyko, 2008) have confirmed the separate species status of a not yet described *Artemia* sp. from a single cyst sample (ARC code 1039) originated from an unknown location in Kazakhstan. In the New World, *A. franciscana* (Kellogg 1906) has a wide natural distribution area including North, Central and South America, whereas *A. persimilis* (Piccinelli and Prosdocimi, 1968) is only found in the extreme south of the continent (Southern Argentina and Chile)(Kappas et al., 2009).

Parthenogenetic populations occur only in the Old World over a vast geographic area, from the Canary Islands in the west to China in the east (Gajardo et al., 2002; McMaster et al., 2007). In Australia, parthenogenetic populations of *Artemia* have been introduced and they may coexist with endemic brine shrimps of the genus *Parartemia* (McMaster et al., 2007).

Currently the biodiversity of the genus *Artemia* is dramatically affected by two main causes, the loss of habitats and the introduction of invasive species (Amat et al., 2007).

In that regard, *A. franciscana*, which is the species commonly used in aquaculture activities, has become an extremely competitive species outside its native range. Introduced populations of *A. franciscana* have been recorded in numerous locations, including Europe, Africa, Southeast Asia, Australia where they have often displaced the autochthonous species (Amat et al., 2005, 2007; Green et al., 2005; Mura et al., 2006; Van Stappen et al. 2007; McMaster et al., 2007).

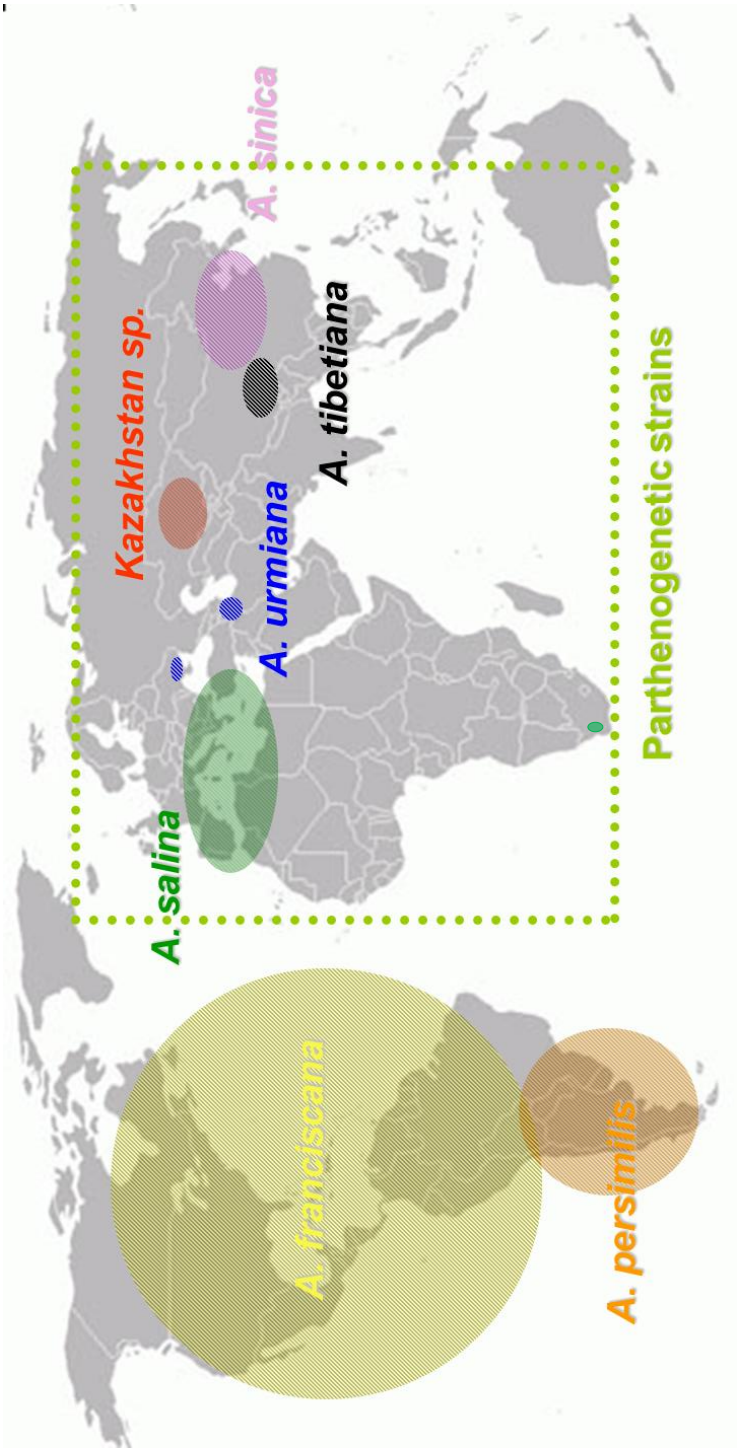

Figure 2. Distribution map of *Artemia* species. Each species is marked with different colours

All sexual *Artemia* species are diploid and they have a chromosome number of 2n=42, with the exception of *A.persimilis* which has an additional chromosome for aneuploidy, 2n=44 (Abatzopoulos et al., 2002b). Parthenogenetic strains are characterized by different ploidy levels (diploid, triploid, tetraploid and pentaploid) (Barigozzi, 1974; Abatzopoulos et al., 2002b). The parthenogenetic diploid lineages are automictic while the polyploidy lineages are apomictic parthenogens (Barigozzi, 1974; Abreu-Grobois, 1987). All parthenogenetic strains are often grouped under the binomen *Artemia parthenogenetica* (Artom, 1931). Since mixed ploidy levels often occur in natural parthenogenetic populations, we have chosen to refer to parthenogens as populations, strains or clones, as suggested by Abatzopoulos et al. (2002b).

Artemia inter- and intra-specific biodiversity has been studied by morphology studies, morphometry, cytogenetics, and over recent years through a variety of molecular markers and techniques (Amat, 1980; Barigozzi et al., 1984, 1987; Gajardo et al., 2002; Kappas et al., 2004; Mura and Brecciaroli, 2004; Mura and Nagorskaya, 2005; Baxevanis et al., 2005, 2006; Qiu et al., 2006; Hou et al., 2006; Muñoz et al. 2008, 2010, 2013; Maniatsi et al., 2011).

Evolutionary relationships between *Artemia* species have been investigated using nuclear and mitochondrial DNA molecular markers in several studies (Baxevanis et al., 2005, 2006; Qiu et al., 2006; Hou et al., 2006; Muñoz et al., 2008, 2010, 2013; Maniatsi et al., 2011). They agree that *A. persimilis* first diverged from the common ancestor of all *Artemia* species between 80-90 MYA at the time of the separation of Africa from South America while Asian species, *A. urmiana*, *A. sinica, A. tibetiana*,

and *Artemia* sp. Kazakhstan diverge more recently (less than 8 MYA). These Asian species may have been involved in the origin of parthenogenetic strains (Baxevanis et al., 2006; Hou et al., 2006; Kappas et al., 2009; Muñoz et al., 2010; Maniatsi et al., 2011).

Molecular data also suggest that the origin of parthenogenesis in *Artemia* is polyphyletic (Baxevanis et al., 2006; Muñoz et al., 2010; Maniatsi et al., 2011) but the phylogenetics of asexual lineages have not yet been fully resolved. Mitochondrial and nuclear phylogenies indicated that diploid and triploid parthenogenetic *Artemia* strains are closely related to *A. urmiana*, *Artemia* sp. Kazakhstan and *A. tibetiana*, ruling out *A. sinica*. In contrast, at least some tetraploid clones would have a separate maternal origin as they are closely related to A. *sinica* (Baxevanis et al., 2006; Muñoz et al., 2010; Maniatsi et al., 2011).

3.4.- Rare males in *Artemia*

Parthenogenetic diploid *Artemia* populations reproduce through automictic parthenogenesis and retain the ability to produce males regularly in low proportions, typically less than 1% in both laboratory and field studies. These are usually known as rare males (Stefani, 1964; Bowen et al., 1978; MacDonald and Browne, 1987; Amat et al., 1991; Cai, 1993; Mura and Nagorskaya, 2005). The mechanisms behind the production of these rare males in parthenogenetic diploid *Artemia* have received some attention and they are thought to be linked to the cytological mechanisms underlying automictic parthenogenesis (Noughé et al., 2015b).

Automictic parthenogenesis involves the reshuffling of allelic variants

within an individual in a modified meiotic process (Abreu-Grobois, 1987) but the cytogenetic mechanisms to restore the diploid condition and, then, those involving the production of rare males in *Artemia* have been uncertain until very recently.

As in birds, *Artemia* females are heterogametic (ZW) while males are homogametic (WW) (Bowen, 1963, 1965; De Vos et al., 2013). On the basis of cytological observations, Stefani (1964) initially proposed that *Artemia* rare males arise as the result of fusion of two haploid Z cell in rare event of terminal fusion while females arise from central fusion event. A pilot study based on allozyme electrophoresis suggested that all the offspring of a female, including rare males, were genetically identical to their mother (Abreu-Grobois and Beardmore, 2001). This would mean that if a female is heterozygote at different loci, this maternal heterozygosity is largely maintained across generations. Since the mechanism proposed by Stefani (1964) would have involved homozygosity at all autosomal loci, not only at the sex locus, they suggested that rare males may be produced from a rare recombination event between the homologous sex chromosomes which induce the segregation of sex loci between first meiotic division products.

Recent work by Noughé et al. (2015b) confirmed the hypothesis of Abreu-Grobois and Beardmore (2001) by studying the patterns of population-wide heterozygosity for 12 microsatellite loci in two natural populations and in strains maintained over 36 generations in the laboratory. Both strains and populations retained heterozygosity. Therefore, automixis with central fusion in combination with low rates of recombination is the reproductive mode of *Artemia* and the occasional

recombination between sex chromosomes in the heterogametic female seems to be the explanation for the origin of rare males. If the sex-determination locus is close to the centromere, it remains heterozygous most of the time, leading to female offspring. When a rare recombination event occurs, it leads to segregation at the sex locus and the production of ZZ males.

Artemia rare males have normal and functional reproductive organs and display normal sexual behaviour (MacDonald and Browne, 1987). They are capable to produce sperm, which is slightly larger than those of sexual males (6.6 μm vs. 4.1 μm), and clasp females (Stefani, 1964; MacDonald and Browne, 1987). The sexual functionality of rare males is less known. Although, rare males have not been shown to fertilize females from their own diploid parthenogenetic lineages (Stefani, 1964; MacDonald and Browne, 1987) or sexual females from *A. franciscana, A. persimilis or A. salina* (MacDonald and Browne, 1987; but see Bowen et al., 1978), they can fertilize sexual females of the closely related species *A. urmiana* (Bowen et al., 1978) and *A. sinica* (Cai, 1993) producing viable offspring, although the data are very limited. In their study, Bowen et al. (1978) documented a transfer of genes from three rare males from Yamaguchi (Japan) parthenogenetic population to an *A. urmiana* female by polymorphism of three genetic markers (one haemoglobin and two esterase isozymes) but they also obtained viable offspring when mating *A. franciscana* females with these rare males.

Although rare males were previously described as meiotic mistakes (MacDonald and Browne, 1987), their production may instead provide a

fitness advantage to the parental females and/or have an evolutionary importance. Fertile matings between rare males and females from close sexual species may be important for the persistence of *Artemia* asexual lineages if rare males are capable to transmit asexuality genes to the offspring, converting a proportion of hybrid offspring to obligate asexuality (contagious parthenogenesis). The coexistence of *Artemia* parthenogenetic lineages with their close sexual relatives makes possible such gene exchange. This mechanism would provide an opportunity for the recurrent emergence of new parthenogenetic lineages, ensuring the longer persistence of asexuality.

For example, recent molecular analysis of polyploidy parthenogenetic *Artemia* strains (Maniatsi et al., 2011) hypothesized that parthenogenetic rare males would be involved into the origin of triploid asexual strains by fertilizing an unreduced ovum.

4.- MOLECULAR MARKERS TO UNDERSTAND THE EVOLUTION OF PARTHENOGENESIS

Contemporary knowledge of the origin and evolution of most asexual clones and the reconstruction of phylogenetic relationships between sexual and asexual taxa is largely based on the use of molecular markers (Simon et al., 2003).

Phylogenetic inferences are used to address several aspects of the evolution of parthenogenesis. First of all, they allow inferring the number of independent events leading to asexuality and distinguishing if parthenogenetic lineages have a monophyletic or polyphyletic origin

(for example identifying the number of maternal lineages in the parthenogenetic strains and their monophyly or not). Second, they are useful to estimate the age of parthenogenetic lineages. Finally, in conjunction with patterns of marker distribution in putative ancestral sexual lineages, they can be used to investigate the possible mechanisms responsible for the loss of sex (Simon et al., 2003).

Evolutionary relationships among organisms can be inferred by constructing a phylogenetic tree. A tree is a graphical representation of evolutionary history of a group of organisms which consists of nodes and branches. Branches are connected by adjacent nodes and each node represents a single taxonomic unit characterized by species, populations or individuals (Graur and Li, 2000). In the context of evolution of parthenogenetic lineages, phylogenetic trees are generally rooted with the closest sexual outgroup to reconstruct the history of the loss of sex and assuming (1) that sexual reproduction is the ancestral state and (2) that the loss of sex is irreversible (Simon et al., 2003; but see Domes et al., 2007). In this regard, Domes et al. (2007) suggested that Crotoniidae mites reevolved sex within the ancient clade of parthenogenetic Camisiidae, possibly as adaptation to certain environmental conditions under which sexual reproductive mode prevails. That is an exceptional case of breaking Dollo's law (Gould, 1970), implying that parthenogenesis is not necessarily an evolutionary dead end.

Genetic markers such as microsatellites and mitochondrial and nuclear DNA sequences can be also used to determine the genotypic identity of populations or individuals and to carry out parentage analysis. For example, microsatellite markers with high level of polymorphism are

powerful tools for assessing genetic relatedness between individuals or closely related taxa (Guichoux et al., 2011; Kalia et al., 2011). By genotyping a few loci, they provide information that allows ruling out parentage even of hybrid individuals (Delmotte et al., 2001; Lutes et al., 2011).

4.1.- Mitochondrial and nuclear DNA sequences.

Both Mitochondrial and nuclear DNA molecular markers are used in molecular ecology and evolutionary analyses but their different features make them more appropriate for different uses.

The **mitochondrial DNA** is a small circular molecule ~17 kb in length that encode the major enzymes for oxidative metabolism and ATP production. The mitochondrial genome in animals typically contains 37 genes (13 protein- coding, two ribosomal, and 22 transfer RNA genes) and one major non-coding region, the displacement loop (D-loop) which is responsible for replication and transcription of the molecule.

Mitochondrial genome is inherited cytoplasmically and maternally. Numerous studies have shown that the molecule evolves rapidly, providing substantial amount of variability within and among closely related species (Crease et al., 1989). Therefore, sections of mtDNA such as cytochrome oxidase gene (COI), 12S and 16S ribosomal DNA are widely used for DNA barcoding and phylogeography studies (Lunt et al., 1996; Hebert et al., 2003).

In the genus *Artemia*, the complete mitochondrial genome was sequenced first in *A. franciscana* (Valverde, 1994) and recently in *A. urmiana* and *A. tibetiana* (Zhang, 2013). In *A. franciscana*, mtDNA has

15,822 base pairs (bp) in total length and includes two ribosomal RNAs (12S and 16S), 22 tRNAs, three subunits of cytochrome c oxidase (CO I, II and III), two subunits of the H+ATP synthase (ATPase 6 and ATPase 8), the cytochrome b (Cyt b), and seven subunits of the NADH dehydrogenase (ND 1 to 6 and 4L) (Valverde, 1994).
In *Artemia*, COI sequences have been used for example to explore the patterns of genetic diversity, phylogenetic relationships and to examine the phylogeography of both parthenogenetic strains and sexual species (Hou et al., 2006; Muñoz et al., 2008, 2010, 2013; Maniatsi et al., 2011).

The **nuclear DNA** is contained within the nucleus of eukaryotic cell and encodes for the majority of the genome of these organisms. The structure of nuclear DNA is linear in each chromosome and adheres to Mendelian inheritance, with information coming from two parents, one male and one female.
Phylogenomic analysis of 62 nuclear protein-coding sequences has revealed all the complex arthropod relationships (Regier et al., 2010). In *Artemia*, mitochondrial and nuclear sequences were used together to assess patterns of congruence and, then, resolve the phylogenetic relationship among sexual species (Baxevanis et al., 2006; Hou et al., 2006; Kappas et al., 2009).

In strictly unisexual lineages the whole genome is inherited as a single linkage group, therefore phylogenies based on maternally inherited (e.g. mtDNA) and nuclear markers should correspond perfectly. Indeed, since recombination does not occur, nuclear and mitochondrial genomes

are inherited as one unit. In contrast, if unisexual lineages result from hybridization with interspecific sexual relatives, or if rare sex occurs within unisexual lineages, incongruence between nuclear and mitochondrial phylogenies should be found, which will provide information on the paternal and maternal origin of the hybrid (Simon et al., 2003).

If genotypic diversity in the nuclear and mitochondrial genomes has arisen after the monophyletic loss of sex, parallel divergence in the two genomes is expected. Furthermore, mitochondrial genomes within the obligate parthenogens should form a monophyletic group. In contrast, if asexuality has arisen polyphyletically, there may be a divergence in the mitochondrial and nuclear genomes. Diversity in each genome will reflect the random capture of genotypic diversity from sexual ancestors (Crease et al., 1989).

4.2.- Microsatellites.

Microsatellites (highly variable short tandem repeat) markers are short and tandemly repeatable sequences of 1–6 nucleotides found at high frequency in the nuclear genomes of most taxa (Selkoe and Toonen, 2006). As such, they are also known as simple sequence repeats (SSR), variable number tandem repeats (VNTR) and short tandem repeats (STR). A microsatellite locus typically varies in length between 5 and 40 repeats; the DNA surrounding a microsatellite locus is termed the flanking region. Because the sequences of flanking regions are generally conserved (i.e. identical) across individuals of the same species and sometimes of different species, a particular microsatellite locus can often

be identified by its flanking sequences (Selkoe and Toonen, 2006; Hodel et al., 2016).

Microsatellites occur at thousands of locations within an organism's genome; additionally, they have a higher mutation rate than other areas of DNA leading to high genetic diversity in the form of alleles with different number of repeats. Microsatellite markers are normally very species-specific and, therefore, they must be independently developed for each organism. These markers have been applied to understand molecular taxonomy, hybridization, sex determination, inter and intraspecific differentiation and phylogenetic reconstruction in a wide range of organisms (Selkoe and Toonen, 2006; Hodel et al., 2016).

In *Artemia*, 10 polymorphic microsatellite markers are available for *A. franciscana* (Muñoz et al., 2008) and 14 for diploid parthenogenetic *Artemia* (Muñoz et al., 2008; Noughé et al., 2015a). They have been useful to investigate parentage of hybrid individuals and to characterize parthenogenetic populations (Maniatsi et al., 2011).

5.- PRINCIPAL OBJECTIVES AND STRUCTURE OF THIS THESIS

This thesis explores the origin and evolution of *Artemia* reproductive and genetic diversity, with a special focus on using molecular markers to understand the mechanisms behind the generation of new parthenogenetic lineages, including hybridization and contagious parthenogenesis and the potential role of rare males. Few experimental systems allow a direct comparison of the genetic and evolutionary

consequences of sex versus asexual reproduction. They are organisms showing the coexistence of different reproductive modes. As shown above, *Artemia* includes gonochoric sexual species with separate males and females, and lineages of obligate parthenogenetic populations of different ploidy levels, which often co-occur (Abatzopoulos et al., 2002b). Diploid parthenogenetic lineages produce occasional fully functional rare males (Stefani, 1964; Bowen et al., 1978; MacDonald and Browne, 1987; Amat et al., 1991; Cai, 1993; Mura and Nagorskaya, 2005), which might be involved in the origin of new parthenogenetic lineages (Simon et al., 2003; Innes and Hebert, 1988; Lynch et al., 2008; Engelstädter et al., 2011; Eads et al., 2012). In addition, in *Artemia* interspecific hybridization, which is known to occur (Bowen et al., 1978; MacDonald and Browne, 1987; Cai 1993; Kappas et al., 2009), could also result in the generation of new parthenogenetic lineages. Such characteristics make the brine shrimp an exceptional model system to investigate evolutionary transitions between reproductive systems and to understand the mechanisms generating genetic diversity of asexual lineages in the genus.

Below I give a brief overview of the main objectives to achieve in this study, followed by the summarized description of their attainments, according to chapters that correspond to already published papers. The methodology used to establish laboratory populations of *Artemia*, to set up the cross-mating experiments, to analyze offspring quality and to perform phylogenetic and paternity analyses are described in detail in each chapter.

These chapters maintain the uniformity requirements of the journals in

which they were published, but they were edited to facilitate their reading and their adaptation to the format of this thesis.

CHAPTER 1 explores how and where asexuality evolved in the *Artemia* genus. Previous analyses suggest that diploid parthenogenetic lineages of *Artemia* originated in an unknown region of Central Asia. Consequently, we examine the genetic diversity of diploid asexual lineages focusing our attention to this specific geographic region. We sequence and analyze mitochondrial and nuclear genes from an extensive set of populations of diploid parthenogenetic *Artemia* and sexual species from Central and East Asia to shed light on their evolutionary origin and the geographic origin of the parental taxa. We use phylogenetic analysis to understand how many times the loss of sex occurred in *Artemia* and to find potential discordances between mitochondrial and nuclear markers to infer the possible genetic mechanisms involved in the transition from sexual reproduction to asexuality.

CHAPTER 2 investigates the occurrence and possible reproductive role of *Artemia* rare males. It is an extensive study whose specific aims are: (i) to describe the frequency of males in numerous populations of diploid parthenogenetic *Artemia* from a wide range of geographical locations and to test whether there was a geographic pattern of their distribution; (ii) to describe rare males morphologically in the context of the variation in closely related sexual Asian *Artemia* species; (iii) to assess the reproductive role of rare males performing

cross-mating experiments with females of sexual Asian related species (*Artemia urmiana, Artemia sinica, Artemia tibetiana*, *Artemia* sp. Kazakhstan); (iv) characterize the viability of F1 hybrid offspring and (v) to confirm genetically both the identity and functionality of rare males using DNA barcoding and microsatellite loci in the parents and in the offspring involved in the cross-mating experiments.

CHAPTER 3 investigates whether *Artemia* has the potential of generating parthenogenetic strains through contagious parthenogenesis. For this purpose, (i) we assess the survival and sex ratio of the hybrid ovoviviparous offspring obtained from the previous crosses (chapter 2) between rare males and Asian sexual species females, (ii) we carry out cross-mating experiments between these F1 hybrid individuals to assess their fertility, (iii) we estimate the viability and the reproductive mode of the resulting F2 offspring; (iv) finally we demonstrate genetically that parthenogenetic F2 individuals are indeed the descendants of the original crosses showing that new parthenogenetic lineages can indeed result from rare males fertilizing sexual females.

Part II

ARTICLES

Chapter I

Origin and genetic diversity of diploid parthenogenetic *Artemia* in Eurasia

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Abstract

There is wide interest in understanding how genetic diversity is generated and maintained in parthenogenetic lineages, as it will help clarify the debate of the evolution and maintenance of sexual reproduction. There are three mechanisms that can be responsible for the generation of genetic diversity of parthenogenetic lineages: contagious parthenogenesis, repeated hybridization and microorganism infections (e.g. *Wolbachia*). Brine shrimps of the genus *Artemia* (Crustacea, Branchiopoda, Anostraca) are a good model system to investigate evolutionary transitions between reproductive systems as they include sexual species and lineages of obligate parthenogenetic populations of different ploidy level, which often co-occur. Diploid parthenogenetic lineages produce occasional fully functional rare males, interspecific hybridization is known to occur, but the mechanisms of origin of asexual lineages are not completely understood. Here we sequenced and analysed fragments of one mitochondrial and two nuclear genes from an extensive set of populations of diploid parthenogenetic *Artemia* and sexual species from Central and East Asia to investigate the evolutionary origin of diploid parthenogenetic *Artemia*, and geographic origin of the parental taxa. Our results indicate that there are at least two, possibly three independent and recent maternal origins of parthenogenetic lineages, related to *A. urmiana* and *Artemia* sp. from Kazakhstan, but that the nuclear genes are very closely related in all the sexual species and parthenogenetic lineages except for *A. sinica*, who presumable took no part on the origin of diploid parthenogenetic strains. Our data cannot rule out either hybridization

between any of the very closely related Asiatic sexual species or rare events of contagious parthenogenesis via rare males as the contributing mechanisms to the generation of genetic diversity in diploid parthenogenetic *Artemia* lineages.

Introduction

There is wide interest in understanding how genetic diversity is generated and maintained in parthenogenetic lineages, as it will help clarify the debate of the evolution and maintenance of sexual reproduction. Many asexual species are genetically diverse and this genetic diversity can to some extent ameliorate the lack of meiotic recombination [1,2]. Several different genetic mechanisms underlie transitions from sexual reproduction to asexuality, and these mechanisms influence in turn the genetic diversity of parthenogenetic lineages and their success and persistence [3,4]. However, some mechanisms of origin of parthenogenetic lineages can be recurrent, resulting in many, repeated non-independent but polyphyletic origins.

One mechanism for the polyphyletic origin of parthenogenetic lineages diversity is contagious parthenogenesis [3], in which parthenogenetically produced functional rare males mate with sexual females and transmit parthenogenesis to their offspring. Some parthenogenetic lineages produce functional rare males or invest in male function [3,5,6]. In the presence of sexual females of related lineages or species, rare males could produce fertile hybrid offspring which would inherit the parthenogenesis-inducing alleles. This mechanism has been best studied in the water flea *Daphnia pulex* [4,7–9], but is also known to occur in the aphid *Myzus persicae* [10] and in the parasitoid wasp *Lisyphlebus fabarum* [11]. The genetic consequence of the spread of asexuality via contagious mechanism is the recurrent origin of new parthenogenetic clones, which will capture some genetic diversity of the

maternal sexual species but also maintain some common genomic background from their parthenogenetic ancestor.

A second mechanism is the recurrent generation of multiple parthenogenetic lineages through recent hybridization between related sexual species [3]. Parthenogenesis can result from hybridization between two co-occurring sexual species in vertebrates [12–14] and in invertebrates [3,15,16]. The repeated origin of hybrid asexuals might generate complex patterns of relationships between the parthenogenetic lineages [17].

A third mechanism of polyphyletic origin is through infection by vertically inherited microorganisms, such as *Wolbachia* [3]. Microorganisms associated with parthenogenesis can alter the reproduction of their host to favour their persistence in populations, for example by feminizing or killing males or inducing parthenogenesis [2,18].

If parthenogenetic lineages arise repeatedly trough these mechanisms or a combination of them, their genetic diversity may be comparable to those of sexual populations [1,19,20]. Such repeated transitions between sexual and asexual lineages can generate many related but highly diverse asexual lineages which can potentially lead to confounding estimates of genetic diversity of parthenogenetic lineages, and conclusions of ancient asexuality [16].

Brine shrimps of the genus *Artemia* (Crustacea, Branchiopoda, Anostraca) are a good model system to investigate evolutionary transitions between reproductive systems as they include sexual species and lineages of obligate parthenogenetic populations of different ploidy

level [21]. Parthenogenetic populations are found only in the Old World, where they co-occur with various sexual species, including *A. salina* (Linnaeus 1758) in the Mediterranean region and South Africa [22], *A. urmiana* (Günther 1899) in and around lake Urmia (Iran) and Crimean salt lakes [23], *A. sinica* in Central and Northern China [24], *A. tibetiana* in the Tibetan plateau [25,26], and likely with a yet undescribed sexual species in Kazakhstan [27,28]. *Artemia* species differ in genetic, morphometric, morphological, life history traits [23, 28], and show reproductive isolation, although this is weaker between Asiatic species [25].

Parthenogenetic diploid *Artemia* populations are automictic and most populations produce fully functional males in low proportions (from 1 to 17 per thousand individuals)[29]. These so called rare males can produce fertile offspring when mating with females of sexual Asiatic species [29]. Assessments of the mitochondrial genetic diversity of Mediterranean parthenogenetic *Artemia* populations suggested that there were at least two maternal origins of diploid parthenogenesis from a group of closely related Central Asiatic sexual species [30]: one of the mitochondrial lineages – largely responsible for the recent expansion of diploid parthenogenetic *Artemia* in the Mediterranean – is closely related to those of a sexual undescribed species from Kazakhstan, and the other, rarer lineage, which is closely related to haplotypes of Iranian *A. urmiana*. The occurrence of two diploid parthenogenetic lineages, and the origin of triploid strains from the common parthenogenetic lineage was also supported by a study of microsatellite and mtDNA sequence diversity of parthenogenetic populations [31]. Nuclear gene sequence

variation such as ITS1 [32], also indicated that there were multiple origins of parthenogenesis amongst the sexual species from Asia including *A. urmiana*, *A. tibetiana* and *A. sinica*, but as the ploidy of the samples was not identified, conclusions could not be drawn regarding the origin of diploid parthenogenetic *Artemia*. However, *A. salina* and the two American species, are only distantly related to parthenogenetic lineages [32].

Although diploid parthenogenetic *Artemia* can be identified by their morphology, a genetic marker to characterise would be very useful. In this respect, a study by Manaffar et al. [33] revealed that the digestion of the fragment of exon-7 of Na^+/K^+ ATPase by *Tru1I* restriction enzyme showed a polymorphism that allowed discriminating between sexual species and parthenogenetic populations. The sexuals resulted to be homozygote whereas the parthenogens were heterozygote in this position.

Little is known about the mechanisms of origin of parthenogenetic lineages from the ancestral sexual condition, although the possibility of an infectious origin of parthenogenetic *Artemia* lineages through *Wolbachia* parasites has been ruled out [34]. Given the functionality of rare males when crossed with Asiatic sexual females, Maccari et al. [29] suggested that they may have an evolutionary role through genetic exchange between parthenogenetic lineages and Asiatic related sexual species. Another possibility would be a hybrid origin between two related sexual species which could give rise to parthenogenetic lineages, especially given the evidence for interspecific hybridization in *Artemia* in natural populations [35] and in the laboratory [25]. The limited analysis

of Asiatic diploid parthenogenetic populations, where the coexistence with closely related sexual species is more likely, has also hampered our understanding of the origin of parthenogenetic lineages.

Here we obtained and analysed sequences from one mitochondrial and two nuclear genes (including the putatively diagnostic marker Na^+/K^+ ATPase) from an extensive set of populations of diploid parthenogenetic *Artemia* and sexual species with emphasis on Central and East Asia in order to gain insights into the evolutionary origin of diploid parthenogenetic *Artemia*, its mode of origin and geographic origin of the parental taxa.

Materials and methods

Samples

Cyst samples from 15 Eurasian populations of diploid parthenogenetic *Artemia* (from here onwards, we will use 'parthenogenetic *Artemia*' or 'parthenogens' to refer to diploid parthenogenetic *Artemia* for simplicity) were obtained from the cyst bank collection of the Instituto de Acuicultura de Torre de la Sal (IATS-CSIC) (Figure 1). Laboratory populations were reared from these cyst samples. We assessed the reproductive mode of each population using a sex ratio criterion [29] and whenever the original cyst samples contained an additional sexual species (see Table 1), we obtained pure laboratory parthenogenetic populations using morphometric methods (for culture conditions and other details see [29]). Cyst samples from Asiatic sexual species were also obtained from the same cyst bank collection, including *A. urmiana* from Urmia lake and from Koyashskoe lake, *A. tibetiana* from four lakes

of the Tibetan plateau (Lagkor Co, Gaize, Hayan, Jingyu), an undescribed sexual *Artemia* population from Kazakhstan (originally Artemia Reference Center code - ARC 1039, unknown locality) and *A. sinica* from Yuncheng (China) (Figure 1).

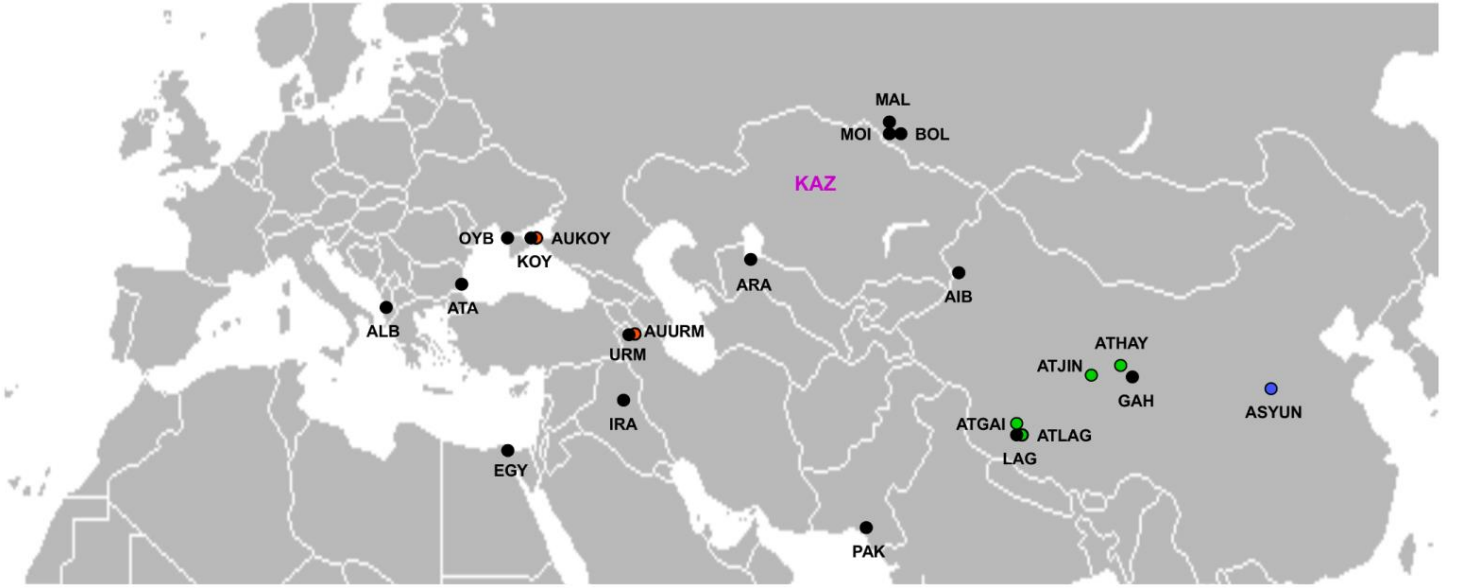

Figure 1. Map of geographic distribution of *Artemia* populations sampled. Black circles represent diploid parthenogenetic populations and coloured ones sexual species. Note that due to its unknown locality, *Artemia* sp. Kazakhstan is represented without circle. See Table 1 for population codes.

Table 1. Detailed information on *Artemia* samples: population name, population codes, location details and additional co-occurring species found in the sample.

	Population	Codes	Coordinates	Other species
Diploid parthenogens	Narte saltern, Albania	ALB	40º34'46''N-19º28'16''E	
	Atanasovko Lake,Bulgaria	ATA	42º34'25''N-27º28'09''E	
	Oybuskoye Lake, Ukraine	OYB	45º16'15"N-33º04'40"E	
	Koyashskoe Lake,Ukraine	KOY	45º02'09"N-36º12'00"E	*A. urmiana*
	Alexandria saltern,Egypt	EGY	31º04'13"N-29º46'57"E	
	Bagdad saltern, Iraq	IRA	33º20'19"N-44º29'32"E	
	Urmia Lake, Iran	URM	37º20'00"N-45º40'00"E	*A. urmiana*
	Aral Sea, Uzbekistan	ARA	45º00'00"N-59º56'00"E	
	Maloje Jarovoe Lake, W.Altai	MAL	52º47'31"N-79º33'39"E	
	Bolshoe Jarovoe Lake, W.Altai	BOL	52º50'N-79º45'E	
	Moimishanskoe Lake, W.Altai	MOI	52º50'N-79º45'E	
	Korangi Creek saltern, Pakistan	PAK	24º47'25"N-67º09'33"E	
	Aibi Lake, China	AIB	44º45'42"N-82º51'54"E	
	Lagkor Co Lake, Tibet	LAG	32º03'N-84º13'E	*A. tibetiana*
	Gahai Lake, China	GAH	36º58'18"N-98º09'53"E	
Sexuals				
A. urmiana	Koyashskoe Lake, Ukraine	AUKOY	45º02'09"N-36º12'00"E	*diploid parthenogenetic*
A. urmiana	Urmia Lake, Iran	AUURM	37º20'00"N-45º40'00"E	*diploid parthenogenetic*
Kazakhstan sp.	*unknown, Kazakhstan*	KAZ	?	
A. tibetiana	Lagkor Co Lake, Tibet	ATLAG	32º03'N-84º13'E	*diploid parthenogenetic*
A. tibetiana	Gaize Lake, Tibet	ATGAI	32º20'N- 84º10'E	
A. tibetiana	Jingyu Lake, Tibet	ATJIN	36º03'N-89º09'E	
A. tibetiana	Hayan Lake, Tibet	ATHAY	36º03'N-100º11'E	
A. sinica	Yuncheng saltern, China	ASYUN	35º00'N-111º00'E	

DNA isolation, polymerase chain reaction, and sequencing

Total DNA was extracted from cysts using a modified HotSHOT protocol [36]. We amplified fragments of one mitochondrial (cytochrome c oxidase subunit I, COI) and two nuclear genes (internal transcribed spacer 1, ITS1, and Na^+/K^+ ATPase).

The COI fragment was amplified using the primers HCO2198 and LCOI490 [37]. 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 (producing 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 72°C, with a final step of 5 min at 72°C.

PCR of the ITS1 region was performed using primers PTF and PTR [38] in a total volume of 30 µl consisting of 3 µl of template DNA, 0.2 mM of each nucleotide, 0.2 µM of each primer, 0.03 U of *Taq* polymerase (Bioline) and 10×Bioline buffer (producing a $MgCl_2$ final concentration of 1.5 mM) using the following conditions: a cycle of 3 min at 95 °C, followed by 35 cycles of 60 s at 95°C, 50 s at 59°C, and 90 s at 72°C, and a final step of 7 min at 72°C.

A fragment of 280-bp, representing exon-7 of Na^+/K^+ ATPase, was amplified using the primers designed by [33]. PCR was performed in a total volume of 20 µl, containing 3µl of template DNA, 0.2 mM of each nucleotide, 0.2 µM of each primer, 0.02 U of *Taq* polymerase (Bioline) and 10×Bioline buffer (producing a $MgCl_2$ final concentration of 2 mM) using the following program: 94°C for 2 min, 32 cycles at 94°C for 25 s

followed by 56°C for 25 s and 72°C for 1 min, and a final extension at 72°for 3 min.

All amplifications were performed on a Verity 96 well thermal cycler (Applied Biosystems). PCR products were purified and sequenced by Macrogen Europe Inc. (Amsterdam, The Netherlands). The electrophoregrams were checked by eye using CodonCode Aligner v. 3.5 (CodonCode Corporation, Dedham, MA). COI and ITS1 sequences generated were deposited in GenBank (for Accession Numbers see Tables 2 and 3) and all alignments are available in Dryad (http://doi.org/10.5061/dryad.kd0k4).

Sequence alignment and phylogenetic analyses

The COI fragment was sequenced in 258 individuals, 165 of which were diploid parthenogens (see Table 2). For the nuclear markers we sequenced a subset of these individuals, 44 for the ITS1 region (two for each population sampled) and 63 for the Na^+/K^+ ATPase fragment (Table 3).

To the COI marker alignment we also added 55 published available sequences from GenBank (parthenogenetic rare males and females KC193638-KC193677, parthenogenetic haplotypes DQ426824-DQ426826, haplotypes from parthenogenetic populations and from *Artemia sp.* Kazakhstan GU591380-GU591389 and *A.tibetiana* EF615588-89). Sequences were aligned using ClustalW in MEGA5 [39] using the default settings and checked by eye. The number of polymorphic and parsimony informative sites was computed with MEGA5.

Table 2. COI samples and haplotypes: sample size; number of haplotypes per population; πJC, corrected nucleotide diversity; *Hd,* gene diversity.

Population code	Sample size	Number of haplotypes	Haplotypes and sample size	*πJC*	*Hd*	*Acc.Num*
Diploid parthenogens						
URM	20	2	APD02(17), APD05(3)	0.0009	0.2684	KF707710-19, KF707765-74
KOY	15	1	APD02(15)	0.0000	0.0000	KF707700-09, KF707805-09
ATA	12	3	APD02(10), APD07(1), APD12(1)	0.0071	0.3182	KF707720-26, KF707800-04
IRA	19	1	APD02(19)	0.0000	0.0000	KF707727-45
EGY	5	2	APD02(3), APD05(2)	0.0020	0.6000	KF707785-89
ALB	10	2	APD02(2), APD05(8)	0.0012	0.3556	KF707790-99
PAK	10	1	APD02(10)	0.0000	0.0000	KF707775-84
OYB	10	2	APD10(3), APD08(7)	0.0008	0.4667	KF707810-19
ARA	6	4	APD02(2), APD11(2), APD13(1),APD14(1)	0.0021	0.8667	KF707820-25
MAL	10	3	APD02(3), APD15(5),APD16(2)	0.0015	0.6889	KF707826-35
BOL	9	3	APD02(7), APD15(1),APD16(1)	0.0007	0.4167	KF707836-44
MOI	10	3	APD02(2),APD18(7), APD19(1)	0.0026	0.5111	KF707865-74
AIB	9	3	APD02(5), APD09(1),APD10(3)	0.0136	0.6389	KF707746-54
GAH	10	1	APD11(10)	0.0000	0.0000	KF707755-64
LAG	10	3	APD02(4), APD05(1),APD17(5)	0.0145	0.6444	KF707845-54
Sexuals						
KAZ	10	4	KAZSEX06(2), KAZSEX05(2), KAZSEX03(4), KAZSEX08(2)	0.0038	0.8000	KF707671-80
AUURM	20	12	AUURM01(1), AUURM02(1), AUURM03(1), AUURM04(7), AUURM05(1), AUURM06(1), AUURM07(1), AUURM08(1), AUURM09(1), AUURM10(2), AUURM11(2), AUURM12(1)	0.0074	0.8790	KF707681-90, KF707875-84
AUKOY	9	2	AUKOY01(5),AUKOY02(4)	0.0027	0.5556	KF707691-99
ATLAG	20	4	AT01(17), AT08(1), AT09(1),AT10(1)	0.0007	0.2842	KF707855-64, KF707919-28
ATGAI	5	1	AT01(5)	0.0000	0.0000	KF707895-99
ATHAY	9	4	AT02(3),AT03(4), AT04(1), AT05(1)	0.0036	0.7500	KF707900-08
ATJIN	10	3	AT05(4), AT06(1), AT06(5)	0.0015	0.6444	KF707909-18
ASYUN	10	2	AS01(6), AS02(4)	0.0017	0.5333	KF707885-90

Table 3. Nuclear loci summary of polymorphic sites in each *Artemia* population. A dash means that heterozygote individuals were found, a forward slash indicate that the position is polymorphic in the population, with both homozygote and heterozygotes found.

		ITS					NA+/K+ ATPase						
		Sample size	**522bp**	**721bp**	**695bp**	***Acc. Num.***	**Sample size**	**26bp**	**56bp**	**80bp**	**95bp**	**140bp**	**152bp**
Diploid parthenogens	**ALB**	2	C	C	T	KF736274,75	2	C	T	T	A	G-T	T
	ATA	2	A	C	T	KF736258,59	2	C	T	T	A	G-T	T
	OYB	2	A	C	T	KF736276,77	3	C	T	T	A	G-T	T
	KOY	2	C-A / A	C	T	KF736255-57	2	C	T-C	T	A	G-T	T
	EGY	2	C	C	T-A	KF736266-69	2	C	T	T	A	G-T	T
	IRA	2	A	C	T	KF736264,65	4	C	T	T	A	G-T	T
	URM	2	C	C	T	KF736253,54	2	C	T-C / T	T-A / T	A	G-T	T
	ARA	2	C / A	C	T	KF736278,79	2	C	T-C	T	A	G-T	T
	MAL	2	C	C	T	KF736280,81	2	C	T-C	T	A	G-T	T
	BOL	2	C	C	T	KF736282,83	2	C	T	T	A	G-T	T
	MOI	2	C	C	T	KF736284,85	3	C	T-C	T-A	A	T / G-T	T
	PAK	2	A	C-T	T	KF736270-73	2	C	T	T	A	G-T	T
	AIB	2	C	C	T	KF736260,61	2	C	T	T	A	G-T	T
	LAG	2	C-A	C	T	KF736286-89	4	C	T	T	A	G-T	T
	GAH	2	C	C	T	KF736262,63	2	C	T-C	T-A	T-A	T	T
Sexuals	**AUKOY**	2	C	C	T	KF736251,52	5	C	T	T	A	T	T
	AUURM	2	C	C	T	KF736249,50	4	C	T	T	A	T	T
	KAZ	2	C	C	T	KF736247,48	6	C	T-C	T-A	A	T	T
	ATLAG	2	C	C	T	KF736290,91	3	C	T	T	A	T	T
	ATGAI	2	C	C	T	KF736294,95	4	C	T	T	A	T	T
	ATJIN	2	C	C	T	KF736291,92	3	C	T	T	A	T	T
	ASYUN	2	C	T	T	KF736296,97	2	T	T	T	A	T	C-T

Patterns of nucleotide diversity, synonymous and non-synonymous substitutions, population haplotype and nucleotide diversity were computed using DnaSP5 [40].

Before phylogenetic reconstruction, sequences were collapsed into haplotypes using FaBox v.1.40 [41]. For both COI and ITS1 markers, phylogenetic analysis was implemented using Maximum Likelihood (ML) approaches in MEGA5 and Bayesian approaches in MrBayes v 3.2.2 [42] on the Cipres Science Gateway portal [43]. We estimated the best-scoring ML tree using the model selected by the inbuilt model generator in MEGA5. The robustness of the branches was assessed with 1000 bootstrap pseudo-replicates. For Bayesian analysis we used the default parameters on the Cipres gateway. In two simultaneous runs, four Markov chains (one cold and three heated) were started from a random tree and run for 1,000,000 generations with sampling frequency every 100 generations. The first 2500 trees were discarded as burn-in.

In addition, we constructed a statistical parsimony haplotype network for COI using TCS v. 1.21 [44] to visualize the genealogical relationships between the mitochondrial haplotypes. For this analysis we used all the COI sequences generated here, two *A. tibetiana* sequences from GenBank (EF615587-8), the sequences from Maccari et al. [29] and Muñoz et al. [30]. For sequences from the latter paper, including Mediterranean populations of diploid parthenogenetic *Artemia*, we reconstructed the sequence of each individual from the paper haplotype information.

Results

Cytochrome oxidase subunit I

The sequence alignment was trimmed to 614 bp long, with all the 313 sequences of the same length. No insertions, deletions or stop codons were present. The COI alignment consisted of 143 variable sites and 133 parsimony informative sites with a total of 144 synonymous and 10 nonsynonymous substitutions.

The sequences generated here collapsed into 45 haplotypes (see Table 2). No haplotype was shared between parthenogens and sexuals, despite both parthenogens and sexuals coexisting in three of the sampled populations. Diploid parthenogenetic populations had a total of 15 haplotypes, 11 of them newly found in this study. APD02, the most common and widespread haplotype, was found in 99 individuals from 13 out of the 15 diploid parthenogenetic populations sampled. The next most common haplotype, APD05 was found in four populations (URM, EGY, ALB and LAG), APD10 in two populations (OYB and AIB), as APD11 (ARA and GAH). Haplotypes APD15, APD16 were found in both populations from the Altai (MAL and BOL). The remaining nine haplotypes were found in single populations.

The sexual populations sequenced here had 30 COI haplotypes. We found four exclusive haplotypes in the undescribed sexual species from Kazahkstan, 12 in *A. urmiana* from Urmia Lake, and two in *A. urmiana* from Koyashskoe Lake, with no shared haplotypes between these *A. urmiana* populations. The populations of *A. tibetiana* had 11 haplotypes. The population of *A. sinica* was characterized by two haplotypes. The highest haplotype diversity (*Hd*) was found in *A. urmiana* from lake

Urmia (0.88) and in the parthenogenetic population from Aral Sea (0.87) (Table 2). Populations from Koyashskoe Lake, Bagdad saltern, Korangi Creek saltern and Gahai Lake amongst the parthenogens and *A. tibetiana* from Gaize Lake among the sexuals were characterized by a single haplotype.

The nucleotide diversity values (π-values) ranged from 0.0000 to 0.0145 (Table 2). The highest value was found in two parthenogenetic populations from Lagkor Co and Aibi Lake, but the sexual populations from Urmia Lake, Kazakhstan and Hayan Lake and the parthenogenetic population from Atanosovko Lake also showed high π-values compared with the rest of the populations.

The ML tree (Figure 2) was obtained using the Tamura-3 parameter (T92) plus gamma model, the one selected by the inbuilt model generator in MEGA5. The tree showed that all diploid parthenogenetic *Artemia* haplotypes, plus the haplotypes of *A. urmiana* populations, *Artemia* sp. Kazakhstan and the haplotypes of *A. tibetiana* from Lagkor Co and Gaize Lake formed a highly supported monophyletic lineage. A group of diploid parthenogenetic *Artemia* haplotypes formed a polyphyletic, not well supported assemblage amongst haplotypes from both *A. urmiana* populations (lineage group A). A second group of haplotypes, including the most common APD02 haplotype, formed a monophyletic, but not highly supported lineage, closely related to *Artemia* sp. Kazahkstan and to the lineage of *A. tibetiana* (which we called lineage group B). The haplotype from Kujalnik (rmKUJ1), obtained in two rare males [29] formed a well supported sister branch to those containing all other parthenogenetic.

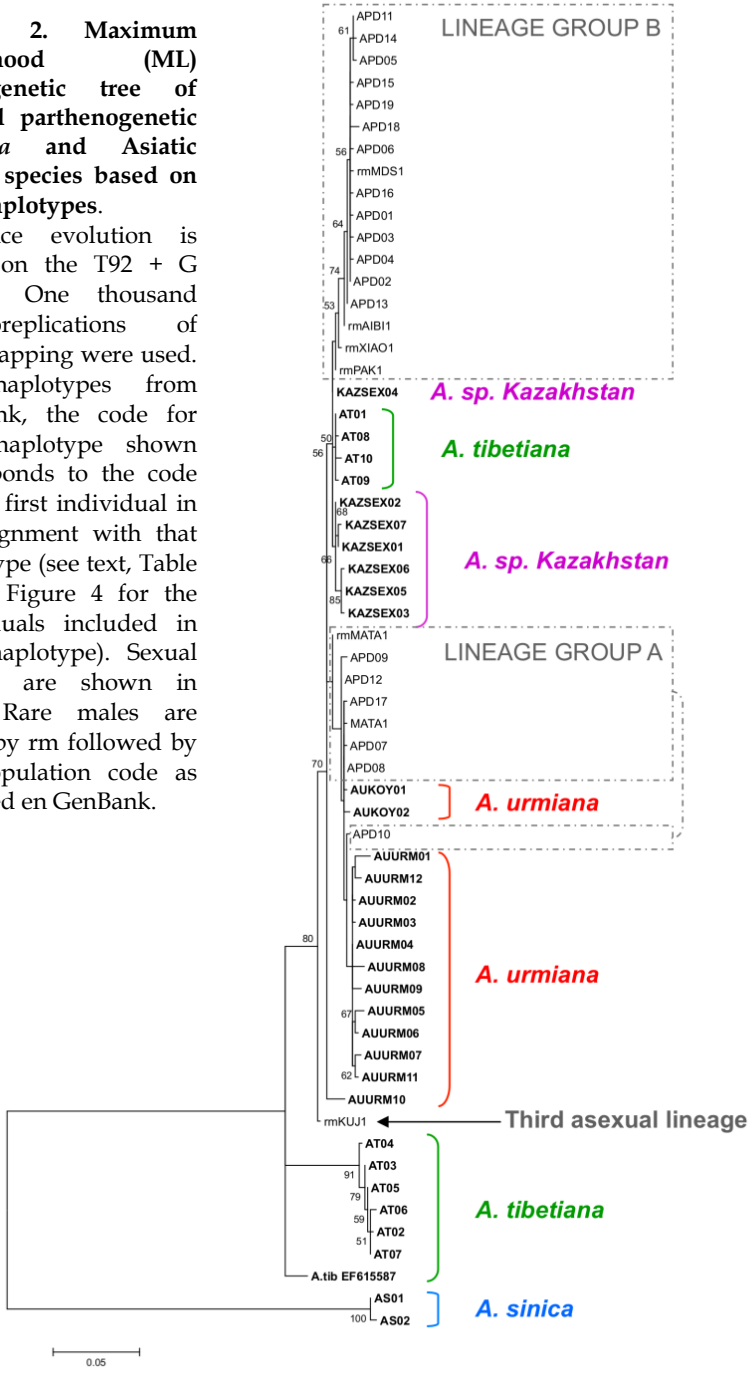

Figure 2. Maximum Likelihood (ML) phylogenetic tree of diploid parthenogenetic *Artemia* and Asiatic sexual species based on COI haplotypes.
Sequence evolution is based on the T92 + G model. One thousand pseudoreplications of bootstrapping were used. For haplotypes from GenBank, the code for each haplotype shown corresponds to the code for the first individual in the alignment with that haplotype (see text, Table 2 and Figure 4 for the individuals included in each haplotype). Sexual species are shown in bold. Rare males are noted by rm followed by the population code as reported en GenBank.

The mtDNA lineages of the other two *A. tibetiana* populations (Hayan and Jingyu Lake) and *A. sinica* were only distantly related to those of diploid parthenogenetic *Artemia*. The Bayesian consensus tree (Figure 3) showed a similar topology, although it resolves the relationships of two *A. tibetiana* lineages. *A. tibetiana* from GenBank (EF615587) forms a highly supported branch with all diploid parthenogens, *A. urmiana*, *Artemia* sp. Kazakhstan and the haplotypes of *A. tibetiana* from Lagkor Co and Gaize Lake. Lineage group A, with the exception of rmMATA1, together with all *A. urmiana* haplotypes forms a well supported lineage. Lineage group B forms a well supported monophyletic lineage and its relationship with *Artemia* sp. Kazakhstan and the haplotypes of *A. tibetiana* from Lagkor Co and Gaize Lake was also highly supported. Further differences with the ML analysis are represented by the position of AURM010, which in the Bayesian analysis falls at the base of the rest of *A. urmiana* haplotypes and Lineage group A, and by the position of rmMATA1 which forms a polytomy more basal in the tree, instead of belonging to lineage group A.

The statistical parsimony network shows the relationship between the mitochondrial haplotypes of parthenogenetic and related sexual species more clearly (Figure 4). There were four unlinked networks. The two haplotypes from *A. sinica* formed a network, the two *A. tibetiana* populations from Hayan and Jinyu Lake resulted in a second haplotype network, and the two *A. tibetiana* sequences from GenBank (EF615587-88) formed a third network. The remaining haplotypes including all diploid parthenogenetic samples, *A. urmiana*, *Artemia* sp. from Kazakhstan and the *A. tibetiana* populations of Lagkor Co and

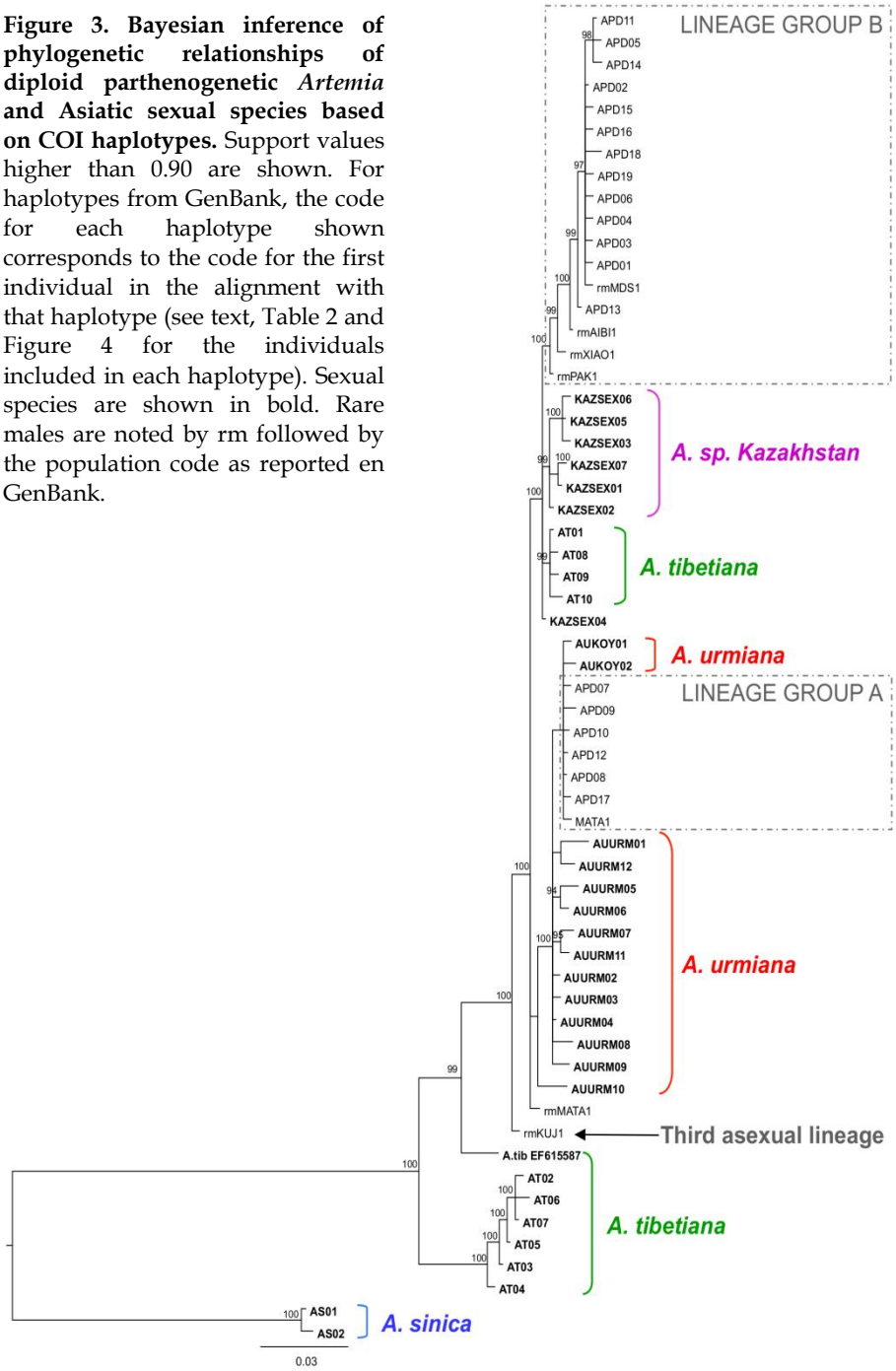

Figure 3. Bayesian inference of phylogenetic relationships of diploid parthenogenetic *Artemia* and Asiatic sexual species based on COI haplotypes. Support values higher than 0.90 are shown. For haplotypes from GenBank, the code for each haplotype shown corresponds to the code for the first individual in the alignment with that haplotype (see text, Table 2 and Figure 4 for the individuals included in each haplotype). Sexual species are shown in bold. Rare males are noted by rm followed by the population code as reported en GenBank.

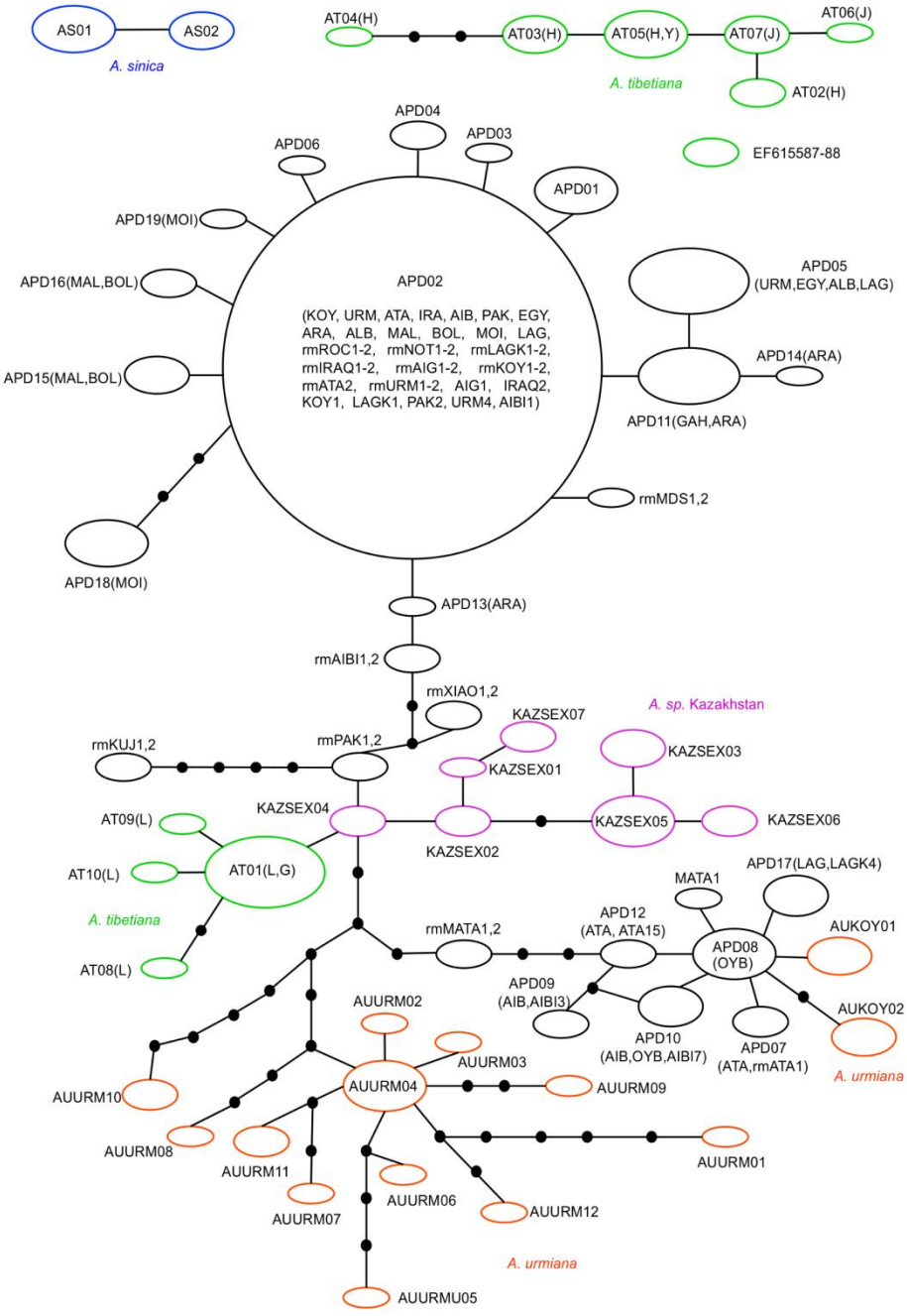

Figure 4. Statistical Parsimony networks showing the nested relationships of diploid parthenogenetic *Artemia* haplotypes and Asiatic sexual species.
For further details see full caption on the next page.

Figure 4. Statistical Parsimony networks showing the nested relationships of diploid parthenogenetic *Artemia* haplotypes and Asiatic sexual species. Black circles represent diploid parthenogenetic *Artemia* haplotypes and coloured circles represent Asiatic sexual species. Circle diameter is proportional to the relative haplotype frequency. Connecting lines indicate single substitutions and small black circles represent putative missing haplotypes. The haplotypes codes correspond to those listed in Table 2 or those from GenBank. Rare males are noted by rm followed by the population code as reported in GenBank.

Gaize Lake were joined in a single network. Haplotypes of diploid parthenogenetic *Artemia* formed three distinct mitochondrial lineage groups as in the phylogenetic reconstructions. Lineage group A, with eight haplotypes, is nested within the diversity of *A. urmiana* haplotypes and most closely related to haplotypes from Koyashkoe Lake population. This is a relatively rare parthenogenetic lineage, but found at very geographically widespread populations (Atanosovsko Lake, Oybuskoye Lake, Lagkor Co Lake, la Mata Lagoon and Aibi Lake parthenogenetic populations). Lineage group B is more common and widespread, and is formed by the common haplotype APD02 and a number of closely related ones forming a star-like network. Lineage group B is closely related to haplotypes from *A. tibetiana* from Lagkor Co and Gaize Lake (AT01, AT08, AT09 and AT10) and *Artemia* sp. from Kazakhstan (KAZSEX01-07), which are also closely related between them. There is no geographic association of the two lineages with a well-defined region because both diploid parthenogenetic haplotype lineage groups coexist in Atanosovsko Lake (ATA), Aibi Lake (AIB) and Lagkor Co Lake (LAG) populations. Some haplotypes found exclusively in rare males from diploid parthenogenetic populations of diverse origins (rmPAK from Korangi Creek in Pakistan; rmXIAO from Xiaotan in

China; rmMATA from La Mata in Spain) appeared in the center of the network, and were more closely related to haplotypes of sexual populations. The haplotype from rare males of Kujalnik (rmKUJ from Kujalnik in Ukraine) formed a separate branch to the rest, and would be a third group of parthenogenetic lineages.

ITS-1

The ITS1 sequences, excluding gaps in the alignment, ranged from 991 (*A. tibetiana*, *Artemia* sp. from Kazakhstan, *A. urmiana* from Koyashskoe lake and all the parthenogens) to 1000 bp (*A. sinica*), including the sequences of *A. urmiana* from Urmia lake which have a variable length (994-999 bp). The final ITS1 alignment was 1002 bp long, with 34 variable sites and 28 parsimony informative sites and collapsed into 14 haplotypes. Evidence of heterozygosity was found in 5 parthenogenetic populations and allele identification in these was straightforward (Table 3).

Prior to the phylogenetic analysis, we collapsed identical haplotypes for each population. Both phylogenetic reconstructions (Maximum Likelihood and Bayesian analysis) had a virtually identical topology and branch support (Figure 5). The ML tree was obtained using the Hasegawa-Kishino-Yano model, the one selected by the inbuilt model generator in MEGA5. It showed *A. sinica* as the most divergent species. The remaining samples were very closely related. The parthenogenetic samples had a total of nine very closely related haplotypes, one of them found in nine populations, was shared with both *Artemia* sp. from Kazakhstan and one of the haplotypes from the Iranian *A. urmiana,*

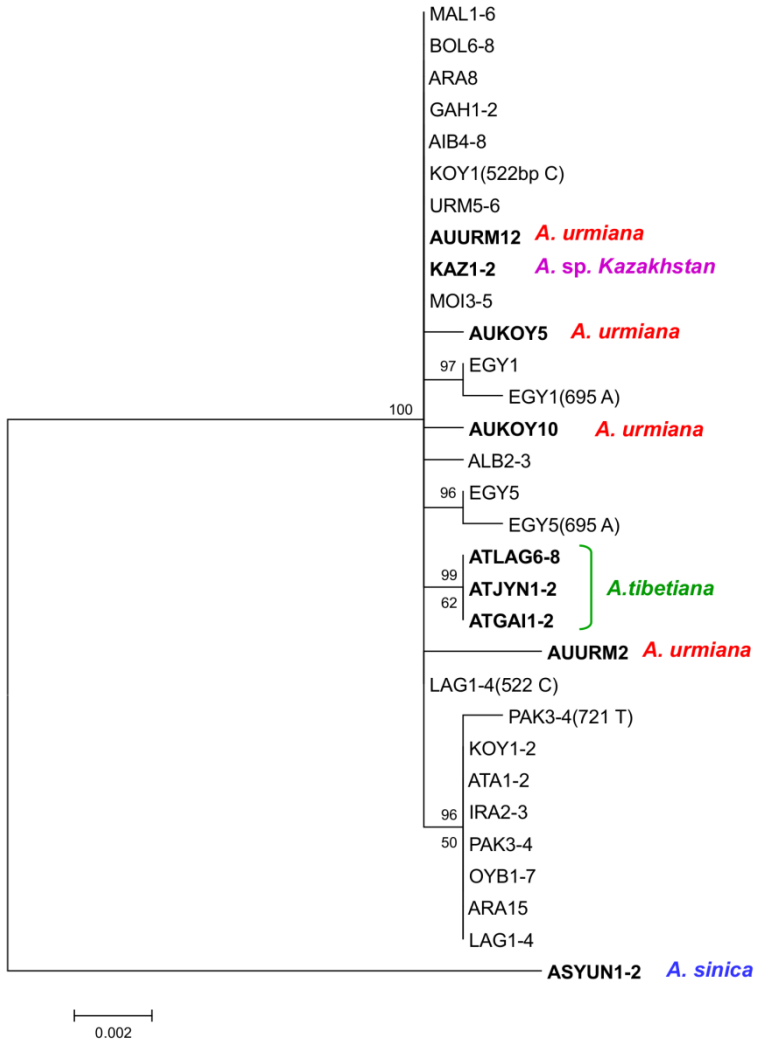

Figure 5. Phylogenetic relationships of diploid parthenogenetic *Artemia* and Asiatic sexual species based on ITS-1 sequences. The topology inferred by Maximum Likelihood (ML) method using HKY model is shown. Bayesian (BA) phylogenetic reconstruction showed a very similar topology. The ML bootstrap values higher than 50 are shown below the branch, and the Bayesian support values over 90% are shown above the branch. Haplotypes found in each population are shown, with population codes corresponding to those listed in Table 3. Sequences corresponding to heterozygous individuals are noted with the polymorphic site in parenthesis.

although this latter haplotype contained an indel. The populations of *A. urmiana* from Koyashskoe Lake and *A. tibetiana* present different haplotypes, although still closely related to the parthenogenetic ones.

Na^+/K^+ ATPase

The Na^+/K^+ ATPase alignment was 160 bp long and consisted of sequences of 63 individuals. The alignment did not contain indels and had nine polymorphic sites (Table 3). Evidence of heterozygosity was found in all parthenogenetic populations and in only the sexual population from Kazakhstan. The populations from Moimishanskoe Lake (Altai), Gahai Lake (China) and Urmia Lake (Iran) share the same alleles at all polymorphic sites with the sexual population from Kazakhstan (see Table 3).

Discussion

In order to shed light on the origin and evolution of parthenogenesis in *Artemia,* we explored the genetic variability of nuclear and mitochondrial DNA of diploid parthenogenetic strains and sexual species, with an emphasis on Asia, the region considered to be the most likely centre of origin of asexual lineages [29–31]. Our analyses confirmed the existence of at least two and possibly three maternal clades of diversity, two of them most related to two different sexual *Artemia* species, *A. urmiana* and *Artemia* sp. Kazakhstan in agreement with Muñoz et al. [30], but also revealed a possibly new lineage of parthenogenetic lineages represented by KUJ [29]. Overall, nuclear genes indicate that diploid parthenogenetic *Artemia* is very closely

related to *A. urmia*na, *Artemia* sp. *Kazakhstan* and *A. tibetiana*, with the exclusion of *A. sinica.* Both nuclear and mitochondrial data for *A. sinica* are very divergent to those of diploid parthenogens, suggesting that this species did not contribute to the genetic diversity of diploid parthenogenetic *Artemia*. Our survey substantially expands our knowledge of its genetic diversity in Eurasia.

Our geographically wider number of *Artemia* populations sampled, inclusion of rare males and samples of a recently found population of *A. urmiana* not sequenced before revealed that the lineages in Muñoz et al [45] are not highly supported phylogenetically, as we found further intermediate haplotypes and also identified the key role of the new *A. urmiana* population from Koyashskoe Lake. Furthermore, we found that the less common mitochondrial group (A) is closely related to haplotypes newly sequenced here from *A. urmiana* from Koyashskoe Lake, but occupies a non-monophyletic position in the network between both *A. urmiana* populations, which appears incompatible with a mutational origin, and points to a possible event of contagious parthenogenesis. In contrast, the most common lineage (B), is monophyletic and closely related both to the haplotypes of *Artemia* sp. from Kazakhstan, and to those of two *A. tibetiana* populations from Lagkor Co and Gaize lakes, which represent a new lineage of *A. tibetiana* (see below). Our analysis also revealed a possibly further lineage, so far only found in rare males from Kujalnik population, indicating that they might be present in some populations at low frequencies, maybe resulting from the emergence of new parthenogenetic lineages [29].

In agreement with previous work [30,38], our results support that the Asiatic sexual species *A. urmiana*, *A. tibetiana* and the undescribed species from Kazakhstan, are closely related such that they might be considered a species complex, despite clear morphological differences [29,46]. This is further supported by experimental crosses showing that, under laboratory conditions there is a proportion of fertile interspecific crosses between these sexual species, indicating weak post mating isolating barriers to gene flow [25].

A. tibetiana contains several divergent, polyphyletic mtDNA lineages, but, in contrast, its nuclear diversity is very homogeneous (monomorphic ITS1 and ATP) and shows little or no differentiation to *A. urmiana* and *Artemia* sp. Kazakhstan. A possibility to explain this pattern is that introgression from other species, in particular from females of *Artemia* sp. Kazakhstan, has resulted on capture of mitochondrial lineages. The genetic diversity of this species needs to be explored further and its taxonomic status might have to be re-evaluated. Given that we have a limited number of samples from *A. tibetiana*, and the richness of hypersaline habitats in Tibet is high [47,48], it is likely that the level of diversity within *A. tibetiana* might still be underestimated. The mitochondrial lineages of *A. tibetiana* are diverse and the genetic diversity of the rest of the Asiatic species appears to be a subset of it, therefore, *A. tibetiana* might have a key role in the origin of the species complex and the origin of parthenogenetic lineages.

Although mitochondrial markers have allowed us to identify the minimum number of maternal origins of each diploid *Artemia* parthenogenetic lineage, nuclear markers should provide information on

both parental species and therefore, shed some light on their modes of origin. For example, diploid parthenogenetic lineages resulting from hybridization between conspecific or interspecific sexuals are expected to have a characteristic signature of high heterozygosity, with diploid asexual lineages containing alleles typical of both parental species [49]. If asexuality arises by contagious parthenogenesis through rare males, we could expect a different maternal origin and possibly distinctive genomic component of parthenogenetic lineages. However, repeated gene flow through contagious parthenogenesis should result in a regular emergence of asexual strains and the genetic differentiation between asexuals and sexuals relatives should be low. Our nuclear analysis shows that ITS-1 from parthenogens is closely related to *Artemia* sp. from Kazakhstan, *A. tibetiana* and *A. urmiana*. Some parthenogens and *Artemia sp.* from Kazakhstan share the same haplotype, whereas *A. sinica* is very divergent. Baxevanis et al. [32] found four parthenogenetic *Artemia* lineages, three of which clustering with *A. urmiana* and *A. tibetiana* and another one more closely related to *A. sinica*. The closely related nature of the sexual species from Asia and the lack of divergence between the investigated nuclear genes, however, make it difficult to assess the mechanism or mechanisms of origin of parthenogenesis. However, our mitochondrial phylogenies do not provide clear evidence of rampant contagious parthenogenesis, as it would result in repeated occurrences of new asexual strains and higher mitochondrial diversity. Moreover, parthenogenetic populations coexisting with the known populations of *A. urmiana* do not have a local origin, as they do not share any haplotypes with the local sexual population. On the contrary, only three mtDNA

lineages are found, one of them a minor lineage identified in rare males. That might indicate either that some occasional contagious parthenogenesis does occur or that these are low frequency parthenogenetic lineages with a higher propensity to produce rare males, and have persisted in populations at low frequency. These events would increase the diversity of parthenogenetic strains but playing little role on the geographical expansion and success of parthenogenetic lineages.

The three mtDNA lineages in diploid parthenogenetic *Artemia* are not differentiated in their nuclear DNA. Although this pattern could result both from repeated hybridization between two similar lineages or from a contagious event between one lineage group and another, the possible existence of contagious parthenogenesis is also supported by microsatellite data. The set of microsatellite loci developed for diploid parthenogenetic *Artemia* [45] did not amplify consistently in all the sexual species from Asia [29,31], suggesting that parthenogenetic strains have enough nuclear distinctiveness, and this may be more consistent with contagious parthenogenesis than with a hybrid origin, although it is possible that different mechanisms underlie the origin of each lineage group.

As we used Manaffar et al.'s [33] primers to amplify and sequence a fragment presumably containing a diagnostic SNP between parthenogenetic and sexual strains, we were able to test their finding on a wider array of samples. Our results indicate that, although most samples from a wide range of parthenogenetic populations do meet this criterion (position 140 in our alignment, see Table 3), we identified some parthenogenetic populations that were homozygous for this position

(GAH and MOI) and do not confirm the universality of the polymorphism at this site to distinguish parthenogenetic and sexual populations.

Our data cannot rule out either hybridization between any of the very closely related Asiatic sexual species, or rare events of contagious parthenogenesis via rare males as the contributing mechanisms to the generation of genetic diversity in diploid parthenogenetic *Artemia* lineages. Although our work has provided information on the origin of diploid parthenogenetic *Artemia*, much is still unknown, and the close relationship of sexual species has hampered this, therefore, more research possibly using genomic approaches is needed to disentangle the evolutionary origin of diploid parthenogenetic *Artemia*.

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Chapter II

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) characterise 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 out 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-fertilising 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). 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 feminising 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; Simon et al., 1999; Rispe 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 haven 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 geographic 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 in Amat (1980). Parthenogenetic females were then allowed to reproduce, and their naupliar or encysted offspring used to obtain pure cultured laboratory parthenogenetic populations.

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	14188	14	0.99
Rocío, Cádiz, Spain[1]	36°51'19"N-06°20'14"W	2001	*A. salina*	12202	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*	12504	12	0.96
Calpe, Alicante, Spain	38°38'37"N-00°03'60"W	1986	-	12000	12	1.00
La Mata, Alicante, Spain[1]	38°02'08"N-00°42'02"W	1989	-	19690	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	-	12320	1	0.08
Aveiro, Portugal	40°38'01"N-08°40'49"W	1992	-	18105	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[1]	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*	12103	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

[1] males of these populations were used in the multivariate discriminant analysis.

Table 1. Continued.

Population	Coordinates	Year	Other species	Individuals sexed	Number of males	Males/1000 individuals
Molentargius, Sardinia, Italy	39°13'51"N-09°12'33"E	2004	*A. salina*	1631	8	4.90
Notteri, Sardinia, Italy[1]	39°07'04"N-09°30'55"E	2009	*A. salina*	5715	16	2.80
Atanosovsko, Bulgaria[1]	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[1]	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	-	12656	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	-	13092	0	0.00
Tenefé, Gran Canaria, Spain	27°48'51"N-15°25'19"W	2005	-	14810	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

[1] males of these populations were used in the multivariate discriminant analysis.

Table 1. Continued.

Population	Coordinates	Year	Other species	Individuals sexed	Number of males	Males/1000 individuals
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	-	10066	10	0.99
Bjurliu, Kazakhstan	51°49'00"N-78°00'00"E	1989	-	18946	181	9.55
Aral Sea, Uzbekistan	44°43'41"N-59°34'22"E	2004	-	1497	3	2.00
Bagdad, Iraq[1]	33°17'06"N-44°15'13"E	2004	-	41568	398	9.57
Urmia, Iran[1]	37°36'20"N-45°28'21"E	1988	*A. urmiana*	4619	78	16.89
Korangi Creek, Pakistan[1]	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[1]	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[1]	36°07'38"N-120°04'36"E	1992	-	16570	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

[1] males of these populations were used in the multivariate discriminant analysis.

Culture conditions

Hatching was induced by incubating cyst samples under standard conditions, in 35 gL^{-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 gL^{-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 geographic locations (Table 1). Individuals were reared until maturity in mass cultures as detailed above and the sex ratio for each population (males per 1,000 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 geographic 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 if 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 hotspots 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 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 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 by eye using CodonCode Aligner v. 3.5 (CodonCode Corporation, Dedham, MA). 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 Neighbor-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).

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

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. 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. 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 two 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 by 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 (Apdq02TAIL, 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 2x QIAGEN® (Qiagen, Hilden, Germany) PCR Master Mix (including 3mM $MgCl_2$, dNTP Mix and HotStarTaq® Polymerase; Qiagen), 2 µL of 10x Primer Mix (2µM 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, 53ºC for 90 s, 72ºC for 90 s, followed by 60ºC 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 geographic 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 significant male ratio hotspots, Urmia Lake, Bagdad Saltern and Bjurliu Lake (Figure 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.

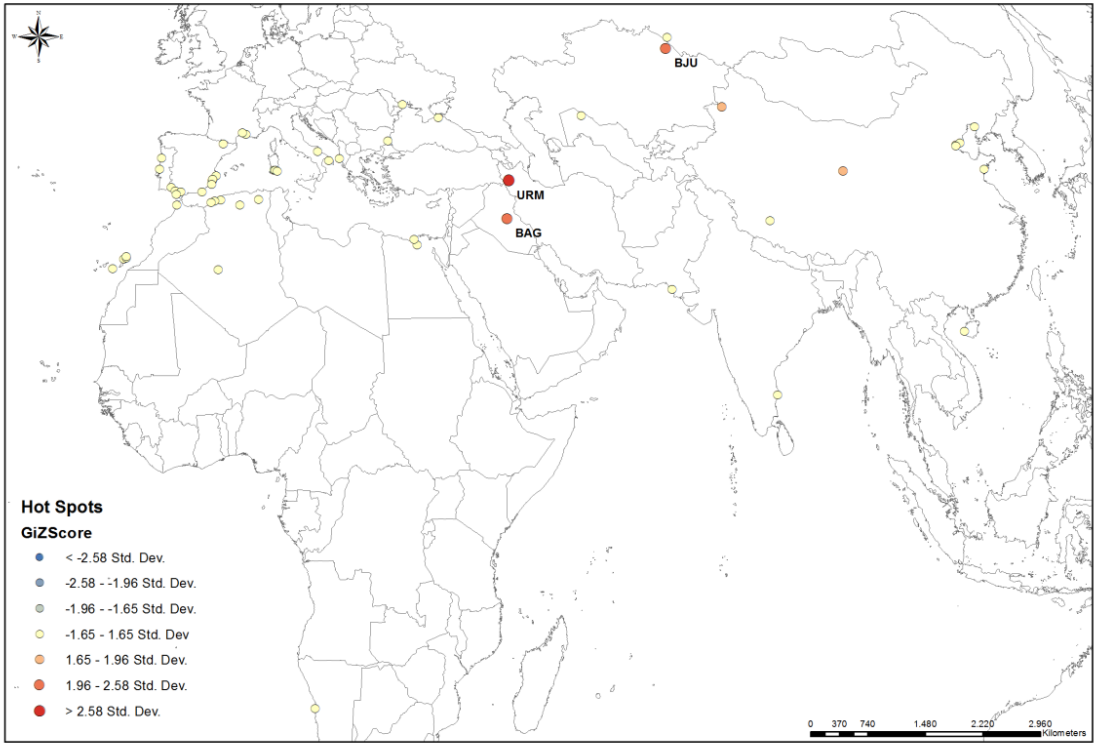

Figure 1. Geographical distribution of diploid parthenogenetic *Artemia* sampling sites. The color codes represent the GIZ score values obtained with the Gi* test, which are related with the frequency of rare males. GIZ score values >1.96 indicate high statistically significant clustering value of rare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

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) (Figure 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 out 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 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.

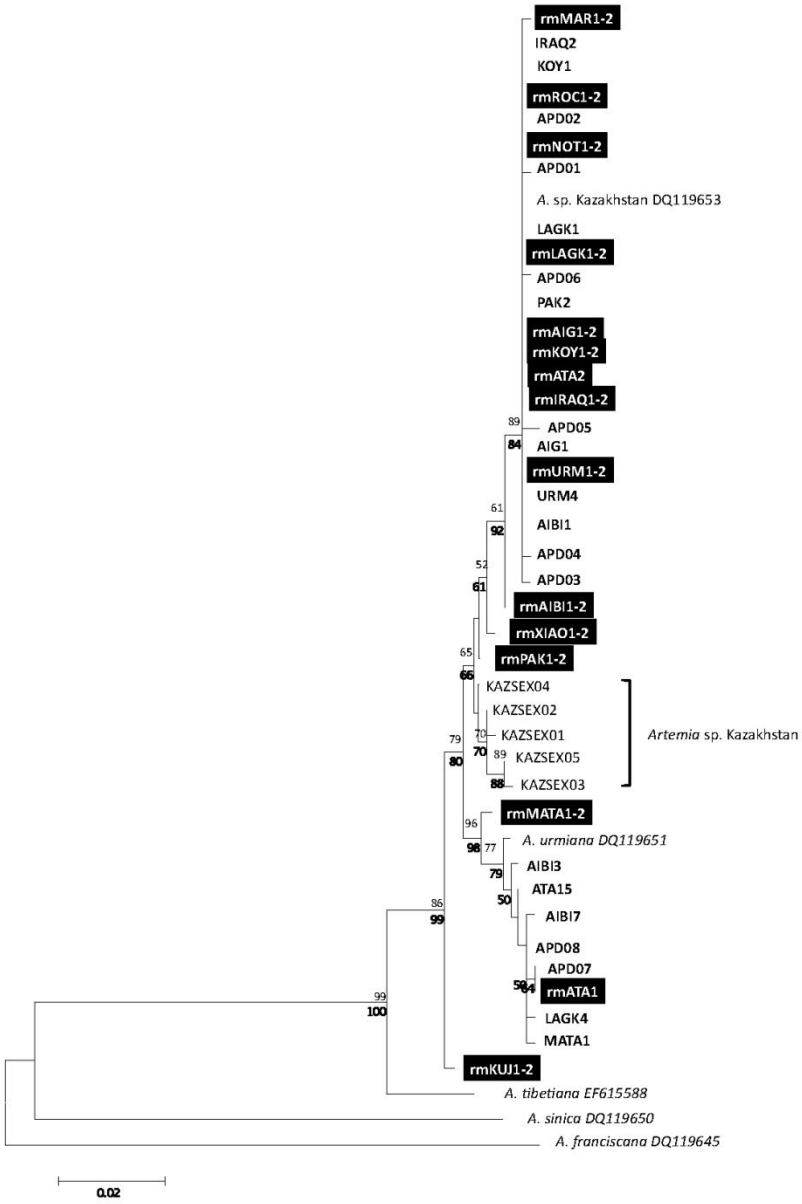

Figure 2. Phylogenetic relationships of diploid parthenogenetic *Artemia* rare male mtDNA haplotypes (which are noted by rm followed by the population code), diploid parthenogenetic female haplotypes (in bold) and Central Asian species based on COI sequences. The neighbour joining (NJ) topology is shown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

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 (Figure 3). The morphometry of the parthenogenetic males was very variable, and their population centroids were located within the limits of the sexual populations.

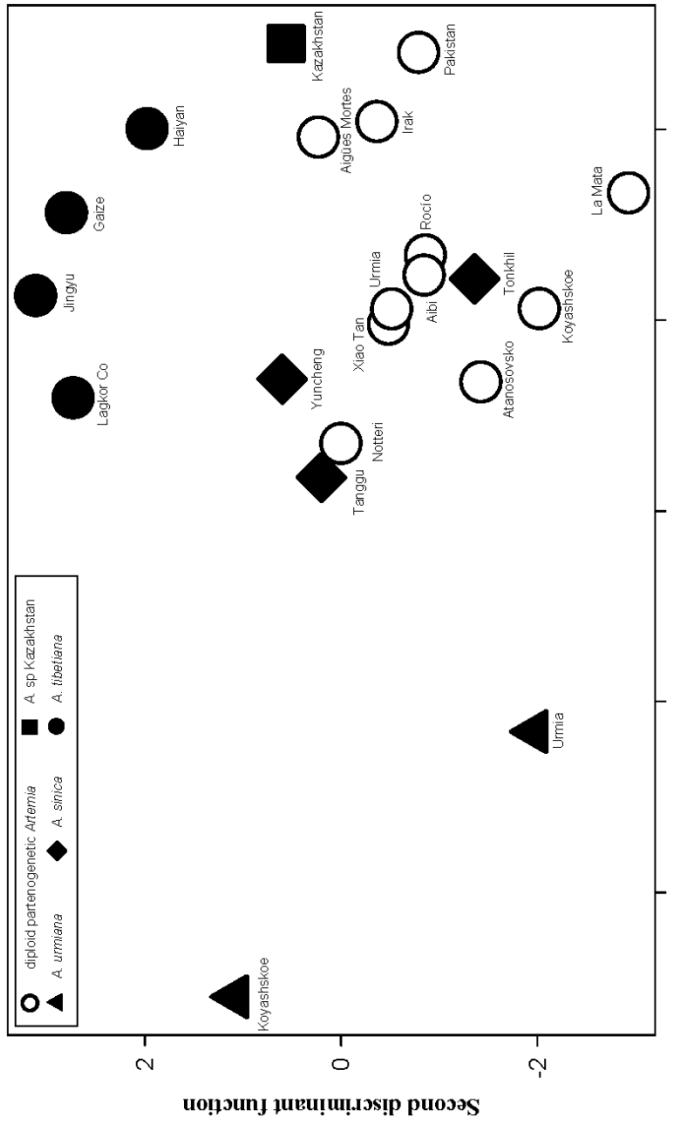

Figure 3. Multivariate discriminant analysis of *Artemia* rare males morphometric traits. Mean values of each *Artemia* population solved for the two first discriminant functions (centroids).

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 out of the four hybrid crosses (rare male x *A. urmiana* and rare male x *A. tibetiana*) than its corresponding controls, but in any case, there were no statistically significant differences between rare male crosses and controls (Table 3).

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 since Normality tests failed in all cases).

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

Crosses involving rare males resulted in viable ovoviviparous and oviparous hybrid offspring (Figure 4). Remarkably, all interspecific crosses between Central Asian sexual females and rare males had a similar or higher F1 offspring quality than controls (intraspecific 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 in ovoviviparous offspring from the crosses between rare males and *A. urmiana* or *A. sinica* females, which were higher in the controls (Figure 4 and Table S1).

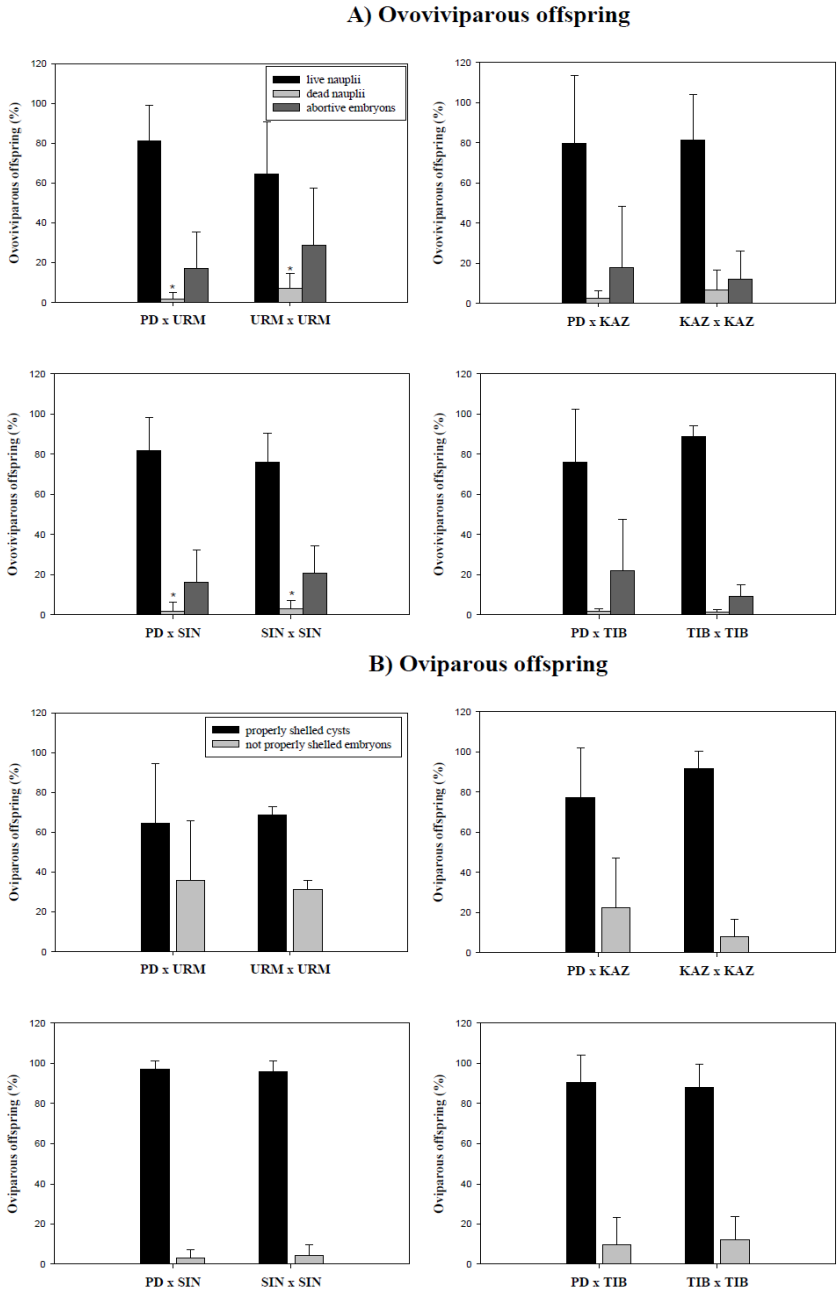

Figure 4. Offspring quality in cross-breeding experiments. For further details see full caption on the next page.

Figure 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).

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 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.

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 x *A. urmiana*	F0 (F-Koy 15)	233-281	207-Ø	170-Ø
	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 x *A. urmiana*	F0 (F-Koy 16)	248-Ø	208-Ø	90-90
	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 x *Artemia* sp. Kazakhstan	F0 (F-Kaz 8)	233-233	213-245	Ø-Ø.
	F0 (M-Iraq 8)	233-**242**	**208-231**	**115**-Ø
	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 x *A. sinica*	F0 (F-sin 7)	Ø-Ø	Ø-Ø	Ø-Ø
	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

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 hotspots, Urmia Lake, Bagdad saltern and Bjurliu Lake. Populations showing a higher ability to produce rare males are therefore found in a geographical region around 40ºN 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 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 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 to 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, while 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 geographic 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 mechanisms 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 out 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 to 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, makes 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 monopolisation (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).

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).

		Normality test	Equal Variance test	t-test (DFs)	Mann-Whitney U test
PDxURM vs URMxURM	live nupliii (%)	failed	passed		p=0.082
	dead naulplii(%)	failed	passed		p=0.009*
	abortive embryons(%)	failed	passed		p=0.325
	properly shelled cyst(%)	failed	passed		p=0.786
	not properly shelled embryons(%)	failed	passed		p=0.786
PDxKAZ vs KAZxKAZ	live nupliii (%)	failed	passed		p=1.000
	dead naulplii(%)	failed	passed		p=0.626
	abortive embryons(%)	failed	passed		p=0.921
	properly shelled cyst(%)	failed	failed		p=0.120
	not properly shelled embryons(%)	failed	failed		p=0.120
PDxSIN vs SINxSIN	live nupliii (%)	failed	passed		p=0.132
	dead naulplii(%)	failed	passed		p=0.020*
	abortive embryons(%)	failed	passed		p=0.112
	properly shelled cyst(%)	failed	failed		p=0.857
	not properly shelled embryons(%)	failed	failed		p=0.857
PDxTIB vs TIBxTIB	live nupliii (%)	passed	failed		p=0.800
	dead naulplii(%)	passed	passed	p=756(4)	
	abortive embryons(%)	passed	failed		p=0.800
	properly shelled cyst(%)	failed	passed		P=0.444
	not properly shelled embryons(%)	failed	passed		P=0.444

Chapter III

Laboratory generation of new parthenogenetic lineages supports contagious parthenogenesis in *Artemia*

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Abstract

Contagious parthenogenesis—a process involving rare functional males produced by a parthenogenetic lineage which mate with coexisting sexual females resulting in fertile parthenogenetic offspring—is one of the most striking mechanisms responsible for the generation of new parthenogenetic lineages. Populations of the parthenogenetic diploid brine shrimp *Artemia* produce fully functional males in low proportions. The evolutionary role of these so-called *Artemia* rare males is, however, unknown. Here we investigate whether new parthenogenetic clones could be obtained in the laboratory by mating these rare males with sexual females. We assessed the survival and sex ratio of the hybrid ovoviviparous offspring from previous crosses between rare males and females from all Asiatic sexual species, carried out cross-mating experiments between F1 hybrid individuals to assess their fertility, and estimated the viability and the reproductive mode of the resulting F2 offspring. Molecular analysis confirmed the parentage of hybrid parthenogenetic F2. Our study documents the first laboratory synthesis of new parthenogenetic lineages in *Artemia* and supports a model for the contagious spread of parthenogenesis. Our results suggest recessive inheritance but further experiments are required to confirm the likelihood of the contagious parthenogenesis model.

Introduction

Parthenogenesis in animals has evolved through different molecular mechanisms that influence the initial genetic variability of parthenogenetic strains and therefore have important implications on their evolutionary success and persistence (*Simon et al., 2003*). One of the most striking mechanisms responsible for the generation of new parthenogenetic lineages is contagious parthenogenesis (*Simon et al., 2003*; *Schön, Martens & van Dijk, 2009*). This involves a parthenogenetic lineage able to produce functional males, which mate with coexisting sexual females producing fertile parthenogenetic hybrid offspring. These new parthenogenetic lineages will combine genetic diversity of the maternal sexual species and their paternal parthenogenetic ancestor, including the genetic fragments linked to the parthenogenesis (*Simon et al., 2003*; *Tucker et al., 2013*).

This mechanism has been documented in aphids and parasitoid wasps (*Schneider et al., 2002*; *Sandrock & Vorburger, 2011*; *Delmotte et al., 2013*), and most extensively in the *Daphnia pulex* species complex (*Innes & Hebert, 1988*; *Paland, Colbourne & Lynch, 2005*). In North American *D. pulex* parthenogenetic lineages, at least two distinct unrecombined haplotypes on chromosome VIII and IX are implied in the sex-limited meiosis suppression (*Lynch et al., 2008*; *Eads et al., 2012*; *Tucker et al., 2013*). These haplotypes leading to obligate parthenogenesis in *D. pulex* stem from a single recent event of hybridization with its sister taxon *D. pulicaria* (*Xu et al., 2013*; *Tucker et al., 2013*). Multiple new parthenogenetic lineages have arisen since this event as males produced

by asexual lineages spread these parthenogenesis-inducing haplotypes by mating with sexual females.

Artemia, an anostracan branchiopod commonly known as brine shrimp, is a typical inhabitant of hypersaline inland lakes and coastal lagoons and salterns. This genus includes sexual species and lineages of obligate parthenogenetic populations of diverse ploidy levels (*Abatzopoulos, 2002*), which makes it a good model system to investigate evolutionary transitions between reproductive systems. Parthenogenetic populations are restricted to the Old World where they co-occur with several sexual species in sympatry in various areas (*Abatzopoulos, 2002*; *Agh et al., 2007*; *Abatzopoulos et al., 2009*; *Maccari et al., 2013*). All strains of *Artemia* can reproduce either ovoviviparously, with the release of free-swimming nauplii broods when they complete their development in the ovisac (therefore, without a dormant phase), or oviparously with the production of broods of diapausing cysts (*Browne, 1980*; *Abatzopoulos, 2002*).

In *Artemia*, both sexual and asexual females are heterogametic (ZW) (*Stefani, 1963*; *Bowen, 1963*; *Bowen, 1965*; *De Vos et al., 2013*). Diploid parthenogenetic lineages reproduce through automictic parthenogenesis, although the cytological details are controversial (*Cuellar, 1987*). It appears that diploidy restoration results in female offspring genetically identical to the mother barring mutation or recombination (*Abreu-Grobois, 1987*; *Stefani, 1960*). Parthenogenetic diploid *Artemia* populations produce fully functional males in low proportions (*Stefani, 1964*; *Bowen et al., 1978*; *MacDonald & Browne, 1987*; *Maccari et al., 2013*). *Abreu-Grobois & Beardmore (2001)* showed that rare

males remain heterozygous at the same allozyme loci as their mothers, suggesting that rare males are produced as a result of rare ZW recombination events. These 'rare males' can generate viable offspring when crossed with females of sexual Asiatic species (*Bowen et al., 1978*; *Cai, 1993*; *Maccari et al., 2013*), to which they are closely related genetically (*Muñoz et al., 2010*; *Maniatsi et al., 2011*; *Maccari, Amat & Gómez, 2013*), but they are reproductively isolated with other more distantly related species (*MacDonald & Browne, 1987*). However, the evolutionary role of rare males in the generation of *Artemia* parthenogenetic lineages is unknown (*Maccari et al., 2013*). The occurrence of contagious parthenogenesis has been suggested in light of the polyphyletic nature of maternal diploid parthenogenetic lineages (*Maccari, Amat & Gómez, 2013*), but we do not know if rare males are able to transmit parthenogenesis to their offspring, a requisite for contagious parthenogenesis. In an early study, *Bowen et al. (1978)* crossed two parthenogenetic rare males, one from Yamaguchi (Japan) and the other one from Madras (India), with one sexual female of *A. urmiana* and one *A. franciscana* respectively, and concluded that parthenogenetic reproduction could not be transmitted through males because they failed to obtain parthenogenetic offspring either in hybrid F1, F2 or F2 backcross.

Laboratory generation and establishment of unisexual lineages can be a useful tool to complement phylogenetic approaches to identify the mechanism involved in the transition from sexual to parthenogenetic reproduction. However, most laboratory hybrids often exhibit low fertility and survival, or show deformation and abnormalities

(*Vrijenhoek, 1989*; *Mantovani et al., 1996*). In vertebrates, the first successful laboratory generation of a unisexual hybrid involved the origin of the hybridogenetic fish *Poeciliopsis monacha-lucida* through crosses of *P. monacha* females with *P. lucida* males (*Schultz, 1973*). Laboratory hybrids of hemiclonal European water frog *R. esculenta* (*Rana ridibunda* x *Rana lessonae*) show faster larval growth, earlier metamorphosis, and higher resistance to hypoxic conditions than their parental species and the equivalent hybrids in nature (*Hotz et al., 1999*). More recently, *Lutes et al. (2011)* generated self-sustaining tetraploid lineages of parthenogenetic lizards by pairing males of diploid sexual species *Aspidoscelis inornata* with females of the triploid parthenogenetic species *Aspidocelis exsanguis*. In invertebrates, the first laboratory generation of clonal hybrids in *D. pulex* was obtained by crossing males from obligately parthenogenetic clones with cyclically parthenogenetic females (*Innes & Hebert, 1988*). In addition, new lineages of thelytokous parthenogenetic lineages have been obtained in the wasp *Lysiphlebus fabarum* and in a South African honeybee, *Apis mellifera capensis* (*Lattorff, Moritz & Fuchs, 2005*; *Sandrock & Vorburger, 2011*).

Here we assess the reproductive role of rare males and investigate whether new parthenogenetic clones could be produced in the laboratory as support for the contagious origin of parthenogenetic lineages in *Artemia*. For this purpose, (1) we assess the survival and sex ratio of the hybrid ovoviviparous offspring obtained from the previous crosses from *Maccari et al. (2013)* between rare males and four Asiatic sexual species, (2) we carry out cross-mating experiments between these F1 hybrid individuals to assess their fertility, (3) we estimate the

viability and the reproductive mode of the resulting F2 offspring; (4) finally we demonstrate genetically that parthenogenetic F2 are indeed the descendants of the original crosses. This study shows that *Artemia* has the potential of generating parthenogenetic strains through contagious parthenogenesis.

Materials and methods

Populations and mating experiments

In a previous study, we set up mating experiments between rare males from the diploid parthenogenetic *Artemia* population from Bagdad (Iraq, hereafter PD) and sexual females from Asiatic *Artemia* species to assess the fertility and the reproductive potential of rare males (*Maccari et al., 2013*). The females used were from the sexual Asiatic populations, *A. urmiana* from Koyashskoe Lake (Ukraine, URM), *A. sinica* from Yuncheng Lake (China, SIN), *A. tibetiana* from Lagkor Co Lake (Tibet, TIB) and *Artemia* sp. from Kazakhstan (*Artemia* Reference Center code – ARC1039, unknown locality, KAZ). These interspecific crosses resulted in viable ovoviviparous and oviparous F1 offspring with similar or higher viability than controls (intraspecific sexual crosses) (*Maccari et al., 2013*).

Survival rate, sex ratio and reproductive performance of hybrid generations

For this study, live nauplii obtained from each ovoviviparous F1 hybrid brood were reared separately in jars containing brine at 80 gL−1 salinity, kept at 20–24 ◦C under mild aeration at a 12D:12L photoperiod and fed a

mixture of *Dunaliella* sp and *Tetraselmis* sp. (1:1) microalgae every other day. When animals showed signs of reproductive maturity they were counted and sexed to estimate survival rates (the proportion of F2 offpring per pair attaining adulthood) and sex ratio (the proportion of males in the F2 offspring per pair). For this procedure the animals were placed in Petri dishes with seawater and anaesthetized with a few drops of freshwater saturated with chloroform and examined carefully under a binocular microscope. We tested for deviations from a 50% sex ratio per cross and per pair using a Chi-square goodness of fit test (Pearson's statistic) (*Wilson & Hardy, 2002*). Statistical analyses were performed with SPSS v. 15.0 (SPSS Inc., Chicago, USA).

Reproductive performance of the F1 hybrid individuals was evaluated in F1×F1 cross fertility tests. For this purpose, 24 randomly size-matched hybrid F1 male–female pairs from each cross were transferred into separate small glass beakers (60 ml) under the culture conditions described above. Lifetime quantitative and qualitative reproductive outputs of each pair were monitored every other day during culture medium renewal events. For each paired F1 female we counted the number of unfertilized and fertilized broods, distinguishing the latter in oviparous and ovoviviparous broods. Eggs from unfertilised broods were identified as they are all smaller and white. In ovoviviparous offspring we also recorded the number of live and dead nauplii, and the number of abortive embryos (pale yellow-orange eggs). When oviparous offspring was produced, we counted the number of normally shelled diapausing cysts (pale grainy surface floating in 200

gl−1 brine), as opposed to abortive, abnormally shelled embryos (bright brown colour cysts sinking in 200 gl−1 brine) (*Maccari et al., 2013*).

Emerged F2 hybrid nauplii were reared until maturity as described above. They were counted and sexed to estimate their survival rate and sex ratio in the F2 generation. Then, males and females were individually isolated in containers until their deaths to check if females could reproduce in isolation, as would be expected in parthenogenetic individuals. It is possible that some parthenogenetic females could be sterile; in this case, our procedure will underestimate the frequency of parthenogenesis. The proportion of parthenogenetic female offspring produced in each cross was tested against the expectations of 25% if governed by a recessive allele in a single gene using a Chi-square goodness of fit test. In addition, to test whether the different crosses produced the same percentage of parthenogenetic female offspring we used a Chi-square homogeneity test.

Paternity analysis of parthenogenetic F2 individuals

(a) Microsatellite analysis

The F2 hybrid generation resulting from crosses between rare males and sexual females from *A. urmiana* and *Artemia* sp. from Kazakhstan included parthenogenetic individuals. In order to rule out contamination and confirm that they were F2 individuals resulting from the original crosses, we screened three microsatellite loci, previously screened in the parental individuals in another study (*Maccari et al., 2013*), in the parthenogenetic F2 animals obtained. Each microsatellite locus (Apdq02TAIL, Apdq03TAIL and Apd05TAIL) (*Muñoz et al., 2008*)

was amplified separately in PCRs performed as described in *Maccari et al. (2013)*. Alleles were scored using the CEQ Fragment Analysis software (Beckman Coulter™) and checked manually. If F2 individuals had a paternal allele in any of the loci this would confirm that they were descendants of the diploid parthenogenetic rare males.

(b)Maternal lineage

The F2 resulting from the rare male x sexual female cross and F1 × F1 cross should carry the maternal DNA of the sexual strain. To establish the maternal lineage of the parthenogenetic F2 offspring, a 709-bp fragment of mitochondrial cytochrome *c* oxidase subunit I (COI) gene region was amplified in the parental (F0) individuals, in the F1 offspring and in the parthenogenetic F2 individuals. Total DNA was extracted and PCR was carried out as described previously (*Maccari et al., 2013*). PCR amplifications were sent to MACROGEN for sequencing, and the resulting electrophoregrams were checked by eye using CodonCode Aligner v. 3.5 (CodonCode Corporation, Dedham, MA).

Results

Survival rate and sex ratio of F1 hybrid offspring

A total of 102 ovoviviparous hybrid F1 broods produced by the crosses between each combination of sexual species with rare males (*Maccari et al., 2013*) were reared to maturity. The live nauplii obtained in each brood were morphologically normal. Survival rates to adulthood were over 50% in all F1 hybrid offspring (Fig. 1), and were highest in the F1 PD×SIN (80%), and lowest in F1 PD×URM and F1 PD×TIB (ca. 56%)(for the codes of the hybrid crosses see Fig. 1). The overall mean sex ratio of

F1 offspring across pairs ranged from 49% males in F1 PD×KAZ cross to 53% males in F1 PD×TIB cross and did not significantly differ from 50% in any cross (Fig. 1).

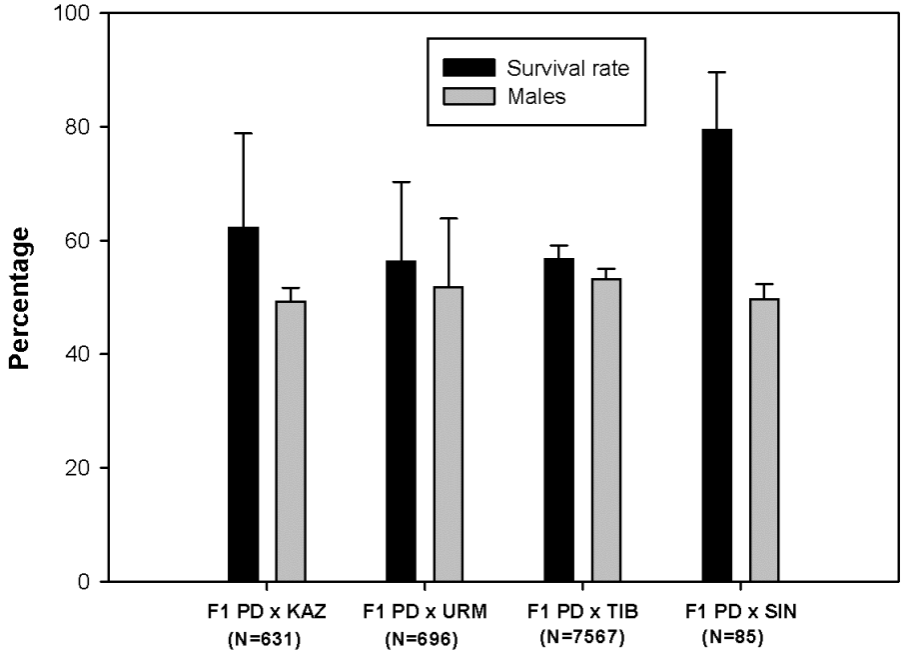

Figure 1 Survival rate and sex ratio (overall percentage of males) in the F1 hybrid offspring from *Artemia* rare males and Asiatic sexual females. F1 hybrids are from parental crosses between *Artemia urmiana* (URM), *A. sinica* (SIN), *A. tibetiana* (TIB), *Artemia* sp. from Kazakhstan (KAZ) and diploid parthenogenetic *Artemia* rare males (PD). Error bars are standard deviations.

Reproductive performance of F1 hybrid offspring

Prior to setting up the crosses, all females were isolated from males for two weeks to ensure that they could not reproduce in isolation (i.e., they were sexual females). No F1 females were able to reproduce when isolated from males. Then, a total of 24 mating pairs (F1 hybrid female×F1 hybrid male) were set up for each F1 produced in each combination of sexual species with rare males. As some individuals died before mating, the final number of experimental pairs ranged from 10 to 22 per cross, which produced a total of 173 fertile and 92 infertile F2 hybrid broods (Table 1). Ovoviviparous and oviparous F2 offspring viability is shown in Fig. 2. The percentage of abortive embryos was high in all crosses (between 70% and 90%), while the proportion of live nauplii in all hybrid ovoviviparous broods was low (from 5% to 25%). In oviparous broods, the proportion of properly shelled cysts ranged from 25% in F2 PD×TIB to 61% in F2 PD×URM.

Table 1 Number of total, fertilized, ovoviviparous and oviparous broods in F1 *Artemia* hybrid offspring. F1 hybrids are from parental crosses between *Artemia urmiana* (URM), *Artemia sinica* (SIN), *Artemia tibetiana* (TIB), *Artemia* sp. from Kazakhstan (KAZ) and diploid parthenogenetic *Artemia* rare males (PD).

Cross	Pairs	Total broods	Fertilized broods	Ovoviviparous broods	Oviparous broods
F1 PD x KAZ	18	80	42	37	5
F1 PD x URM	16	48	26	22	4
F1 PD x TIB	10	33	18	4	14
F1 PD x SIN	22	104	87	40	47

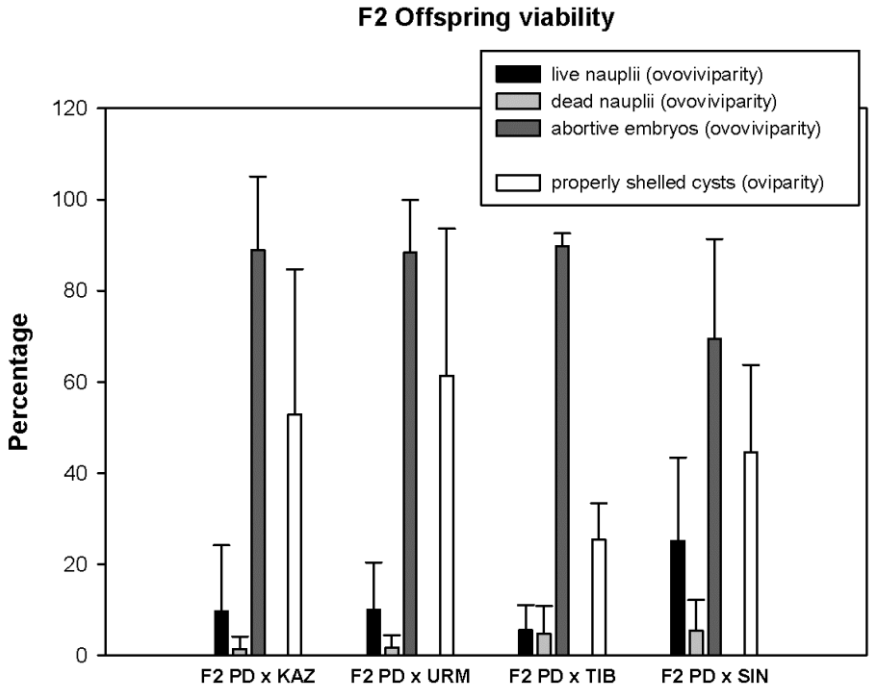

Figure 2 Reproductive traits (offspring quantity and quality) in F2 hybrids between *Artemia* rare males and Asiatic sexual females. The viability of ovoviviparous and oviparous broods is shown. Error bars are standard deviations.

Survival rate and sex ratio of F2 hybrid offspring

A total of 103 F2 ovoviviparous broods were recorded (Table 1), of which 35 broods from 27 pairs, characterized by the greatest number of nauplii, were followed to assess the survival rate and the sex ratio of the F2 offspring. F2 nauplii were morphologically normal but they had low survival rates when compared to F1 nauplii (Fig. 3). No F2 hybrid offspring produced by the crosses between rare male and *A. tibetiana* survived to maturity. The F2 PD×KAZ had the highest survival rate, about 37%, followed by the F2 PD×SIN (34%) and F2 PD×URM (24%).

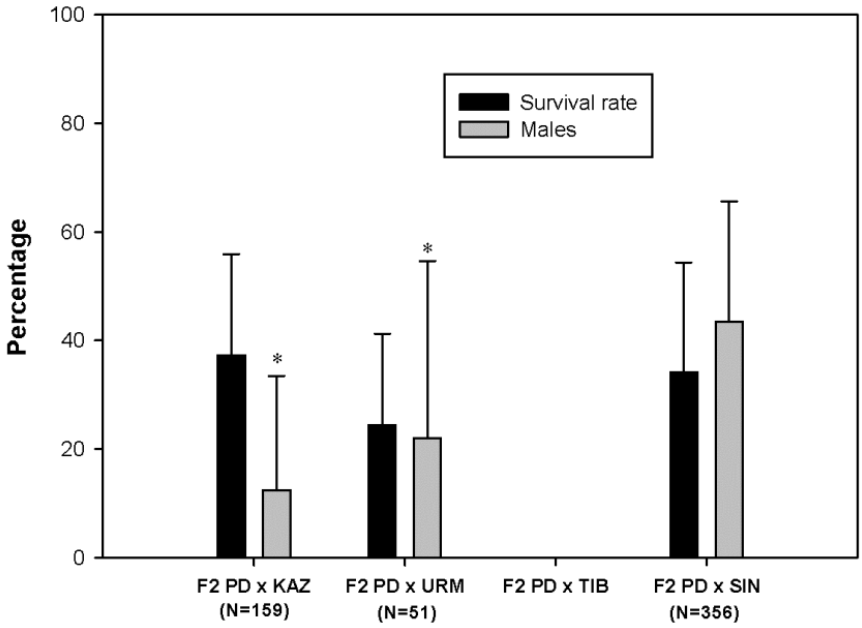

Figure 3 Survival rate and sex ratio (overall percentage of males) in the F2 hybrid offspring from *Artemia* rare males and Asiatic sexual females. F2 hybrids are from crosses between F1 hybrid individuals which are obtained in the crosses between *Artemia urmiana* (URM), *A. sinica* (SIN), *A. tibetiana* (TIB), *Artemia* sp. from Kazakhstan (KAZ) and diploid parthenogenetic *Artemia* rare males (PD). Error bars are standard deviations. Asterisks ($P \leq 0.05$) indicate significant differences from 50% sex ratio (Chi-square goodness of fit test was employed).

The overall mean sex ratio across pairs was significantly female-biased in F2 PD×KAZ and F2 PD×URM crosses (12% and 22% of males respectively; $\chi 2 = 111.25$ and $\chi 2 = 16.49$, 1 df, $p < 0.05$), but was non-significantly different from 50% in the F2 PD × SIN (43% of males; $\chi 2 = 0$, 1 df, $p < 0.05$) (Fig. 3). Furthermore, we observed differences in the sex ratio of the F2 offspring among different pairs from the same cross, in particular for F2 PD × KAZ and F2 PD × URM crosses (see Table 2). In the cross F2 PD × KAZ, which higher sample sizes, one pair produced

offspring with an even sex ratio (pair 3) while the remaining five pairs had were female biased offspring (see Table 2).

Generation of hybrid parthenogenetic individuals

Some females isolated from males of all F2 hybrid offspring analysed (when males were present) reproduced parthenogenetically in two of the three crosses. Specifically, 12 out of 41 isolated females (29.27%) were parthenogenetic in F2 PD × KAZ (four out of the five offspring analysed, Table 2), and two out of 36 (5.56%) isolated females in F2 PD×URM (two of five offspring analysed, Table 2).

The percentages of parthenogenetic female offspring in the F2 crosses were significantly different from each other ($\chi 2 = 7.24$, 1 df, $p < 0.05$), and only that one in F2 PD × KAZ did not differ significantly from the expectations of 25% ($\chi 2 = 0.4$, 1 df, $p > 0.05$) under expectations of a recessive allele in a single locus determining parthenogenesis. In all but one case, parthenogenetic females were produced in offspring with significantly female-biased sex ratios (Table 2). None of the 21 F2 PD×SIN offspring included females that could reproduce parthenogenetically.

Table 2 Sex ratio and parthenogenetic females found in F2 PD × KAZ, F2 PD × URM and F2 PD× SIN *Artemia* offspring. Asterisks ($P \leq 0.05$) indicate significant differences from 50% sex ratio (number of males/total individuals) (Chi-square goodness of fit test was employed). All females obtained were isolated until their deaths to determine their mode of reproduction.

	Pair	Females	Males	Total	Sex ratio (%)	Parthenogenetic females / analysed females	Parthenogenetic females (%)
F2 PD x KAZ	*1*	10	0	10	0.00**	3/10	30
	2	10	2	12	16.67*	1/10	10
	3	7	8	15	53.33	0/7	0
	4	20	0	20	0.00**	6/10	60
	5	68	2	70	2.86**	2/4	50
	6	31	1	32	3.13**	-	-
Total		**146**	**13**	**159**		**12/41**	**29.27**
F2 PD x URM	*1*	16	3	19	15.79**	0/16	0
	2	2	4	6	66.67	0/2	0
	3	2	0	2	0.00	0/2	0
	4	3	1	4	25.00	1/3	33.33
	5	2	1	3	33.33	-	-
	6	2	0	2	0.00	-	-
	7	13	2	15	13.37**	1/13	7.69
Total		**40**	**11**	**51**		**2/36**	**5.56**
F2 PD x SIN	*1*	15	13	28	46.43	0/15	0
	2	13	24	37	64.86	0/13	0
	3	6	3	9	33.33	0/6	0
	4	1	3	4	75.00	0/1	0
	5	14	12	26	46.15	0/14	0
	6	10	10	20	50.00	0/10	0
	7	20	18	38	47.37	0/20	0
	8	23	24	47	51.06	0/23	0
	9	30	41	71	57.75	0/30	0
	10	5	8	13	61.54	0/5	0
	11	16	0	16	0.00**	0/16	0
	12	7	0	7	0.00**	0/7	0
	13	4	1	5	20.00	0/4	0
	14	14	21	35	60.00	0/14	0
Total		**178**	**178**	**356**		**0**	**0**

Paternity analysis

In order to examine the parentage of newly generated hybrid parthenogenetic individuals we integrated the information from the mitochondrial COI and from microsatellites markers (Table 3). Six of the 10 analysed females from pair 4 of the cross F2 PD×KAZ were parthenogenetic and produced F3 clones. As expected, all of them shared their mtDNA haplotype with their sexual grandmother, and amplified one paternal allele in the two informative microsatellite loci, confirming that they were the offspring of the rare male used in the crosses. The F3 generation was overall composed by females and by two rare males with the same genotype as their F2 mothers.

The F2 offspring of two pairs from the crosses PD×URM (pairs 4 and 7), composed of three and 13 females respectively, included a parthenogenetic female that produced F3 parthenogenetic clones. In both cases, the F2 parthenogenetic female shared its COI haplotype with its sexual grandmother. In one cross, one paternal allele was detected in the F2 hybrid female at each of the three microsatellite loci; in the other cross, the parthenogenetic female inherited one paternal allele at the two informative loci. Most individuals of the F3 generation, composed of females and one rare male in both crosses, have the same genotype as their F2 mothers, with a few exceptions that lacked one of the maternal alleles, suggesting some level of recombination consistent with automixis parthenogenesis.

Table 3 Mitochondrial cytochrome c oxidase subunit I (COI) and microsatellite loci analyses for parental individuals (F0) and for parthenogenetic F2 and F3 offspring obtained from the hybrid *Artemia* crosses. Genotypes for three microsatellite loci (allele sizes in base pairs) are shown. Diagnostic alleles, that is, alleles present in the rare male grandfather and not in the grandmother are shown in bold in the grandfather and in the F2 and F3 offspring. 'O' indicates the presence of null alleles; 'm' indicates a rare male. COI haplotypes as named in GenBank are shown. KAZSEX03: GU591387; APD02: DQ426825; AUKOY02: KF707698; AUKOY01: KF707699.

	Sample code	Apd02	Apd03	Apd05	COI
Rare male x Artemia sp. Kazakhstan	**F0 (F-Kaz 8)**	233-233	213-245	Ø-Ø	KAZSEX03
	F0 (M-Iraq 8)	233-**242**	208-**231**	115-Ø	APD02
	F2-8-2-2	233-233	**231**-245	Ø-Ø	KAZSEX03
	F2-8-2-3	233-**242**	**231**-245	Ø-Ø	KAZSEX03
	F2-8-2-4	233-**242**	**231**-245	Ø-Ø	KAZSEX03
	F2-8-2-5	233-**242**	**231**-245	Ø-Ø	KAZSEX03
	F2-8-2-6	242-**242**	**231**-245	Ø-Ø	KAZSEX03
	F2-8-2-8	233-**242**	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-2-3	233-233	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-2-5	233-233	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-2-10	233-233	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-2-12m	233-233	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-6-3	**242-242**	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-6-4	**242-242**	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-6-5	**242-242**	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-6-7m	**242-242**	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-8-1	233-**242**	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-8-2	233-**242**	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-8-3	233-**242**	**231**-245	Ø-Ø	KAZSEX03
	F3-8-2-8-4	233-**242**	**231**-245	Ø-Ø	KAZSEX03
Rare male x A. urmiana	**F0 (F-Koy 15)**	233-281	207-Ø	170-Ø	AUKOY02
	F0 (M-Iraq 15)	**254**-233	**216**-231	115-**185**	APD02
	F2-15-8-A	**254-254**	207-**216**	**185**	AUKOY02
	F3-15-8-A-1	**254-254**	**216**	**185**	AUKOY02
	F3-15-8-A-4	**254-254**	207-**216**	**185**	AUKOY02
	F3-15-8-A-5	**254-254**	207-**216**	**185**	AUKOY02
	F3-15-8-A-6	**254-254**	207-**216**	**185**	AUKOY02
	F3-15-8-A-7m	**254-254**	207	**185**	AUKOY02

Table 3 Continued

	Sample code	Apd02	Apd03	Apd05	COI
Rare male x A. urmiana	**F0 (F-Koy 16)**	248-Ø	208-Ø	90-90	AUKOY01
	F0 (M-Iraq 16)	233-**251**	216-230	**117**-189	APD02
	F2-16-7-4	248-**251**	Ø-Ø	90-**117**	AUKOY01
	F3-16-7-4-1	248-**251**	Ø-Ø	90-**117**	AUKOY01
	F3-16-7-4-2	248-**251**	Ø-Ø	90-90	AUKOY01
	F3-16-7-4-3	248-**251**	Ø-Ø	90-**117**	AUKOY01
	F3-16-7-4-5	248-**251**	Ø-Ø	90-**117**	AUKOY01
	F3-16-7-4-7m	248-**251**	Ø-Ø	90-**117**	AUKOY01

Discussion

This study reports for the first time the laboratory generation of parthenogenetic *Artemia* lineages through hybridization via rare males, i.e., through contagious parthenogenesis (*Simon et al., 2003*), shedding light on the possible evolutionary role of parthenogenetically produced males and the genetic basis of parthenogenesis in this genus.

Contagious parthenogenesis may have important evolutionary consequences as it results in the repeated generation of new asexual genotypes, increasing the genetic diversity in parthenogens. This may counteract the loss of asexual genotypes resulting from the accumulation of deleterious mutations (Muller's ratchet) or gene conversion (*Tucker et al., 2013*) and could contribute to the evolutionary success of parthenogenesis (*Simon et al., 2003*).

The occurrence of contagious parthenogenesis relies on regular or occasional hybridization with absence of complete reproductive isolation between parthenogenetically produced males and closely related sexual females (*Simon et al., 2003*). In a previous study, we

showed the absence of prezygotic isolation between rare males and Asiatic sexual *Artemia* species since these males often coexist in the same environment of a sexual species (*Abatzopoulos et al., 2006*; *Agh et al., 2007*; *Agh et al., 2009*; *Shadrin, Anufriieva & Galagovets, 2012; Van Stappen et al., 2007; Van Stappen, 2008; Zheng & Sun, 2013)*, show normal pairing behaviour and are fully functional and capable of fertilizing eggs from females of sexual Asiatic *Artemia* species producing viable hybrid offspring (*Maccari et al., 2013*). Under laboratory conditions, each combination of sexual species with rare males produced morphologically normal, viable sexual hybrid F1. Their survival rate to adulthood was over 50% for all the hybrid populations, a high value if compared to survival of F1 of intraspecific crosses of the different *Artemia* species (*Browne & Wanigasekera, 2000*).

We found that females constitute approximately 50% of each F1 hybrid population, an even sex ratio that usually characterizes *Artemia* sexual populations, and this was confirmed by their inability to reproduce without males. These results ruled out a dominant gene as the genetic basis of parthenogenesis. Although all laboratory F1 lines were found to combine ovoviviparous and oviparous reproduction, we observed a strong reduction in the reproductive output in all crosses when compared with the reproductive performance of the parental crosses (*Maccari et al., 2013*). Ovoviviparous broods were mostly made up by abortive embryos (more than 80%) in all the crosses and live nauplii represented only 25% of the offspring in the cross F2 PD × SIN, and less than 10% in all the other crosses (F2 PD × KAZ, F2 PD × URM and F2 PD × TIB). Oviparity, the production of dormant encysted

embryos that are resistant to extreme environmental conditions, was represented by a variable quantity of properly shelled embryos, only 25% in the F2 PD × TIB increasing up to 61% in F2 PD × URM. Similarly, a decline in nauplii F2 production occurs in the interspecific crosses between *A. tibetiana* and *A. sinica* (*Van Stappen et al., 2003*).

In contrast to the high survival rates of F1 hybrids, hybrid breakdown was evident in the F2 generation. Nauplii from the F2 generations had low survival rates and were completely inviable in the F2 PD × TIB generation. The lower fertility level of F1 laboratory populations and the reduced viability of F2 hybrid individuals suggest partial genetic incompatibility between parthenogenetic males and sexual females. However, the production of some viable offspring both in F1 and F2 in all hybrid crosses is not so surprising given the recent evolutionary origin of diploid parthenogenetic lineages (Holocene) (*Muñoz et al., 2010*; *Maccari, Amat & Gómez, 2013*).

In two of the three F2 generations (F2 PD × KAZ and F2 PD × URM) we identified 14 hybrid females that upon reaching maturity were capable of parthenogenetic reproduction. Surprisingly, these parthenogenetic females were produced by pairs yielding strongly female biased F2 offspring. Genetic analysis confirmed the parentage of the parthenogenetic lineages found as the F2 individuals inherited the COI haplotype from the sexual grandmother but included some paternal alleles at nuclear markers, showing that they were the offspring of the rare male used in the crosses. Our results contrast with previous observations suggesting that rare males in the genus *Artemia* are not capable to transmit parthenogenesis-inducing alleles (*Bowen et al., 1978*).

The production of parthenogenetic individuals only in the second generation, suggests that the parthenogenesis-inducing alleles are recessive in *Artemia*. A single-locus recessive inheritance of obligate parthenogenesis also occurs in *Apis mellifera capensis* and in *Lysiphlebus fabarum* (*Sandrock & Vorburger, 2011*; *Lattorff, Moritz & Fuchs, 2005*; *Lattorff et al., 2007*). This is in contrast with *D. pulex*, where the sex-limited meiosis suppression genes are dominant and the asexual clones arise in the first generation (*Innes & Hebert, 1988*). If a single recessive locus was responsible for parthenogenesis and there was no differential viability in *Artemia*, a 25% of parthenogenetic females would be expected in the F2 generation. The proportion of isolated females that reproduced parthenogenetically differed between the crosses. In the cross F2 PD × KAZ, the overall proportion of parthenogenetic F2 females was 29.27%, not significantly different from 25%, whereas in the cross F2 PD × URM this was much lower (5.56%) and significantly different from the expectations for a single recessive locus. These results suggest either differences in the mechanism underlying parthenogenesis between populations, or increased incompatibilities between PD and URM resulting in viability differences linked to the putative locus associated to parthenogenesis. The latter is supported by the lower viability of F2 PD × URM nauplii. The finding of parthenogenetic females only in sex-biased broods suggests that the inheritance of parthenogenesis has a more complex genetic basis, however. Given that females are heterogametic (WZ) (*Bowen, 1963*; *Bowen, 1965*; *Stefani, 1963*) and that F1 females are sexual, we can rule out complete sex-linkage (Z-linkage) of the parthenogenesis determining gene, otherwise

parthenogenesis should be apparent in the F1, given that all F1 females are WZ with their Z chromosome presumably inherited from their asexual father. Sex-biased sex ratios are not uncommon in hybrid offspring and can be due to the evolution of sex-ratio distorters and counter evolution of suppressor genes in different lineages (*Hurst & Pomiankowski, 1991*). Our data suggests an interaction between a sex ratio distorter (possibly sex-linked) and a parthenogenetically determining factor. Alternatively, the same gene determining parthenogenesis could act as a sex ratio distorter in heterozygous F1 females, increasing the likelihood of transmission of the W chromosome. Our results do not support differential male mortality, as there was no correlation between brood survival and sex ratio (data not shown). These interpretations must be taken with caution given the limitations of our experimental design and data, as we analysed F2 broods where there was a larger number of nauplii, the survival of the F2 was low, and we cannot rule out some effect of differential sterility. These factors might have biased our conclusions regarding the genetic basis of parthenogenesis. Therefore, to fully understand the genetic basis of parthenogenesis in *Artemia* additional crosses and a large set of marker loci will be necessary.

The ability of sexual females of *A. urmiana* and *Artemia* sp. from Kazakhstan to generate parthenogenetic clones when crossed with rare males is not surprising, as the two main mitochondrial haplogroups of diploid parthenogenetic *Artemia* lineages are related to these species (*Muñoz et al., 2010*; *Maniatsi et al., 2011*; *Maccari, Amat & Gómez, 2013*). However, the more distantly related *A. sinica* (*Baxevanis, Kappas &*

Abatzopoulos, 2006; *Hou et al., 2006*) did not produce any parthenogenetic offspring, despite high survival rate in the F2, suggesting that the specific genomic background affect the expression of the gene inducing parthenogenesis. Although repeated gene flow between sexual females and asexual males through contagious parthenogenesis would be expected to result in a regular emergence of asexual strains with diverse maternal origins, the fact that just two, possibly three, maternal origins of parthenogenetic lineages have been identified (*Muñoz et al., 2010*; *Maniatsi et al., 2011*; *Maccari, Amat & Gómez, 2013*) indicate that the incidence of contagious parthenogenesis, if this is the mechanism of origin, must be extremely low in natural environments. Indeed, the rare males must be present in the population at the same time as the sexual females of the related species, and given that both parthenogenetic and sexual species often have different ecological requirements, they may overlap just during part of each season (*Amat et al., 1991*; *Ghomari et al., 2011*). In addition, the percentage of rare male production by diploid parthenogenetic females is very low, about 1–16 in 1000 (*Maccari et al., 2013*). Then, as the parthenogenesis occurs in the second generation (i.e., is based on a recessive trait), a F1 × F1 mating must occur for parthenogenesis to appear in the offspring. Finally, F2 survival is very reduced, overall making the origin of a parthenogenetic lineage an unlikely event in the wild.

Our study is the first to generate new parthenogenetic lineages in *Artemia* by mating rare males from parthenogenetic genotypes with sexual females, providing evidence that contagious parthenogenesis can potentially occur in the genus *Artemia*. This conclusion does not rule out

that other mechanisms (spontaneous origin or hybridisation) might have been also responsible for the origin of parthenogenetic lineages. Demonstration of contagious parthenogenesis as the mechanism underlying parthenogenesis in *Artemia* in the wild will necessitate the use of genomic tools. Further studies on hybrid fitness would be necessary to estimate the strength of reproductive isolation and to compare the reproductive performance of laboratory-produced parthenogenetic clones with the parental parthenogenetic strains. The origin of independently reproducing parthenogenetic clones in the laboratory raises the question of the survival of these clones when competing with sympatric sexual species.

Given that many parthenogenetic organisms produce males occasionally (*van der Kooi & Schwander, 2014*) and such males are still able to maintain their functionality, the occurrence of contagious parthenogenesis could be more widespread than currently acknowledged.

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Supplemental Information

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Table S1. Survival rate and sex ratio in the F1 hybrid *Artemia* offspring.
F1 hybrids are from parental crosses between *Artemia urmiana* (URM), *A. sinica* (SIN), *A. tibetiana* (TIB), *Artemia* sp. from Kazakhstan (KAZ) and diploid parthenogenetic *Artemia* rare males (PD). Live, dead and abortive individuals and number of males and females of individuals reaching maturity are given.

F1 PD x KAZ							
Female code	**Live nauplii**	**Dead nauplii**	**Abortive**	**Total**	**females**	**males**	**total**
A	175	0	3	178	47	42	89
B	133	20	184	337	51	48	99
	0	25	176	201			
C	204	0	8	212	53	61	114
	203	0	71	274	44	48	92
D	122	4	1	127	32	29	61
E	206	0	2	208	87	89	176

F1 PD x TIB							
Female code	**Live nauplii**	**Dead nauplii**	**Abortive**	**Total**	**females**	**males**	**total**
A	89	1	4	94	25	27	52
B	60	3	42	105	15	18	33

Table S1. Continued.

F1 PD x URM Female code	Live nauplii	Dead nauplii	Abortive	Total	females	males	total
A	47	0	0	47	7	18	25
B	90	0	3	93	31	38	69
C	28	0	20	48	8	11	19
	87	0	27	114	22	34	56
D	28	0	5	33	8	4	12
E	143	0	0	143	47	41	88
	115	0	11	126	15	19	34
F	116	0	10	126	22	56	78
G	63	3	7	73	21	25	46
	81	0	3	84	29	45	74
	127	0	21	148	51	47	98
	118	2	8	128	14	32	46
H	40	0	3	43	13	7	20
	36	0	5	41	4	2	6
	121	0	17	138	17	24	41
	106	56	0	162	32	31	63
I	27	0	0	27	3	7	10
	15	0	14	29	3	2	5
L	31	3	51	85	11	3	14
	8	0	67	75	2	1	3
	78	0	30	108	18	20	38
M	150	2	17	169	52	56	108
N	115	0	23	138	33	26	59
	46	0	127	173	20	17	37
O	262	0	12	274	63	48	111
	67	35	0	102	15	5	20

Table S1. Continued.

F1 PD x SIN Female code	Live nauplii	Dead nauplii	Abortive	Total	females	males	total
A	76	0	2	78	33	31	64
	28	4	29	61	10	10	20
B	102	0	0	102	43	39	82
C	41	32	1	74	17	16	33
	92	0	18	110	38	36	74
D	4	0	166	170	1	1	2
	205	31	58	294	84	80	164
	225	0	68	293	93	87	180
E	94	0	6	100	38	36	74
	129	0	79	208	50	50	100
	183	47	23	253	78	76	154
F	65	0	0	65	28	25	53
	125	2	8	135	52	47	99
G	126	1	0	127	49	46	95
H	138	0	44	182	55	51	106
I	285	0	0	285	116	112	228
L	212	0	2	214	81	78	159
M	74	0	16	90	30	28	58
	165	1	9	175	77	70	147
	178	0	34	212	76	97	173
N	57	0	5	62	24	22	46
	181	0	7	188	72	67	139
	243	0	4	247	97	90	187
	276	0	25	301	121	116	237
O	65	0	6	71	26	25	51
	142	0	0	142	62	59	121

Table S1. Continued.

F1 PD x SIN Female code	Live nauplii	Dead nauplii	Abortive	Total	females	males	total
P	0	8	70	78			
	150	0	6	156	58	59	117
	185	0	1	186	31	44	75
	187	0	16	203	32	40	72
Q	30	0	121	151	16	13	29
	183	3	15	201	75	71	146
	212	0	57	269	26	27	53
	234	0	54	288	80	99	179
	232	4	25	261	93	93	186
R	38	2	23	63	16	16	32
	9	0	77	86	1	2	3
S	24	0	11	35	12	10	22
	138	0	5	143	54	64	118
	173	0	5	178	69	66	135
	39	0	91	130	15	15	30
	182	0	31	213	88	79	167
T	163	0	6	169	59	42	101
	193	2	12	207	99	84	183
	175	0	27	202	65	67	132
U	73	0	2	75	32	37	69
	158	0	7	165	77	74	151
	254	1	17	272	103	103	206
V	61	2	9	72	31	26	57
	133	0	14	147	64	63	127
X	69	0	18	87	34	34	68
Y	80	0	1	81	41	34	75
	132	0	28	160	51	49	100
	241	0	18	259	99	104	203
	243	0	18	261	101	97	198
	327	2	21	350	132	140	272

Table S2. Viability, survival rate and sex ratio of F2 hybrid *Artemia* offspring: Number of live, dead, abortive individuals and number of males and females of individuals reaching maturity in ovoviviparous broods are given. Number of properly and improperly shelled embryos in oviparous broods are given.

F2 PD x KAZ Female code	Live nauplii	Dead nauplii	Abortive	Total	females	males	total	Female code	properly shelled cyst	bad cyst	Total
A	1	0	53	54				A	26	0	26
	1	3	106	110							
	1	0	149	150							
B	1	0	107	108				B	3	63	66
	2	2	157	161							
	7	1	182	190							
C	4	1	37	42				C	81	97	178
	1	0	68	69							
	5	7	149	161							
D	7	2	149	158							
	2	0	194	196							
	1	0	247	248							
	7	2	198	207							
E	1	0	48	49							
	5	0	164	169							
F	7	0	59	66							
	48	12	137	197	10	0	10				

Table S2. Continued.

F2 PD x KAZ Female code	Live nauplii	Dead nauplii	Abortive	Total	females	males	total	Female code	properly shelled cyst	bad cyst	Total
G	2	0	171	173							
	7	0	168	175							
H	0	2	71	73							
	43	12	129	184	10	2	12				
	1	0	206	207							
I	2	0	125	127				**I**	88	93	181
	1	0	163	164							
	28	4	145	177	7	8	15				
L	1	0	83	84							
	1	1	172	174							
M	89	22	87	198	20	0	20				
N	61	0	167	228	4	0	4				
	114	0	106	220	64	2	66				
O	0	1	73	74							
P	48	0	111	159	31	1	32	**P**	139	52	191

Table S2. Continued.

F2 PD x URM Female code	Live nauplii	Dead nauplii	Abortive	Total	females	males	total	Female code	properly shelled cyst	Bad cyst
A	1	0	74	75				A	32	60
	0	2	122	124					43	62
									50	76
B	0	2	168	170				B	69	13
C	7	0	92	99						
	11	3	124	138						
	5	0	130	135						
	1	0	170	171						
D	46	0	143	189	16	3	19			
E	4	0	123	127						
F	45	0	86	131	2	4	6			
G	1,00	0,00	50	51						
H	3	1	164	168						
I	2	0	83	85						

Table S2. Continued.

F2 PD x URM Female code	Live nauplii	Dead nauplii	Abortive	Total	females	males	total	Female code	properly shelled cyst	Bad cyst	Total
L	6	2	56	64							
	17	1	47	65	2	0	2				
M	1	0	98	99							
	19	4	132	155	3	1	4				
N	14	1	112	127	2	1	3				
0	9	2	74	85							
P	24	12	87	123	2	0	2				
Q	1	0	102	103							
R	28	9	102	139	13	2	15				

Table S2. Continued.

F2 Pd x SIN											
Female code	Live nauplii	Dead nauplii	Abortive	Total	females	males	total	Female code	properly shelled cyst	Bad cyst	Total
A	86	1	82	169	4	4	8	A	96	73	169
	143	17	82	242	6	6	12		154	125	279
	22	3	142	167	5	3	8				
B	123	5	68	196	6	15	21	B	145	39	184
	51	154	101	306	7	9	16				
C	89	0	89	178	6	3	9	C	70	88	158
	0	1	95	96							
	52	7	42	101	1	3	4				
D	1	0	243	244				D			
	10	2	112	124							
E								E	34	82	116
									87	54	141
									93	70	163
									111	67	178
									52	31	83
									93	64	157
F	25	2	148	175	1	1	2	F	5	69	74
	36	2	182	220	4	5	9		34	67	101

Table S2. Continued.

F2 Pd x SIN Female code	Live nauplii	Dead nauplii	Abortive	Total	females	males	total	Female code	properly shelled cyst	Bad cyst	Total
F	44	4	141	189	9	6	15				
	64	19	91	174	14	12	26				
G	38	51	199	288				**G**	5	164	169
H	44	0	214	258	10	10	20	**H**	30	118	148
	67	16	168	251					47	79	126
I	1	0	227	228				**I**	27	77	104
	0	5	254	259					22	75	97
									14	72	86
L	36	0	110	146	5	6	11	**L**	9	130	139
	64	3	171	238	15	12	27		158	76	234
M	139	24	130	293	23	24	47	**M**	80	119	199
									10	136	146
									197	74	271
N	98	4	42	144	8	17	25	**N**	73	86	159
	134	19	45	198	22	24	46		116	47	163
	148	25	64	237							

Table S2. Continued.

F2 Pd x SIN												
Female code	Live nauplii	Dead nauplii	Abortive	Total	females	males	total	Female code	properly shelled cyst	Bad cyst	Total
O	26	13	78	117				O	12	117	129
									77	51	128
									175	47	222
P	11	3	74	88				P	31	64	95
	19	25	147	191					47	69	116
	196	0	3	199							
Q	29	6	188	223	5	8	13	Q	82	147	229
R	69	8	144	221				R	128	95	223
									129	118	247
									223	16	239
S	6	0	121	127				S			
	18	0	228	246	16	0	16				
	22	1	161	184							
	1	2	171	174							
T								T	35	182	217
									74	185	259
									191	77	268
									140	87	227

Table S2. Continued.

F2 Pd x SIN											
Female code	Live nauplii	Dead nauplii	Abortive	Total	females	males	total	Female code	properly shelled cyst	Bad cyst	Total
U	7	0	191	198				U	70	86	156
V	15	0	201	216	7	0	7	V	105	109	214
									183	70	253
									86	79	165
									141	84	225
Z	32	1	185	218	4	1	5	Z	18	161	179
	24	0	220	244					117	112	229
J	121	22	97	240	14	21	35	J	181	70	251
									208	41	249

Table S2. Continued.

F2 PD x TIB											
Female code	Live nauplii	Dead nauplii	Abortive	Total	females	males	total	Female code	properly shelled cyst	bad cyst	Total
A	0	10	69	79	0	0	0	A	12	42	54
B	2	5	72	79	0	0	0	B	21	97	118
C								C	24	63	87
									12	82	94
D	5	0	67	72	0	0	0	D	15	32	47
									20	87	107
E								E	18	26	44
F								F	9	55	64
G								G	33	79	112
H	7	0	49	56	0	0	0	H	31	64	95
									27	103	130
I								I	11	50	61
									23	64	87
L								L	28	53	81

Part III

GENERAL DISCUSSION AND CONCLUSIONS

RESULTS AND GENERAL DISCUSSION

In this thesis we addressed the question of how genetic diversity is generated and maintained in diploid *Artemia* parthenogenetic lineages.

We focused our attention on two mechanisms which may occur in the genus: i) the generation of parthenogenetic populations through hybridization between two related sexual species (*Artemia urmiana, Artemia sinica, Artemia tibetiana*, *Artemia* sp. Kazakhstan); ii) contagious parthenogenesis in which parthenogenetically produced functional males mate with sexual females and transmit parthenogenesis to their offspring.

In order to gain insight into the evolutionary origin of diploid parthenogenetic *Artemia*, we tried two different but complementary approaches. On one hand, we used nuclear and mitochondrial markers to explore the phylogenetic relationship between diploid asexual populations and Asian sexual relatives, to understand how many times parthenogenesis has arisen and to infer the possible genetic mechanisms involved in the evolution of diploid parthenogenetic lineages (Chapter 1); on the other hand, we established laboratory cross-mating experiments between rare males and females of sexual Asian related species to investigate the reproductive role of rare males and to understand if they have the potential of generating parthenogenetic strains (Chapters 2 and 3).

The results obtained were discussed in detail in each chapter. In this section we will summarize them in a general discussion.

The genus *Artemia* has featured in the literature extensively and different studies have been investigating the phylogenetic relationships among sexual species and those between parthenogenetic lineages and sexual relatives (Abatzopoulos et al. 2002a; Gajardo et al., 2002; Baxevanis et al., 2005, 2006; Qiu et al., 2006; Hou et al., 2006; Muñoz et al., 2008, 2010, 2013; Maniatsi et al., 2011). In a previous study based on allozymically calibrated molecular clock, Abreu–Grobois (1987) evaluated the degree of interspecific divergences of the genus. He indicated that the first evolutionary event of the genus was the separation of New and Old World sexual species. This was followed by the separation of *A. franciscana* and *A. persimilis* in the New World and the divergence of *A. salina* and *A. urmiana* lines in the Old World. He speculated that the parthenogenetic lineage branched from the Old World sexual ancestor appearing in the Mediterranean Basin between 3 and 6 MYA, event that may have coincided with a dramatic increase in salinity and subdivision of habitats in this region during the Messinian salinity crisis (Krijgsman et al, 1999). Later, a study based on mtDNA sequences divergence (Perez et al., 1994) claimed a substantially more ancient origin of parthenogenetic *Artemia* (30-40 MYA).

More recently, Baxevanis et al. (2006) challenged this evolutionary hypothesis by analysing ITS1 nuclear sequences and 16S mtDNA. They inferred that, although the South American species *A. persimilis* diverged from the common ancestor of all *Artemia* species between 80-90 MYA, at the time of separation of Africa from South America, *A. franciscana* formed a sister clade to all Asian *Artemia* and found at least four independent origins of parthenogenetic forms, all related to Asian

species (*A. urmiana*, A.*sinica*, *A. tibetiana*). The ploidy of asexual samples was not identified and they could not discriminate between different hypotheses on the evolution of parthenogenesis.

Muñoz et al. (2010) explored the mitochondrial genetic diversity of Mediterranean parthenogenetic diploid *Artemia* including in the analysis all the Asian *Artemia* sexual species, also *Artemia* sp. from Kazakhstan, which was not previously investigated. Their results indicated two maternal origins for diploid parthenogenetic *Artemia*, one closely related to the Kazakhstan native population and the other to one population of *Artemia urmiana*. They strongly suggested that the origin of parthenogenesis in *Artemia* was much more recent, possibly even during the Holocene, and that it occurred in Central Asia.

Successively, in a study based on a combination of microsatellites and mtDNA sequences, Maniatsi et al. (2011) found that diploid, triploid and tetraploid strains had different evolutionary origins. They indicated that diploid and triploid clones are maternally related to *A. urmiana*, whereas the tetraploid one has an independent origin related to *A. sinica.* In addition, they suggested that the triploid taxa might be derived from a diploid parthenogenetic ancestor through fertilization of an unreduced asexual ovum or through fertilization by rare males of an unreduced sexual ovum. However the Kazakhstan native population was not included in this study. Moreover pooled cyst samples were used for flow cytometry analyses, potentially confounding cyst endopolyploidy with population level ploidy variation.

In this study, phylogenetic analyses were designed to better understand the origin and evolution of diploid asexual lineages in the *Artemia*

genus. More specifically, we assessed the robustness of previous phylogenies using an extensive collection of strains and, sequencing nuclear and mitochondrial markers, we tried to investigate if new asexual clones originated spontaneously from sexual species and, in this case, which sexual species were involved, if they originated through contagious asexuality or through hybridization between sexual species. For this purpose, we explored the genetic variability of nuclear and mitochondrial DNA of diploid parthenogenetic populations from different geographic locations of Central and East Asia, the region considered to be the most likely centre of asexual diploid origin. We also sequenced different populations of all Asian sexual species, including a new population of *A. urmiana* from Crimea (Koyashskoe Lake) and four different populations of *A. tibetiana*. Finally, for the first time, we included in the phylogenetic analysis sequences from rare males.

This survey substantially expands our knowledge of diploid genetic diversity in Eurasia and allows inferring the possible mechanisms generating genetic diversity of asexual lineages in the genus. The mitochondrial tree (COI sequences) was well supported phylogenetically and revealed three maternal clades of diversity in diploid parthenogenetic *Artemia*. The most common lineage is monophyletic and closely related not only to the haplotypes of the Kazakhstan population but also to haplotypes of two *A. tibetiana* populations. The less common lineage forms a polyphyletic clade, closely related to haplotypes of the newly sequenced population of *A. urmiana* from Koyashskoe Lake (Crimea). We also found a third minor lineage, which is present only in rare males from the Kujalnic (Ukraine)

population. These three maternal clades are not differentiated in their nuclear DNA (ITS sequences) since our results show that diploid parthenogens cluster very closely to all the three Asian species, *Artemia* sp. from Kazakhstan, *A. tibetiana* and *A. urmiana*. That may be explained by repeated hybridization between sexual similar lineage groups or by contagious events between one lineage group and another.

Parthenogenetic populations do not display very high mitochondrial diversity, what we would expect for repeated events of contagious origin. Moreover, parthenogenetic populations coexisting with the sexual *A. urmiana* do not have a local origin. For this reason, we did not find a strong evidence of rampant contagious parthenogenesis. However, the polyphyletic origin of the second asexual clade and the existence of a third rare clade only in rare males, may point to events of occasional contagious parthenogenesis which may occur in some populations at low frequencies, and have a high chance of not being successful from an evolutionary viewpoint.

Our study also reveals a new lineage of *A. tibetiana*, not identified before. Despite its exceptional mitochondrial genetic diversity, *Artemia tibetiana* is instead very homogeneous in nuclear genes. Possible explanations may be the introgression of genes from females of the Kazakhstan population and a hybrid origin of this species. Nuclear genes show that three species, *A. urmiana*, *Artemia* sp. Kazakhstan and *A. tibetiana* are very closely related so that they might be considered a species complex. In this regard, further investigation on the genetic diversity of *Artemia tibetiana* would be necessary to know if this species might be involved in the origin of the species complex and in the origin of parthenogenesis.

Finally, in accordance with previous studies (Muñoz et al., 2010; Maniatsi et al., 2011), both phylogenetic trees based on ITS and COI sequences, indicated that *A. sinica* do not contribute to the genetic diversity of diploid parthenogenetic *Artemia*.

Many researches have confirmed the occurrence of rare males in various obligate parthenogenetic animal species (Stefani, 1964; Blackman, 1972; Bowen et al., 1978; Plantard et al., 1998; Pongratz et al., 1998; Butlin et al., 1998; Martens, 1998; Rispe et al., 1999; Simon et al., 1999; Delmotte et al., 2001) but little is known about their population frequencies or their mechanism of origin.

Rare males are often functional and can mate with sexual females of related species but they cannot fertilize conspecific females as these females are parthenogenetic. So, why do parthenogenetic females still produce some males? Are these rare males a form of evolutionary atavism or do they have an evolutionary role?

It has been demonstrated that matings between parthenogenetically produced males and females from sexual lineages may generate both sexual and parthenogenetic lineages (Lynch, 1984; Innes and Hebert, 1988; Rispe et al., 1999; Simon et al., 1999; Delmotte et al., 2001; Paland et al., 2005; Engelstädter et al., 2011). In these cases, the occurrence of contagious parthenogenesis could be an efficient process to slow down the accumulation of deleterious mutations and to generate a substantial amount of genetic diversity in asexual lineages, potentially contributing to their persistence.

We still did not know whether contagious asexuality is possible in *Artemia*. In fact, very limited information has been available to understand the reproductive and evolutionary role of *Artemia* rare males (Bowen et al., 1978; MacDonald and Browne, 1987).

In order to evaluate the fertility and the reproductive potential of rare males in *Artemia*, we first investigated their occurrence in different diploid parthenogenetic *Artemia* populations of all over Eurasia (Chapter 2). In our extensive study, their presence was confirmed in 50 of 54 sampled populations, with a total number of 415 666 individuals sexed, indicating that male production is a general feature in diploid parthenogenetic *Artemia*, with the possible exception of the Westernmost populations. The populations with a higher ability to produce rare males were found indeed between the Mediterranean–Caspian Basins region and the salt lakes region in Kazakhstan, the region indicated as the most probable centre of origin of parthenogenesis.

DNA barcoding confirmed that males found were rare males rather than sexual strains in low frequencies. Rare male mtDNA haplotypes were either identical to those found in the parthenogenetic females from the same populations or they were closely related to them. These findings allow us to hypothesize that some rare lineages in these populations might have a higher propensity to produce rare males. This is in agreement to a study by MacDonald and Browne (1987), which found intra-population variability in the propensity to generate of rare males in *Artemia*.

Rare males were also described morphologically in the context of the variability of closely related sexual *Artemia* species. They showed higher

morphological variability than males from Asian sexual species. That may be due to heterogeneous geographical origin of parthenogenetic lineages and the inability for them to interbreed. In addition, there was not an association between haplotype group and their morphological resemblance to either *A. urmiana* or *Artemia* sp. Kazakhstan. It means that, for example, a rare male with a haplotype closely related to *A. urmiana* did not appear morphologically similar to *A. urmiana* males.

To assess the reproductive role of rare males, we performed cross-mating experiments with females of sexual Asian related species (*Artemia urmiana, Artemia sinica, Artemia tibetiana*, *Artemia* sp. Kazakhstan) (Chapter 2). We found that rare males were fully functional and capable to fertilizing eggs from all Asian sexual females. Indeed, we produced more than 250 hybrid broods that resulted in viable ovoviviparous and oviparous F1 offspring with similar or higher quality than controls (intraspecific crosses).

A panel of three microsatellite markers was screened in rare males, in the sexual females mated to them and in their F1 offspring, to find evidence that rare males contributed to the genetic material of the progeny. As these microsatellite markers were originally developed for diploid parthenogenetic strains, they amplified well in the rare male fathers but we found evidence of null alleles in the mothers for one or more of the analyzed loci. Despite this, they were very useful to demonstrate that *Artemia* rare males underwent meiotic reduction (producing haploid sperm) and were able to transmit their alleles to their offspring.

Next, we investigated whether *Artemia* had the potential of generating parthenogenetic strains through contagious parthenogenesis (Chapter 3). A requisite for this mechanism is the ability of rare males to transmit asexuality to their offspring. To test this hypothesis, live nauplii obtained from each ovoviviparous brood, achieved from crosses between rare males and Asian sexual species females, were reared in the laboratory to adulthood, then counted and sexed to estimate survival rates and sex ratio.

We found that the survival of hybrid F1 offspring was very high, and their sex-ratio was close to 1:1, an even sex ratio that usually characterizes *Artemia* sexual populations. Indeed, F1 females were unable to reproduce asexually when isolated. Then, we carried out cross-mating experiments between these F1 hybrid individuals (F1 hybrid females x F1 hybrid males) to assess their fertility, to estimate the viability of the resulting F2 offspring and to investigate their reproductive mode. Although all laboratory F1 hybrid lines were found to combine ovoviviparous and oviparous reproduction, a strong fitness decline of their reproductive performance was apparent. Overall, nauplii from F2 generations had low survival rates, and were completely unviable in the F2 generation obtained from rare males and *A. tibetiana* matings. In two of the F2 generations obtained, those from the crosses between rare males and *Artemia* sp. Kazakhstan and *A. urmiana*, we identified morphologically females that were able to reproduce parthenogenetically. Genetic analysis based on a combination of microsatellites and mtDNA sequences confirmed that the new parthenogenetic individuals were effectively generated from the

crossing with rare males. They showed to have inherited COI mtDNA haplotype from the sexual grandmother and alleles at nuclear markers from the asexual grandfather.

Our study documents the first laboratory generation of new parthenogenetic lineages in *Artemia* and supports a model for the contagious spread of parthenogenesis.

We found many surprising results in these experiments. The production of parthenogenetic individuals only in the second generation suggests a recessive inheritance of obligate parthenogenesis in *Artemia*. That also ruled out complete sex linkage (Z linkage) of the asexuality inducing alleles because the F1 females, which are the heterogametic sex, are not parthenogenetic. Moreover, the proportions of parthenogenetic females isolated in the F2 generations from the two crosses were very different. It was not significantly different from 25% in the rare males x *Artemia* sp. Kazakhstan cross F2 progeny, but much lower in the rare males x *A. urmiana* cross F2 progeny. This means that asexuality is not determined by a single recessive locus but it is likely that more genes are involved. In addition, we isolated new parthenogenetic females only in sex-biased broods. That induces to consider that there is an interaction between sex ratio distorters and a parthenogenetically determining locus or loci.

Our study is the first one to generate new parthenogenetic lineages in *Artemia* by mating rare males with some Old World sexual species females, providing evidence that contagious parthenogenesis may occur in the genus *Artemia*, particularly in populations inhabiting conspicuous biotopes in this Old World.

FUTURE DIRECTIONS

This study contributes to draft the evolutionary relationships of diploid parthenogens and their closest Asian sexual relatives in the genus *Artemia*. It confirms that asexuality has arisen many times, and reveals that different mechanisms, such as rare events of hybridization between sexual species, or by means of contagious asexuality through clonal rare males, may occur to generate and increase the genetic diversity of diploid parthenogenetic *Artemia* lineages. Our work also demonstrates the functionality of rare male in *Artemia* and their possible evolutionary role. The cross mating experiments designed have demonstrated that rare males are functional, that successfully mate with females of sexual relatives, that produce reduced gametes and that are capable to transmit parthenogenetic genes to their offspring. This is good evidence that contagious parthenogenesis may occur in *Artemia*.

From these findings, our study opens the door to many other possible investigations. First of all, laboratory crosses between sexual Asian species remain to be investigated in order to verify if parthenogenetic populations may be originated by the hybridization of those. In the future, a full use of genomic tools might help to resolve *Artemia* phylogenetic relationships, to better understand the details of the origin and genetic basis of asexuality and to demonstrate the actual evidence of contagious parthenogenesis and possible events of hybridization in the wild.

Further research may be led to unravel the genetic basis of the variation in male production rates among and within populations and to understand why there is a geographic variation in rare male frequency.

It would be also interesting to investigate the potential interactions between genetic and environmental factors that may be involved into rare male production. Indeed, as in many other cyclical parthenogenetic animal species, environmental triggers, such as stressful conditions, may be important for the switch from asexual to sexual reproduction.

Moreover, the genes involved in the transitions to asexuality are still unknown in *Artemia*. Our results suggest that sex-limited meiosis suppression might have a complex genetic basis. Additional crosses and genomic resources loci could be useful to individuate how many and which genes are responsible for the loss of sex, and to fully understand the mechanisms by which these genes cause reproductive transitions.

Future studies could focus on the discovery of ecological interactions between parthenogenetic and sexual relatives when they coexist. For example, parthenogens producing rare males might not take the full demographic advantage of avoiding the cost of males. Although it might be regarded as very low investment, when there are highly competitive conditions under resource limitation the cost of sex for parthenogens may be important.

Finally, it would be very important to unveil ecological requirements of hybrid and parental taxa, which would allow estimating the strength of reproductive isolation comparing the biological fitness of both parthenogenetic and sexual populations.

CONCLUSIONS

The main conclusions obtained from the body of research presented in this Thesis are as follows:

1) Mitochondrial and nuclear genetic diversity supporting phylogenetic reconstructions suggests that the three Asian species, *Artemia* sp. from Kazakhstan, *Artemia tibetiana* and *Artemia urmiana* are closely related and may be considered a species complex; on the other hand, the genetic diversity of *Artemia tibetiana* points to a hybrid origin of this species. All of them are involved in the origin of parthenogenesis.

2) Phylogenetic analyses on genetic diversity in diploid parthenogenetic *Artemia* populations confirm the multiple origin of asexuality in the genus. Automictic parthenogenesis has arisen at least three times independently.

3) Mitochondrial and nuclear genetic diversity of diploid parthenogenetic *Artemia* do not reveal the mechanisms underlying the origin of each group, but they suggest occasional events of contagious parthenogenesis.

4) Nuclear and mitochondrial data sequences confirm that *Artemia sinica* did not contribute to the genetic diversity of diploid parthenogenetic *Artemia* populations.

5) Male production in small frequencies is a general feature of diploid parthenogenetic *Artemia*. There is a large population variation in male frequencies, but populations with a higher ability to produce rare males were found in the region indicated as the most probable centre of origin of parthenogenesis.

6) Rare males are fully functional. They undergo meiotic reduction, producing haploid sperm and are capable to fertilize eggs from all Asian sexual *Artemia* females. Crosses between rare males and Asian sexual *Artemia* females produce viable sexual hybrid progeny in the first generation, what supports an incomplete reproductive isolation between parthenogenetic and all sexual Asian species.

7) Rare males are capable to transmit asexuality to their offspring, converting a proportion of hybrid progeny to obligate asexuality. Crosses between rare males and *A. urmiana* and *Artemia* sp. Kazakhstan produce new parthenogenetic lineages in the second generation (F2).

8) There is a recessive inheritance of obligate parthenogenesis in *Artemia*. There is not sex linkage (Z linkage) of asexuality inducing alleles, but sex limited meiosis suppressor is conferred by a recessive allele at possibly more than one locus.

9) The gene flow between sexual and parthenogenetic lineages allows asexuality genes to spread into the sexual species and, that way, parthenogens assimilate the diversity of sexual species into a diverse clone assemblage. This is important for the persistence of parthenogenetic populations by increasing their genetic diversity and slowing the accumulation of deleterious mutations in parthenogenetic strains.

10) Finally, we discuss the need to use genomic tools to further understand the genetic basis of parthenogenesis in *Artemia*.

SPANISH SUMMARY

RESUMEN

Introducción

El modo de reproducción de una especie determina su diversidad genética y, a su vez, su éxito ecológico y evolutivo (Normarck et al., 2003; Simon et al., 2003; De Meeûs et al, 2007). En una población sexual, la recombinación meiótica permite que nuevas combinaciones de genes se formen y destruyan constantemente. De hecho, las poblaciones sexuales son generalmente más diversas genéticamente en comparación con las poblaciones asexuales. Por el contrario, en un linaje estrictamente asexual, donde se supone que la mutación (con la mayoría de mutantes deletéreos) sea la única fuente de diversidad genética, se espera que la diversidad clonal de la población se reduzca en cada generación. Por esto las especies asexuales suelen ser consideradas ramas evolutivas sin salida, lo que hace presuponer que tengan una breve vida evolutiva y se extingan a corto plazo (10^4 - 10^5 generaciones) (Lynch and Gabriel, 1990). A pesar de ello, diversos estudios han demostrado que la diversidad genética de las poblaciones asexuales puede ser comparable a la de las poblaciones sexuales, si se generan repetidamente o si se producen a través de mecanismos distintos (Schwander et al., 2011, Delmotte et al., 2001, 2002, 2003). En estos casos las poblaciones asexuales producirán linajes asexuales polifiléticos muy diversos.

Por ello es muy importante conocer el origen y la evolución de los linajes asexuales, y comprender cómo se genera y preserva la diversidad genética en dichos linajes. Esto nos permitirá conocer la adaptabilidad ecológica y la competitividad de las poblaciones asexuales frente a las especies sexuales emparentadas, y evaluar su potencial

evolutivo (Bell, 1982; Simon et al. 2003).

Artemia (Crustacea, Anostraca) es un organismo cosmopolita que vive en ecosistemas hipersalinos litorales y continentales de todo el mundo, excepto en la Antártida (Triantaphyllidis et al., 1998; Van Stappen 2002). Su importancia procede tanto de su uso práctico en acuicultura como de su aplicación científica como especie modelo en una gran variedad de investigaciones genéticas y ecológicas. Otra cualidad de este organismo, que lo hace muy interesante desde un punto de vista evolutivo, se debe a la existencia de varias especies sexuales y distintos linajes partenogenéticos de diversa ploidía (diploides, triploides, tetraploides) dentro del género (Abatzopoulos 2002), que con frecuencia coexisten. Esto nos da una oportunidad única de estudiar su diversidad genética, el origen de los linajes partenogenéticos y sus interacciones evolutivas con especies sexuales.

Las poblaciones partenogenéticas diploides de *Artemia* en particular, uno de sus linajes mas extendidos biogeográficamente, son muy interesantes por varios aspectos. Las cepas o estirpes asexuales poliploides se reproducen por apomixis, ello implica que las divisiones de los ovocitos serán mitóticas, y que los descendientes serán verdaderos clones de la madre. Por su parte, los linajes partenogenéticos diploides se reproducen por partenogénesis automíctica. La meiosis y la recombinación génica pueden ocurrir, y se han identificado distintos mecanismos citológicos que permiten restaurar la diploidía del ovocito. Cada uno de estos mecanismos tiene un impacto diferente en la diversidad genética de la población, ya que pueden mantener o eliminar

la variación genética de una generación a otra, con consecuencias evolutivas muy diferentes para las poblaciones partenogenéticas (Pearcy et al., 2006; Noughé et al., 2015b).

Un aspecto potencialmente muy importante en las poblaciones partenogenéticas diploides de *Artemia* es que en estas, ocasionalmente, se encuentran machos raros, que son viables y fértiles. Aunque estos machos no tienen ninguna utilidad reproductiva para las hembras partenogenéticas (Stefani, 1960; MacDonald and Browne, 1987), podrían fecundar a las hembras de las poblaciones bisexuales asiáticas *A. urmiana, A. tibetiana, A. sinica*, originando una descendencia híbrida bisexual (Bowen et al. 1978), pero trasmitiéndole los genes causantes de la partenogénesis. Este fenómeno sería sumamente interesante, pues podría explicar el origen polifilético de la partenogénesis, a condición de que los cruces fértiles de los machos raros con las hembras sexuales produjeran nuevos clones partenogenéticos en la descendencia híbrida. Mecanismos similares se han descrito en otros organismos asexuales (Blackman, 1972; Sandrock and Vorburger, 2011; Xu et al., 2013).

El origen de los linajes partenogenéticos diploides ha sido muy debatido. Estudios genéticos recientes han establecido que las especies sexuales evolutivamente mas próximas al linaje partenogenético diploide forman un grupo monofilético de especies de Asia Central, (*A. urmiana*, *A. tibetiana*, y una especie aun no descrita de Kazajistán) (Baxevanis et al 2006; Muñoz et al. 2010; Maniatzi et al. 2011). Un estudio sobre la diversidad genética mitocondrial del linaje partenogenético diploide ha apoyado la existencia de, por lo menos, dos orígenes maternos: uno de los dos linajes mitocondriales, el mas común, está muy

estrechamente emparentado con la especie no descrita de Kazajstán, y el otro, un linaje más raro, está mas relacionado con la especie sexual *A. urmiana* (Muñoz et al. 2010). La existencia de estos dos linajes partenogenéticos diploides, y el origen de las cepas triploides del linaje común partenogenético, han hallado su apoyo en un estudio sobre la diversidad nuclear y mitocondrial de las cepas partenogenéticas de *Artemia* (Maniatsi et al. 2011). El origen biogeográfico de las cepas partenogenéticas diploides habría ocurrido recientemente en algún punto de Asia Central, y desde allí este linaje se habría extendido rápidamente a toda su distribución actual en Europa, África, Asia y Australia (Muñoz et al., 2010). Sin embargo, se desconoce la diversidad genética de las formas sexuales y partenogenéticas asiáticas.

Existe muy poca información sobre el modo de origen de la partenogénesis en *Artemia*. La posibilidad de un origen infeccioso producido por parásitos del genero *Wolbachia* ha sido recientemente descartada (Maniatsi et al. 2010). Otras posibilidades serian: 1) un origen híbrido, por el que la hibridación de dos especies sexuales emparentadas pudo dar origen a linajes partenogenéticos. Existen datos sobre hibridación entre especies de *Artemia* en la naturaleza y en el laboratorio (Abatzopoulos et al. 2002; Kappas et al. 2009); 2) un origen espontáneo, por el que una cepa partenogenética surgiría espontáneamente a partir de una sola de las especies sexuales 3) un origen contagioso, según el que podrían originarse nuevos linajes partenogenéticos cuando machos de origen partenogenético (machos raros) fecundaran hembras de especies sexuales emparentadas, trasmitiéndoles los genes causantes de la partenogénesis (Simon et al. 2003).

Esta tesis explora el origen y la evolución de la diversidad reproductiva y genética de *Artemia*, con especial énfasis en el uso de marcadores moleculares, y con la intención de comprender los mecanismos subyacentes en la generación de nuevos linajes partenogenéticos, especialmente los de hibridación y partenogénesis contagiosa, a partir del papel potencial ofrecido por los machos raros.

Los estudios realizados se exponen en los tres capítulos que conforman la base de la presente Tesis, y que plantean los siguientes objetivos particulares.

Objetivos

Capítulo I: Analizar la diversidad genética de las poblaciones sexuales y partenogenéticas asiáticas del genero *Artemia* mediante el uso de marcadores nucleares y mitocondriales. De esta manera se pretende caracterizar en detalle las relaciones filogenéticas de las cepas partenogenéticas y sus potenciales ancestros sexuales, y obtener información sobre los posibles mecanismos de origen de estas estirpes partenogenéticas.

Capítulo II: Investigar el papel evolutivo de los machos raros de *Artemia*. Para abordar este tema se ha procedido a: 1) cuantificar la presencia de machos raros en numerosas poblaciones de *Artemia* partenogenética diploide, identificando, si existe, un modelo de distribución geográfica de estas frecuencias, 2) describir morfológicamente estos machos raros en el contexto de la variabilidad morfológica presente en las especies sexuales asiáticas emparentadas, 3) evaluar el papel reproductivo de los

machos raros mediante experimentos de cruzamiento interespecífico entre estos y las hembras de las especies sexuales asiáticas relacionadas (*Artemia urmiana, Artemia sinica, Artemia tibetiana, Artemia* sp. Kazajistán), 4) caracterizar la viabilidad de la descendencia híbrida F1, 5) confirmar genéticamente la identidad y la funcionalidad de los machos raros por medio de DNA barcoding y analisis de microsatélites.

Capítulo III: Investigar si en *Artemia* existe la posibilidad de que se generen nuevas cepas partenogenéticas por origen contagioso. Para ello se ha procedido a: 1) evaluar la tasa de supervivencia y proporción de sexos en los descendientes híbridos (F1) obtenidos de los cruces entre machos raros y hembras sexuales asiáticas, 2) realizar experimentos de cruzamiento entre especímenes híbridos de la F1, 3) estimar la viabilidad y el modo reproductivo de los descendientes en la F2, 4) demostrar genéticamente que los individuos partenogenéticos obtenidos en la generación híbrida F2 descienden de los cruces originales entre machos raros y las hembras sexuales asiáticas.

Material y métodos generales

Muestras y cultivos

Las poblaciones de *Artemia* objeto de nuestros estudios se han obtenido de la extensa colección de muestras de quistes mantenidas en el banco de quistes del IATS-CSIC. Los quistes se han procesado según el protocolo descrito por Vanhaecke & Sorgeloos (1980). A partir de los nauplios procedentes de la eclosión de estos quistes originales se han obtenido poblaciones adultas, mantenidas en cultivo bajo condiciones

estandarizadas (salinidad 80 gL^{-1}, temperatura 20-24º C, fotoperíodo 12:12 h).

Machos raros, frecuencias y análisis morfométrico.

Las poblaciones partenogenéticas diploides adultas se han utilizado tanto para cuantificar la presencia de machos raros en estas poblaciones de distinto origen geográfico como para aislar los machos raros necesarios para su análisis morfométrico. Los individuos necesarios para ambos estudios (identificación de los machos raros en las muestras y medición de sus caracteres morfológicos) se han anestesiado previamente en agua de mar, mediante la adición de unas gotas de agua destilada saturada de cloroformo. Se han identificado y medido utilizando una lupa binocular provista de ocular micrométrico.

Tras cuantificar la frecuencia de aparición de los machos raros en cada población partenogenética diploide, se han tratado los datos mediante análisis estadísticos (Moran's Index y Gi test of Getis Ord) con el fin de caracterizar la existencia de un patrón geográfico de distribución de estas frecuencias, y para identificar las zonas geográficas con mayor presencia de machos raros.

El estudio morfométrico de los machos raros ha consistido en la medición de 12 parámetros: longitud total, longitud del abdomen, anchura del abdomen, anchura de la cabeza, distancia máxima entre ojos, diámetro máximo de los ojos, longitud de las antenas, longitud de la furca, número de sedas en cada rama de la furca, anchura del segmento genital y proporción de la longitud abdominal respecto a la longitud total del individuo. Los datos morfométricos medidos en los

machos raros y en los machos de las especies sexuales asiáticas (procedentes de la base de datos morfológicos mantenida en el IATS) se han tratando mediante un análisis discriminante multivariante (Hontoria y Amat., 1992) usando el programa estadístico SPSS 15.0.

Experimentos de cruzamientos interespecíficos.

Se han dispuesto experimentos de cruzamiento interespecífico entre los machos raros y hembras de las distintas especies sexuales para obtener generaciones híbridas (F1 y F2). La población partenogenética diploide de Bagdad (Irak) se ha elegido como recurso de machos raros, debido a la alta incidencia de estos en aquella población y a la mayor disponibilidad de quistes. Las hembras utilizadas se seleccionaron entre las poblaciones sexuales asiáticas, *A. urmiana* del lago Koyashskoe (Ucrania), *A. sinica* del lago Yuncheng (China), *A. tibetiana* del lago Lagkor Co (Tibet) y *Artemia* sp. de Kazajistan. Para los cruces se han elegido hembras vírgenes (emparejadas con machos raros cuando aún eran inmaduras sexualmente) o mantenidas aisladas durante las dos semanas previas a los experimentos.

La eficacia biológica de las generaciones híbridas F1 y F2 se ha descrito contrastando el tipo de reproducción: ovoviviparismo / oviparismo. En la reproducción ovovivípara se ha determinado la calidad de la descendencia ovovivípara (presencia relativa de nauplios vivos, nauplios muertos y huevos no fecundados). La calidad de la descendencia ovípara se ha caracterizado por la presencia relativa de quistes bien corionados, portadores de embriones viables, frente a

quistes mal corionados, que encierran embriones abortivos o no desarrollados.

Las puestas de nauplios vivos obtenidas en ambas descendencias híbridas F1 y F2 se han cultivado hasta el estado adulto para estimar las tasas de supervivencia y la proporción de sexos.

Para comprobar y evaluar la aparición de nuevas cepas partenogenéticas por origen contagioso, las hembras de la generación híbrida F2 se han aislado, se han diferenciado morfológicamente, y se ha controlado su modo de reproducción. Todos los datos obtenidos se han tratado estadísticamente con tests específicos utilizando el programa SPSS 15.0.

Caracterización genética

La diversidad genética de las poblaciones sexuales y partenogenéticas asiáticas se ha analizado mediante el uso de marcadores mitocondriales (COI) y nucleares (ITS1 y Na^+/K^+ATPasa). Los marcadores mitocondriales se heredan citoplásmicamente y proporcionan información sobre la genealogía maternal. Los marcadores nucleares se heredan de ambos padres, y mediante ellos se pueden identificar incongruencias debidas, por ejemplo, a hibridación. Los marcadores genéticos de alta variabilidad (microsatélites) se han empleado para genotipar los machos raros, las hembras sexuales emparejadas y la descendencia de los cruces híbridos.

El protocolo concreto del estudio genetico consiste en: 1) extracción y purificación de ADN total a partir de ejemplares adultos fijados en alcohol absoluto, o a partir de quistes, 2) selección de los cebadores para

las regiones de ADN que tienen que ser analizadas, 3) amplificación mediante PCR, 4) purificación del producto de la PCR, 5) secuenciación o genotipado en secuenciadores automáticos BEQMAN Coulter, 6) elaboración de los datos obtenidos mediante programas de análisis filogenéticos, y análisis estadísticos. Los datos obtenidos son analizados con el uso de diversos programas y recursos informáticos. Los principales programas de análisis genéticos y filogenéticos que se utilizaron son: CODONCODE para editar secuencias, MEGA, MRBAYES y FIGTREE para analizar secuencias y crear árboles filogenéticos, DNAsp para analizar la diversidad genética de las poblaciones, TCS para crear Networks.

Resultados principales y discusión

Análisis filogenéticos

Este estudio investiga las relaciones filogenéticas existentes entre las cepas partenogenéticas diploides de *Artemia* y sus potenciales ancestros sexuales e intenta identificar los posibles mecanismos de origen de la partenogénesis en el género (origen espontáneo de la partenogénesis, origen híbrido y/o contagioso). Utilizando marcadores nucleares y mitocondriales se ha analizado la diversidad genética de numerosas poblaciones partenogenéticas diploides de *Artemia* nativas de diferentes localidades geográficas de Asia Central y Oriental, región considerada como el centro más probable de origen de la partenogénesis (Muñoz et al., 2010). También hemos secuenciado diferentes poblaciones de todas las especies sexuales asiáticas emparentadas con aquellas, incluyendo

una nueva población de *A. urmiana* hallada en Crimea (Lago Koyashskoe) y cuatro poblaciones diferentes de *A. tibetiana*. Por primera vez se han incluido secuencias de machos raros en el análisis filogenético.

La diversidad mitocondrial de las cepas partenogenéticas diploides (secuencias de COI) muestra tres linajes distintos. El linaje más común es monofilético, y está estrechamente relacionado tanto con los haplotipos de la especie de Kazajistán como con los haplotipos de dos poblaciones de *A. tibetiana*. El linaje menos común forma un grupo polifilético, que està estrechamente emparentado con los haplotipos de la nueva población secuenciada de *A. urmiana* del lago Koyashskoe (Crimea). Además se ha encontrado un nuevo tercer linaje, presente sólo en los machos raros de la población de Kujalnic (Ucrania). Estos tres linajes no se diferencian en el ADN nuclear (secuencias ITS), con lo que estos resultados evidencian que todas las poblaciones partenogenéticas diploides están estrechamente emparentadas con las tres especies sexuales asiáticas, *Artemia* sp. Kazajistán, *A. tibetiana* y *A. urmiana*. Esto podría explicarse por eventos de hibridación producidos entre las especies sexuales o por eventos de partenogénesis contagiosa sucedidos entre un linaje y otro. Las poblaciones partenogenéticas diploides de *Artemia* no muestran una diversidad mitocondrial muy alta, lo que cabría esperar en una situación de repetidos orígenes producidos por partenogénesis contagiosa. Además, las poblaciones partenogenéticas simpátricas con la especie sexual *A. urmiana* no tienen un origen local. Sin embargo, el origen polifilético del segundo linaje asexual y la existencia del tercer linaje, identificado solo en machos raros, apuntan a

episodios ocasionales de partenogénesis contagiosa, que pueden ocurrir con frecuencias bajas en algunas poblaciones, y que podrían no tener una elevada probabilidad de éxito evolutivo.

Nuestro estudio también revela un nuevo linaje de *A. tibetiana*, no identificado anteriormente. A pesar de su excepcional diversidad mitocondrial, *Artemia tibetiana* es, en cambio, muy homogénea en sus genes nucleares. Esto podría deberse a una introgresión de genes por parte de las hembras sexuales de *Artemia* sp. de Kazajistan y a un origen híbrido de la especie *A. tibetiana*.

En general los genes nucleares muestran que las tres especies sexuales, *A. urmiana*, *Artemia* sp. Kazajistán y *A. tibetiana* están muy relacionadas entre si, hasta tal punto que pueden considerarse un complejo de especies. Finalmente, de acuerdo con estudios previos (Muñoz et al., 2010; Maniatsi et al., 2011), nuestros resultados indican que la especie *A. sinica* no contribuye a la diversidad genética de las cepas partenogenéticas diploides de *Artemia*.

Papel reproductivo de los machos raros de Artemia.

Para poder investigar el papel reproductivo, y el potencial evolutivo de los machos raros de *Artemia*, en primer lugar se ha cuantificando su presencia en 54 poblaciones de *Artemia* partenogenética diploide a lo largo de toda su distribución geográfica (Eurasia). Se han examinado 415.666 individuos, registrando la presencia de estos machos en 50 de las 54 poblaciones analizadas. Nuestros resultados indican que la producción de machos raros es una característica general en *Artemia*

partenogenética diploide, con la excepción de las poblaciones más occidentales. Además, las poblaciones con mayor capacidad para producir machos raros se han encontrado entre la región de las cuencas del Mediterráneo-Caspio y la región de los lagos salados en Kazajistán, el área geográfica indicada como el centro de origen más probable de la partenogénesis en *Artemia* (Muñoz et al., 2010). El análisis del ADN mitocondrial de los machos raros encontrados también nos ha permitido confirmar su identidad genética. Los haplotipos de los machos raros son idénticos a los encontrados en las hembras partenogenéticas de las mismas poblaciones, o están estrechamente relacionados con ellos. Estos resultados nos permiten plantear la hipótesis de que algunos linajes mitocondriales raros en las poblaciones partenogenéticas diploides podrían tener una mayor propensión a producir machos raros. Esta hipótesis encuentra apoyo en un estudio de MacDonald y Browne (1987), que evidencia una variabilidad intra-poblacional en la propensión a generar machos raros en una misma población de *Artemia* partenogenética diploide.

Los machos raros de A*rtemia* también se han descrito morfológicamente en el contexto de la variabilidad morfológica de los machos de las especies sexuales asiáticas emparentadas. Los resultados muestran una mayor variabilidad morfológica comparada con la de los machos de las especies sexuales asiáticas. Esto puede explicarse de acuerdo con el origen geográfico heterogéneo de los linajes partenogenéticos, y con el hecho de que las cepas partenogenéticas no se cruzan entre ellas. Además, nuestros resultados no han detectado ninguna correlación entre los grupos de haplotipos y el parecido morfológico con los machos

de *A. urmiana* o los de *Artemia* sp. Kazajistán. Es decir que, por ejemplo, un macho raro con un haplotipo estrechamente relacionado con la especie sexual *A. urmiana* no se parece morfológicamente a los machos de *A. urmiana*.

Para evaluar el papel reproductivo de los machos raros, se realizaron experimentos de cruzamiento interespecífico entre ellos y las hembras de las especies sexuales asiáticas relacionadas (*Artemia urmiana, Artemia sinica, Artemia tibetiana, Artemia* sp. Kazajistan). Nuestro estudio confirma que los machos raros son completamente funcionales y capaces de fertilizar los huevos de las hembras de todas las especies sexuales asiáticas. Se han obtenido más de 250 puestas de descendencias híbridas (F1), que presentan una viabilidad similar o superior a la de los controles (cruces intraespecíficos). La funcionalidad de los machos raros se ha confirmado también genéticamente mediante un panel de tres marcadores, que se han amplificado en los machos raros, en las hembras sexuales emparejadas y en su descendencia hibrida F1. Los resultados evidencian que los machos raros producen gametos haploides y que contribuyen al material genético de la progenie, transmitiendo sus alelos a los descendientes.

Potencial evolutivo de los machos raros de Artemia.

Nuestro estudio también se ha propuesto investigar si *Artemia* tiene el potencial de generar cepas partenogenéticas mediante el proceso de la partenogénesis contagiosa. Un requisito para desarrollar este mecanismo precisa de la capacidad de los machos raros de transmitir los genes de la asexualidad a su descendencia. Para probar esta hipótesis,

los nauplios vivos obtenidos de las puestas ovovivíparas híbridas F1 (obtenidas a partir de cruzamientos entre machos raros y hembras sexuales asiáticas) se mantuvieron en cultivo en el laboratorio hasta la edad adulta, tras lo que se cuantificaron y sexaron para estimar las tasas de supervivencia y la proporción entre sexos.

Los resultados muestran que la supervivencia de la descendencia híbrida F1 es muy alta, y que la proporción de sexos en cada puesta se acerca a 1: 1, proporción que usualmente caracteriza a las puestas de las poblaciones sexuales de *Artemia*. Las hembras de las F1 no pudieron reproducirse asexualmente cuando se aislaron de sus machos.

Seguidamente se procedido a cruzar individuos híbridos F1 (hembras híbridas F1 x machos híbridos F1) para evaluar la fertilidad y la viabilidad de la descendencia F2 resultante. Se evidenció que todos los cruzamientos híbridos F1 producen puestas ovovivíparas y ovíparas, aunque la viabilidad de los híbridos F2 resultó, en todos casos, de menor calidad. Los nauplios vivos de la generación F2 de todos los cruzamientos híbridos (F1) presentan bajas tasas de supervivencia, y en la generación F2 obtenida de los cruzamientos entre machos raros y hembras de *A. tibetiana* resultan completamente inviables.

Entre los especímenes adultos de las generaciones híbridas F2 obtenidas de los cruzamientos entre machos raros y hembras de *Artemia* sp. Kazajistan y hembras de *A. urmiana* se identificaron morfológicamente hembras que fueron capaces de reproducirse partenogenéticamente.

El análisis genético, basado en una combinación de microsatélites y secuencias de ADN mitocondrial, ha confirmado que estas hembras partenogenéticas se generaron efectivamente a partir de los

cruzamientos iniciales con machos raros, y que no procedían de un error de contaminación de muestras de laboratorio. Las hembras partenogenéticas examinadas presentan los haplotipos de ADN mitocondrial (COI) de la hembra abuela sexual, y alelos en los marcadores nucleares (microsatélites) del abuelo macho partenogenético.

Nuestro estudio documenta por primera vez la generación de nuevos linajes partenogenéticos de *Artemia* en laboratorio, y apoya la posibilidad de un origen contagioso de la partenogénesis en este género. Otros resultados han sido sorprendentes en estos experimentos. La producción exclusiva de hembras partenogenéticas en la segunda generación sugiere una herencia recesiva de la partenogénesis en *Artemia*. Lo que también descarta la hipótesis de que los alelos que inducen la partenogénesis estén asociados únicamente a los cromosomas sexuales. De hecho, en *Artemia* las hembras son el sexo heterogamético, pero en la generación hibrida F1 las hembras no son partenogenéticas.

Además, en las descendencias híbridas F2, las proporciones de hembras partenogenéticas halladas en los distintos cruzamientos son muy diferentes. Los análisis estadísticos indican que la proporción de hembras partenogenéticas producidas en el cruzamiento entre machos raros y hembras de *Artemia* sp. Kazajistan no es significativamente diferente del 25%, mientras que en el cruzamiento entre machos raros y hembras de *A. urmiana* esta proporción resulta mucho menor. Esto significa que la partenogénesis en *Artemia* no puede ser determinada por un solo locus recesivo (lo que cabria esperar si las proporciones de las hembras partenogenéticas fueran siempre un 25% de las hembras totales

en las F2). Es probable que más genes estén involucrados en el proceso de transición de la reproducción sexual a la partenogenética. El hecho de hallar nuevas hembras partenogenéticas sólo en las puestas sexualmente sesgadas (en las que dominan las hembras), nos induce a considerar que existe una interacción entre distorsionadores de la segregación sexual, de la proporción entre sexos y de los factores que determinan la partenogénesis.

Nuestro estudio es el primero en generar nuevos linajes partenogenéticos en *Artemia* mediante cruzamientos interespecíficos entre los machos raros de origen partenogenético y hembras de algunas de las especies sexuales emparentadas del Viejo Mundo, y aporta evidencia de que la partenogénesis contagiosa puede ocurrir en el género *Artemia*, particularmente en poblaciones que habitan biotopos hipersalinos conspicuos en el Viejo Mundo.

Conclusiones

Las principales conclusiones obtenidas del trabajo de investigación presentado en esta Tesis son las siguientes:

1) El análisis filogenético de la diversidad genética mitocondrial y nuclear de las poblaciones partenogenéticas diploides y de las especies sexuales asiáticas emparentadas con ellas sugiere que *Artemia* sp. Kazajistan, *Artemia tibetiana* y *Artemia urmiana* están estrechamente relacionadas y pueden considerarse un complejo de especies. Todas ellas están involucradas en el origen de la partenogénesis en el género.

2) La diversidad genética de *Artemia tibetiana* apunta a un origen híbrido de esta especie.

3) Los análisis filogenéticos de la diversidad genética en las poblaciones de *Artemia* partenogenética diploide confirman un origen múltiple de la partenogénesis en el género, en el que la partenogénesis automíctica ha surgido al menos tres veces de forma independiente.

4) La diversidad genética mitocondrial y nuclear en las poblaciones de *Artemia* partenogenética diploide no revela los mecanismos subyacentes en el origen de cada grupo, si no que apuntan a eventos ocasionales de partenogénesis contagiosa.

5) Las secuencias de datos nucleares y mitocondriales confirman que *Artemia sinica* no contribuye a la diversidad genética de las poblaciones de *Artemia* partenogenética diploide.

6) La producción de machos raros es una característica general de las poblaciones de *Artemia* partenogenética diploide. Su frecuencia es baja, aunque las poblaciones con mayor predisposición a producir machos raros se encontraron en la región geográfica sugerida como el centro de origen más probable de la partenogénesis en el género.

7) Los machos raros son completamente funcionales, producen espermatozoides haploides y son capaces de fertilizar los huevos de las hembras de todas las especies sexuales asiáticas de *Artemia*. Los cruzamientos entre machos raros y hembras de las especies sexuales asiáticas de *Artemia* producen una progenie sexual híbrida muy viable en la primera generación (F1), lo que apoya la existencia de un aislamiento reproductivo incompleto entre las poblaciones partenogenéticas y todas las especies sexuales asiáticas.

8) Los machos raros son capaces de transmitir la asexualidad a sus descendientes, convirtiendo a una cierta proporción de su progenie

híbrida en individuos partenogenéticos. Los cruzamientos entre machos raros y hembras sexuales de *A. urmiana* y de *Artemi*a sp Kazajistan producen nuevos linajes partenogenéticos en la segunda generación (F2).

9) La partenogénesis en *Artemia* se rige por una herencia recesiva. Los factores que inducen la partenogénesis no están asociados a los cromosomas sexuales (ligamiento al cromosoma Z) sino que, posiblemente, están asociados a más loci recesivos.

10) El flujo genético entre los linajes sexuales y partenogenéticos en *Artemia* permite que los genes responsables de la asexualidad se difundan en las especies sexuales, y que los nuevos linajes partenogenéticos asimilen gran parte de la diversidad procedente de una especie sexual produciendo nuevos clones. Este hecho es de capital importancia para la persistencia de las poblaciones partenogenéticas ya que, de este modo, se incrementa la diversidad genética de los linajes partenogenéticos, y se elimina la acumulación de mutaciones perjudiciales en las cepas partenogenéticas.

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