Stick insects: parthenogenesis, polyploidy and beyond

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Introduction

Metasexual animals

The most common way animals reproduce is bisexuality, namely through the mixis of male and female gametes. Bisexual reproduction relies on the Mendelian mechanism, although several additional modes also occur. In turn, the Mendelian inheritance of genetic variability stands on the meiotic process, which entrains recombination and chromosome reshuffling within the frame of a balanced segregation: any departure from these standard features will of necessity have a bearing on the genetic structure at both individual and population level. Sexual systems are typically eu-Mendelian when an equal complement of chromosomes is inherited from both parents and it is also likely to be transmitted to the following generations the same way (Normark 2006).

Some genetic systems clearly derive from the sexual one but do not completely maintain the above defined symmetries: if they skip any of them, they are termed asymmetric and typically give rise to thelytoky, haplodiploidy and/or parent-specific gene expression (Normark 2006). Collectively, those sex-derived asymmetric reproductive systems have been defined as *metasexual*, leaving the term *asexual* to those genetic systems/reproductive modes which did not evolve from the Mendelian mechanism and do not make use of gametes (Scali *et al.* 2003 and quotations therein).

The widespread identification of asexuality with the lack of recombination and/or fertilization and also the assumption that unisexuals invariably lack both of them, thus being therefore barred from any genetic variance and evolutionary potential, is ill-adviced (e.g., Bell 1982; Baxevanis *et al.* 2006): as a matter of fact, it is increasingly clear that many all-female taxa are not invariant genetic replicas as they should be if really asexual (McKinnel 1985; Hughes 1989), since most of the truly investigated thelytokous animals have shown a variety of genetic mechanisms producing new clones. This is obtained either by real recombination, as in automicts and even in some apomicts, or by mutation and new genome

incorporations. As a consequence, several metasexual organisms can maintain significant levels of genetic diversity (Turner *et al.*, 1983; Densmore *et al.* 1989; Quattro *et al.* 1992; Haddal *et al.* 1994; Tinti & Scali 1996; Johnson *et al.* 1999; Halkett *et al.* 2005). Polyploidy also adds to metasexual animal diversification, so that unisexual taxa are often a "colourful" bunch of clones with very good colonizing abilities (Hughes 1989), far from asexual uniformity. In addition, their egg maturation processes and sex differentiation bear unmistakable traits of sexuality (see below, *Sexual and metasexual stick insects*). On the whole, it sounds a bit artful to consider an egg a gamete only when fertilized and not a gamete if unfertilized (Bell 1982), as it occurs in haplo-diploid systems or facultative parthenogenesis. This assumptions would therefore lead to accept that a female is "sexual" or "asexual" on the basis of her laid eggs' destiny.

Cytogenetic and genetic analyses have been performed in depth on a variety of metasexual animals, particularly after the adoption of gene-enzyme systems' electrophoresis and nucleotide sequencing, to better understand the origin and evaluate the amount of genetic diversity. In all cases, more genetic variation than expected was found in parthenogenetic or gynogenetic (pseudogamic) taxa. Genetically diversified parthenogenetic/gynogenetic groups other than the stick insects (phasmids) include freshwather flatworms (Pongratz *et al.* 2003), oligochaetes – especially *Octolasion tyrtaeum* and *Lumbricillus lineatus* (Viktorov 1997), several hybrid and non-hybrid freshwater snails (Stoddart 1983; Johnson *et al.* 1999), the millipede *Nemasoma varicorne* (Jensen *et al.* 2002), several crustacean complexes within branchiopods, cladocerans, ostracods, and isopods (Christensen *et al.* 1987; Dufresne & Hebert 1994; Shön *et al.* 2003; Baxevanis *et al.* 2006), *Pycnoscelus* cockroaches (Parker & Niklasson 2000), the orthopteran *Warramaba* complex (Kearny *et al.* 2003), several species of aphids (Delmotte *et al.* 2002; Halkett *et al.* 2005), some simuliid (White 1970) and lonchopterids (Niklasson *et al.* 2004) among the Diptera, a few moths (Lokki *et al.* 1975; Suomalainen *et al.* 1981; Harshman and Futuyma 1985) and several weevils (Lokki *et al.* 1976; Stenberg *et al.* 2003). Within vertebrates, examples are found among freshwater fishes (poeciliids, the cyprinids *Rutilus* (= *Tropidophoxinellus*) *alburnoides* and *Phoxinus eos-neogaeus*, the cobitid *Cobitis*), the urodeles (*Ambystoma*) and the reptiles (the teiids *Aspidoscelis* and *Cnemidophorus*, the gekkonid *Heteronotia* and the lacertid *Lacerta*; Dawley & Bogart 1989; Densmore *et al.* 1989; Hedges *et al.* 1992; Quattro *et al.* 1992; Haddal *et al.* 1994; Martins *et al.* 1998; Vrijenhoek 1998; Alves *et al.* 1999).

Parthenogenesis and polyploidy

Parthenogenetic or gynogenetic animals, particularly the apomictic ones, are often polyploid (see Otto & Whitton 2000 for a comprehensive list; also Pongratz *et al.* 2003; Stenberg *et al.* 2003; Halkett *et al.* 2005; Gómez-Zurita *et al.* 2006). The

connection between parthenogenesis/gynogenesis and polyploidy is to be found in the advantages obtained by parthenogens in escaping chromosome pairing constraints and/or cytogenetic disruption (due to sex-chromosomes and their dosage compensation) in the polyploid germ line and embryo cells, particularly in cases of uneven ploidy (Orr 1990). As a correlate, polyploidy is more common in animals with a genetic sex-determination than in those with defined heterochromosomes (Omodeo 1951, 1953; Dufresne & Hebert 1994; Evans *et al.* 2004; Holloway *et al.* 2006; Scali 2009a).

Furthermore, compared to diploids, both autopolyploids and allopolyploids can take advantage from higher mutational rate and the consequent increase of heterozygosity. However, the highest short-term advantage is experienced by allopolyploids, where a considerable rate of heterozygous loci is immediately obtained through hybridity. An additional general advantage of polyploids appears to reside in their better buffering ability in maintaining functional alleles when non-functional mutations tend to accumulate in long persisting partheno-genetic strains (Lokki 1976a, b).

Geographic parthenogenesis

Most parthenogens are found on a geographic area different and generally wider than their sexual relatives. This common feature was first defined as "geographical parthenogenesis" by Vandel (1928). Association of parthenogens to glacial regression, confining their Mendelian ancestors in refugia of non-glaciated areas and to peripheral, more disturbed habitats with lower biotic diversity were most often observed (Suomalainen *et al.* 1987; Hughes 1989, 1996; Jensen *et al.* 2002; Kearny & Moussalli 2003; Kearny *et al.* 2003; Kearny 2005). The overall genetic properties of parthenogens could account for the displacement or extinction of their ancestors. When polyploids are mainly distributed in the marginal areas of the diploids' range, the term "geographic polyploidy" has sometimes been preferred (Stenberg *et al.* 2003). However, the distribution pattern of some parthenogenetic complexes, other than reflecting competitions with sexual parentals, might also be the outcome of chance and historical accidents (Law & Crespi 2002; Pongratz *et al.* 2003).

Sexual and metasexual stick insects

Phasmida (Otte & Brock 2005; = Phasmatodea or Cheleutoptera of previous literature) is an orthopteroid insect order to which the ca. 3000 known species of stick insects are referred.

Among the Phasmida, about 10% of the named taxa, patchily scattered in different families, are parthenogenetic. Phasmid parthenogenesis is thelytokous

(i.e. it produces all-female offspring). The most divergent condition from the typical eu-Mendelian origin is provided by apomictic parthenogens, where meiosis is deeply modified or completely replaced by a mitotic division and fertilization is skipped altogether. Several intermediate mechanisms closer to eu-Mendelian reproduction are also known to occur in these insects.

Accurate investigations on karyotype structure, reproduction and phyletic relationships of the holomediterranean genus *Bacillus* (Fig. 1), revealed the occurrence of a wide variety of reproductive modes such as facultative and obligate parthenogenesis, hybridogenesis and androgenesis, within a general frame of interracial and interspecific hybridizations leading to both diploid and polyploid taxa (Scali *et al.* 2003). More recent analyses of the Iberian genus *Pijnackeria* and of the Moroccan *Clonopsis* (Fig. 1) are also suggestive of a similar array of reproductive modes and species interactions (Passamonti *et al.* 2004; Ghiselli *et al.* 2007; Milani *et al.* 2009; Scali 2009a, b).

Bacillus Berthold. The genus includes the following taxa:

- the western Mediterranean facultative parthenogen *B. rossius* (Rossi), 2n = 35/36, X0/XX. North-African races are strictly bisexual, while the two Italian sub-species (*B. rossius rossius* and *B. rossius redtenbacheri*) are represented by either bisexual or all-female populations
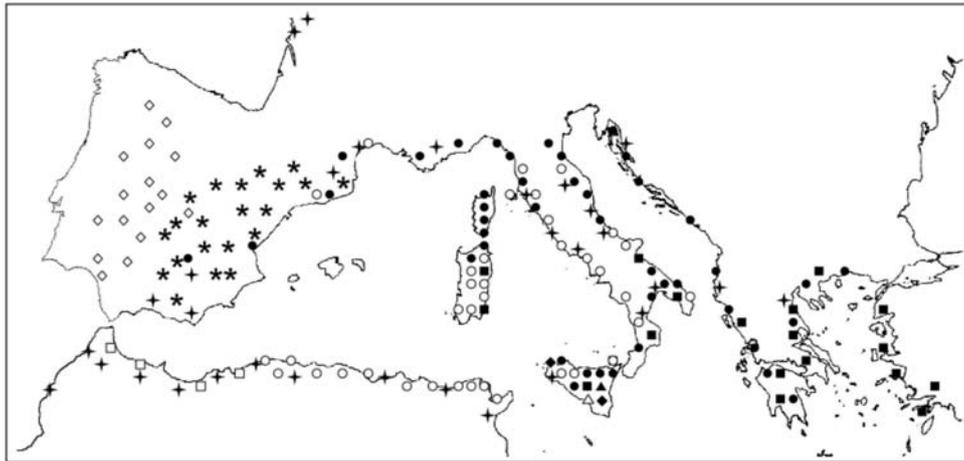

Fig. 1 Map showing the overall distribution of the circum-mediterranean stick insect taxa. Open circles refer to bisexual, the solid ones to parthenogenetic *Bacillus rossius*. In Sicily, solid diamonds indicate *B. grandii*; open or solid triangles *B. whitei* and *B. lynceorum*, respectively; solid squares refer to *B. atticus*. Open squares indicate amphigonic *Clonopsis* species, four-arm stars the obligate parthenogens of the genus (only *C. gallica* outside Africa). Open diamonds mark the range of *Leptynia* species and the five-arm stars the range of *Pijnackeria*.

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- the bisexual *B. grandii* Nascetti & Bullini, 2n = 33/34, X0/XX, endemic to tiny areas of Sicily and formally split into three subspecies, namely *B. grandii grandii*, *B. grandii benazzii* and *B. grandii maretimi*

- the central/eastern Mediterranean *B. atticus* Brunner (Fig. 1), an all-female, obligate parthenogenetic complex, differentiated into three karyological and allozymic races (*B. atticus atticus*, 2n = 34, XX; *B. atticus cyprius*, 2n = 32, XX; *B. atticus carius*, both 2n = 34, XX, and, mainly, 3n = 48-51, XXX)

B. rossius redtenbacheri, *B. grandii grandii* and *B. atticus atticus* are the ancestors of the Sicilian thelytokous species hybrids (Fig. 2): the diploid *B. whitei* Nascetti & Bullini (= *rossius redtenbacheri/ grandii grandii*, 2n = 35, XX) and the triploid *B. lynceorum* Bullini, Nascetti & Bianchi Bullini (= *rossius redtenbacheri/ grandii grandii/ atticus atticus*, 3n = 51, XXX).

Both *B. whitei* and *B. lynceorum* exploit an apomictic meiotic mechanism, that still allows for some recombination during the highly modified prophase I, during which an extra-doubling of DNA occurs, so that several different clones are produced. In addition, also racial hybrids, such as the automictic *Bacillus atticus*, particularly the triploids, are very rich of clones, which obviously demonstrate high genetic differentiation within the taxon (review in Scali *et al.*, 2003).

In addition to species hybrid parthenogens, all-female populations of *B. rossius* and *B. grandii* males hybridized, in Sicily again, to produce two different hybridogenetic strains: *B. rossius redtenbacheri-grandii grandii* and *B. rossius*
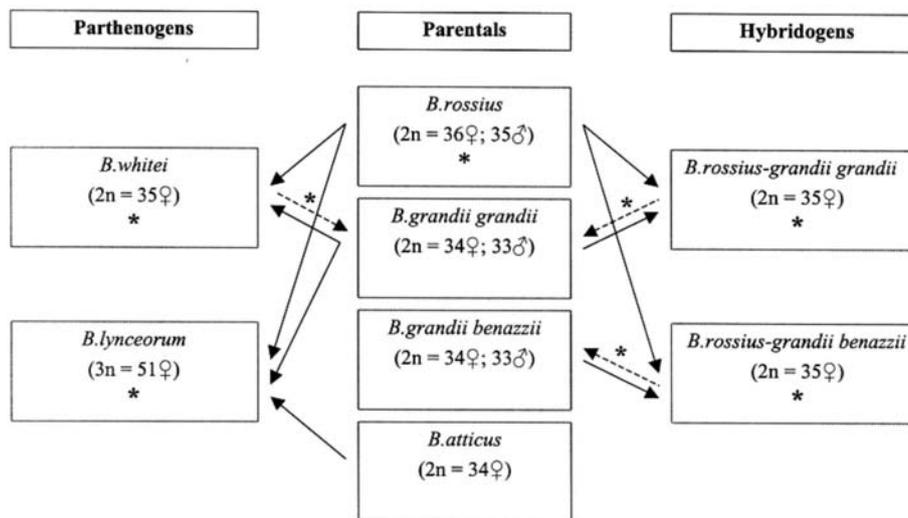

Fig. 2 Reproductive relationships among *Bacillus* taxa, a clear case of reticulate evolution. Stars mark taxa with *rossius* mitochondrial genome, while sketched arrows the observed reversions from hybrid to the non-hybrid paternal ancestor (from Scali *et al.*, 2003).

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redtenbacheri-grandii benazzii, which pass on to their progeny an invariant maternal *rossius redtenbacheri* haploset, while renewing at each generation (hemiclonal reproduction) the paternal contribution to their genome (either *grandii grandii*, for the southern hybridogen, or *grandii benazzii*, for the northern one). Through hybridogenesis, offspring of both sexes is produced, but males are almost invariably lethal and always sterile. Mitochondrial DNA analysis (see below) showed that *Bacillus* species hybrids and hybridogens arose through asymmetrical crosses, with *B. rossius* always being the maternal parent (Mantovani & Scali 1992; Mantovani *et al.* 1999).

It has also been shown that by shifting to androgenesis, hybridogenetic females can escape hybridity to step back in just one generation to Mendelian reproduction with the full genetic diversification of the fathering species. This can occur since phasmid eggs are physiologically polyspermic and when syngamy with the egg pronucleus fails, two male pronuclei may fuse instead and develop into embryos of either sex with nuclear gene composition exclusively from the fathering species. This androgenetic progeny will then reproduce as a eu-Mendelian species. Androgenesis actually frees hybridogenetic strains from being just sexual parasites of the fathering species *B. grandii*; their hybridogenetic derivation is clear, however, since they possess *grandii* nuclear alleles alongside with *rossius* mitochondrial genome. It was right this peculiar genetic feature that allowed a sound assessment of the occurrence of androgenetic specimens in the field (Tinti & Scali 1996; Mantovani *et al.* 2001). Surprisingly enough, instances of reversion from a hybrid structure to the fathering species have been obtained even in *B. whitei*, thus demonstrating both the availability of androgenesis to a parthenogenetic species and sperm access to parthenogenetic eggs.

On the whole, *Bacillus* taxa appear to experience a wide array of reproductive modes: eu-Mendelian reproduction, facultative and obligate parthenogenesis, hybridogenesis, androgenesis and reversion from hybridogenesis or parthenogenesis to bisexual reproduction. A complex net of reproductive and phyletic interactions was thus established to represent a clear instance of "reticulate evolution" (Fig. 2; Mantovani *et al.* 1999).

Pijnackeria Scali. Two species, *Leptynia attenuata* and *L. hispanica*, were originally recognized by Pantel (1890) among Iberian stick insects. Detailed analyses of recently collected samples revealed that *L. hispanica* as described by Pantel is in fact an all-female taxon and that the syntopic males assigned to it actually belonged to a taxon, undescribed at the time (now *L. montana* Scali, 1996) (Scali 2009b). Furthermore, within each of the two nominal species the real occurrence of several bisexual taxa was afterwards ascertained (Scali 1996; Bianchi & Meliado 1998; Passamonti *et al.* 1999, 2004). Therefore, on the basis of morphological, chromosomal, allozymic and mitochondrial gene evidence, the nominal genus *Leptynia* had to be revised to include only the "*attenuata*"-like taxa, while all the "*hispanica*"-like species were attributed to the new genus

Pijnackeria (Scali 2009b). The *Leptynia* taxa are all bisexual and followed a chromosomal differentiation pathway which also entrained shifts to an XX/XY sex-chromosome formula from the original XX/X0, perfectly matching the evolution described in the Australian *Didymuria violescens* species-complex (Craddock 1970, 1975). On the other hand, *Pijnackeria* includes both bisexual and parthenogenetic taxa: bisexual species invariably show 38/37 chromosomes
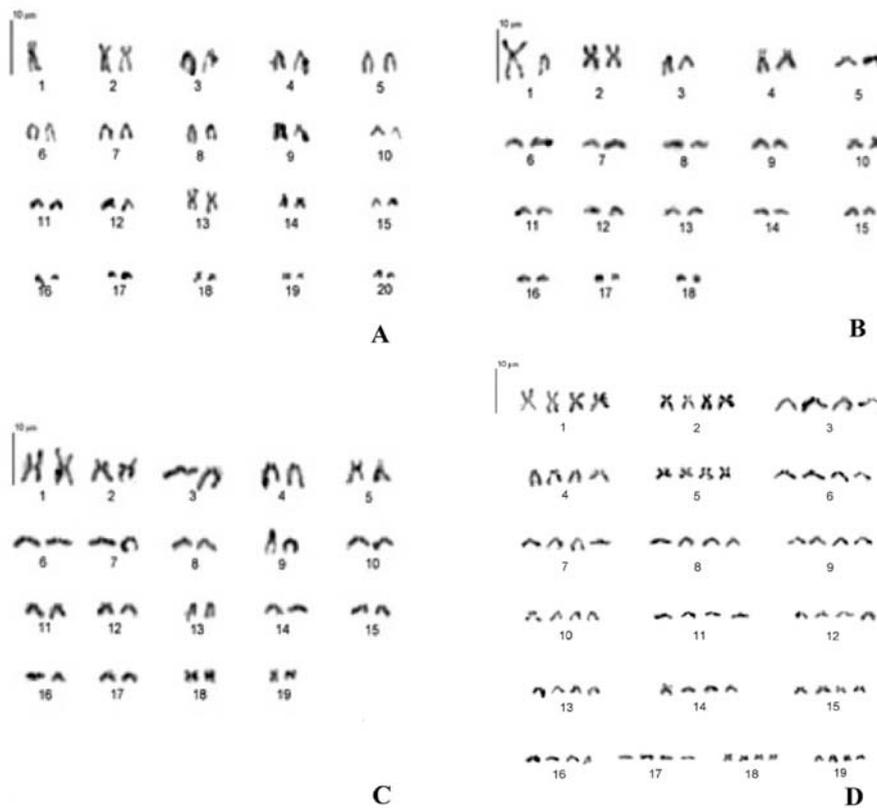

Fig. 3 A - Male karyotype of *Leptynia sp.* with 2n = 40/39, XX/X0. This southern taxon is basal to all other *Leptynia* species and together with *L. caprai* shows the highest chromosome number. B - Male karyotype of *Leptynia attenuata* (2n = 36, XX/XY) from Saõ Fiel (Northern Portugal). The species has the lowest chromosome number and its sex-chromosome formula, shifted from the usual XX/X0 to XX/XY. C - Female karyotype of *Pijnackeria species D* (Sierra de Cazorla), with 2n = 38/37, XX/X0; this is the maternal ancestor of the tetraploid *P. hispanica*. All bisexual species of *Pijnackeria* have the same number of chromosomes and rather similar karyotypes. D - Tetraploid karyotype (4n = 76, XXXX) of the parthenogenetic *Pijnackeria hispanica*: within each quartet the chromosomes are very similar, thus the hybrid derivation of this parthenogen cannot be argued from the karyotype, but only from mitochondrial and nuclear genes (from Scali, 2009b).

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with the usual XX/X0 sex-chromosome constitution; the two all-female taxa include a new triploid species, 3n = 57, XXX, and the tetraploid *P. hispanica* (4n = 76, XXXX, Fig. 3).

To disentangle the relationship network within *Pijnackeria*, the recombinant DNA technology was used (Ghiselli *et al.* 2007). Sequence analysis of the mitochondrial *cox2* gene splits the whole sample set into 6 distinct groups, wholly reflecting their geographical distribution, namely, four groups of diploid taxa (A-D, not *sensu* Nascetti *et al.* 1983), one of triploids and one of tetraploids (Fig. 4). As the sequences derived from triploids were identical to those from the diploid bisexual *Pijnackeria sp. C*, the latter is to be regarded as the maternal ancestor of the triploids. Furthermore, the haplotypes derived from tetraploids were very similar to those of the bisexual *Pijnckeria sp. D*, which is therefore the maternal ancestor of tetraploids. These observations also support the hybrid origin of both parthenogens, although only for the triploids the hybrid condition can be recognised from karyotype. Haplotypes from triploids are almost invariant and contrast with the more varied sequences from the tetraploids; this finding may be tentatively ascribed to the much older age of tetraploids as compared to triploids: the 2.86 $\pm$ 1.01 MYA old tetraploid taxon certainly had many more chances for mutations to occur and accumulate in the *cox2* gene than the very recent triploid parthenogen. Of course the higher ploidy level also allowed a larger accumulation of mutational changes.
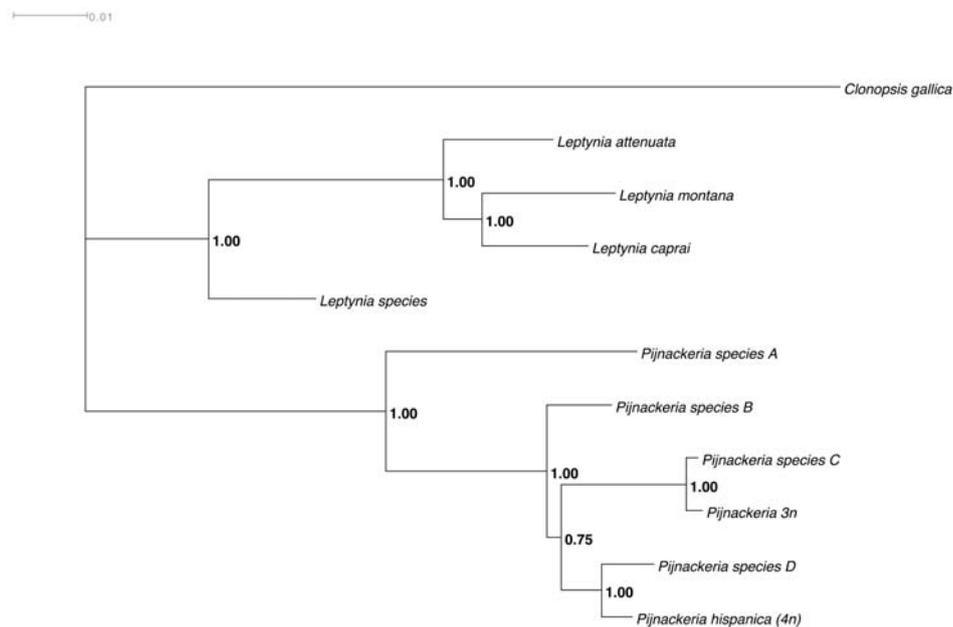

Fig. 4 Phylogenetic tree of Iberian stick insects, obtained from *cox2* mitochondrial gene sequences (Mr Bayes 3.1, 10,000,000 generations), giving an updated systematic status of the Iberian taxa (from Scali, 2009b).

Pijnackeria parthenogens' distribution follows a geographic parthenogenesis pattern and their ancestors' situation directly reminds the one noticed for the relic *B. grandii* distribution when compared to the ranges of *B. whitei* and *B. lynceorum* (Scali *et al.* 2003): the parental species have been apparently displaced by their hybrid descendants, with the paternal one being completely outcompeted and the maternal one very reduced in range. Owing to the very wide ranges of both *Pijnackeria* polyploid parthenogens, it seems likely that hybridity and uniparental reproduction have conferred them a physiological robustness and a strong colonizing ability, thus allowing the exploitation of remarkably different niches.

Furthermore, in the attempt to trace the putative paternal ancestor(s) of the two parthenogenetic taxa, 80 clones of the nuclear *elongation factor-1α* genes have been PCR amplified. Quite unexpectedly, no sequence cluster related to the maternal ancestor *Pijnackeria sp. D* was found in the tetraploid parthenogens. While triploids could be easily explained as a species hybrid originated through the backcross of a diploid hybrid parthenogen to the fathering taxon, the tetraploid parthenogens require a different explanation (Ghiselli *et al.*, 2007). It has been suggested that a diploid androgen, escaping its former hybrid constitution, was an intermediate step to *Pijnackeria* tetraploids and that such an androgen could have originated an all female parthenogen through unreduced tetraploid eggs. In other words, since no elongation *factor-1α* alleles of tetraploids appear to derive from their maternal ancestor (which however contributed the mitochondria), they might derive from a different paternal genome(s) through androgenesis. Although other routes to *Pijnackeria* tetraploids are possible, the presented one is the only one based on known reproductive features of phasmids.

***Clonopsis* Pantel.** Of recent, new *Clonopsis* stick insects have been discovered in the Moroccan Rif area, so that at present the genus is known to include bisexual, parthenogenetic and androgenetic taxa (Bullini & Nascetti 1987; Milani *et al.* 2009a; Scali & Milani 2009). The three sexual species do not appear chromosomally related, while they certainly are so as for body and egg morphology. On the other hand, the karyotypes of the eu-Mendelian *C. felicitatis* and those of *C. gallica* and *C. soumiae* parthenogens form a numerically polyploid series (2n = 36, 3n = 54, 4n = 72, respectively); quite surprisingly, all karyotypes are structurally diploid though, and appear to share very similar chromosomes (Milani *et al.*, 2009a). A close phyletic relationship among *C. felicitatis*, *C. gallica* and *C. soumiae*, as well as with two all-male androgenetic taxa, *C. androgenes-35* and *C. androgenes-53*, is further supported by the analysis of mitochondrial *cox2* haplotypes (Milani *et al.* 2007).

Clonopsis species are apparently restricted to North Africa, with the only exception of *C. gallica* (Charpentier), which, in spite of its rather low fecundity (about 80 eggs per female; Pantel 1890, 1915), has a very wide range, spreading eastwards from Morocco to Tunisia and, across Gibraltar, to the whole Southern

Europe (Iberian Peninsula, France, Italy, Eastern Adriatic coasts, Greece).

For many years *C. gallica* was the only species of the genus analyzed at the level of chromosomes, but whether it was structurally diploid or triploid remained an open question, since the investigated samples did not support a sound arrangement of chromosomes in pairs or triplets, while revealing the occurrence of chromosome variability, from 54 to 57, with some unique elements (Bullini & Bianchi Bullini 1971). On the whole, allozyme analyses of *C. gallica* suggested hybrid constitution and an apomictic mechanism of reproduction owing to the occurrence of fixed heterozygosities at a few loci in several populations, but this finding was at variance with the considerably high number of clones, often differentiated at several loci, which alternatively supported either recombination events or multiple hybridizations (Bullini & Nascetti 1987; Bullini 1994).

The discrepancy between the polyploid number and the diploid structure of *C. gallica* and *C. soumiae* chromosomes is now being evaluated, also suggesting the karyotype evolution mechanisms likely involved, (Milani *et al.*, 2009b). Furthermore, the contrasting genetic features of *C. gallica* were explained by actually investigating its egg maturation process: it was found that two different apomictic mechanisms are at work, one replaced meiosis by a unique mitotic division, the other kept a meiotic-like pattern, still allowing some recombination, in the very same way as *Bacillus* and other phasmid apomicts do (Scali *et al.* 2009). The combined outcomes of the cytological mechanisms and the hypothesized derivation of the parthenogens appear to account for the whole karyological and genetic data sets now available for *Clonopsis*.

Centrosome dynamics and γ-tubulin(s) in stick insects

Besides acquiring the right chromosome number, a parthenogenetic egg has to build the first embryonic spindle without sperm contribution to start development. Spindle microtubules (MTs) play a key role in chromosome segregation during mitosis and meiosis. The main MT organizing centre (MTOC) of animal cells is the centrosome, generally made of two orthogonally arranged centrioles surrounded by numerous proteins, known as pericentriolar material, from which MTs grow. The centrosome duplicates during the interphase of the cell-cycle and, at the following prophase, each duplicated centrosome (diplosome) moves apart and emanates the aster and hemispindle MTs. The latter join up to build the MT apparatus, which operates the correct chromosome segregation (Compton 1998).

Within the centrosome, much attention has been given to the role of γ-tubulin, an ubiquitous, highly conserved protein mainly found in the pericentriolar material of the MTOCs, where it starts MT nucleation (see Marescalchi *et al.* 2002a and references therein).

During insect gametogenesis, the spermatocyte MTOC partially dis-assembles: the centrosome loses its pericentriolar material, including γ-tubulin,

while the centrioles are maintained; during the interphase between the first and second meiotic division, no centriole doubling takes place, so that only a single centriole is present in round spermatids at the beginning of spermatozoon differentiation and only one centriole is found in the neck region of differentiating spermatozoa. On the other hand, in the female germ line, at the onset of meiosis, the centrosome loses the centrioles, but keeps its pericentriolar material including γ-tubulin, which, however, disperses throughout the egg cytoplasm: these dispersed centrosome components are not capable of centrosome reorganization and duplication (Callaini *et al.* 1999; Krioutchkova & Onishchenko 1999).

In sexually reproducing insects, the centrosome is typically reassembled soon after sperm penetration into the egg by the gathering of the pericentriolar material, including γ-tubulin, from the egg cytoplasm, around the single, sperm-derived centriole (Schatten 1994; Riparbelli *et al.* 1997; Callaini *et al.* 1999; Cowan & Hyman 2006). In fertilized eggs, the reconstitution of the centrosome prompts the nucleation of the MTs of the sperm-aster. This is followed by centriole/centrosome duplication, which eventually plays a major role in the rejoining and fusing of the pronuclei. As a consequence, the sperm-derived centrosome has a key function in the syngamy and the onset of embryo development.

On the other hand, the most clear and common example of unconventional contribution to the zygote centrosome by the two gametes is parthenogenetic development (Simon *et al.* 2003), which has been analysed particularly well in insects (Suomalainen *et al.* 1987; Normark 2003).

The spindle of dividing *Bacillus* spermatogonia and spermatocytes reveals centrioles and spots of γ-tubulin in the centrosome area and later, in spermatids, γ-tubulin is gradually reduced and then lost altogether in mature spermatozoa as it is the rule in animals. However, unexpectedly, also the centriole is no longer detectable in the neck region of spermatozoa. Therefore nor γ-tubulin nor centriole are transferred to the egg by the sperm head and, as a consequence, no sperm aster forms. It has also been found that in bisexual *Bacillus* oocytes, γ-tubulin disperses and the centrosome, together with centrioles, is lost as is the general rule in animals, but, at the end of meiosis, the pronucleus autonomously starts nucleating MTs just before syngamy. In other words, in bisexual *Bacillus,* syngamy appears to occur without centriole and aster contribution by sperm to the zygotic spindle: actually only a tangle of MTs is nucleated around sperm-head chromatin (Marescalchi *et al.* 2002a).

Fertilized *Bacillus* eggs were found to contain several spermatozoa (physiological polyspermy): within few hours the tails of those sperm cells disintegrate, while most heads evolve into pronuclei and eventually become surrounded by newly nucleated tufts of MTs. At syngamy, one sperm-derived pronucleus, with its MT felt, fuses with the egg pronucleus, which, independently from sperm, developed the same way its own MT bundle: their fusion originates

the zygotic spindle. Thus, the fertilization process is quite peculiar since it has abolished the sperm centriole and the deriving spermaster contribution to the egg.

When no sperm at all is at hand, the parthenogenetic egg's ability to derive the first embryonic spindle wholly from MTs nucleated around its pronuclear chromatin is of paramount importance, since it makes up for the complete lack of sperm contribution to the zygotic MTOC (Marescalchi *et al.* 2002a). This flexibility in building the first spindle is likely a major pre-adaptation of phasmid species to parthenogenesis. In both fertilized and parthenogenetic germ "anlagen", γ-tubulin foci can only be immuno-labelled when the embryo development has gone through its first third: this finding clearly suggests that centrioles are built *de novo* later on during embryogenesis (Marescalchi *et al.* 2002a and unpublished).

The absence of asters at spindle poles of both somatic and germ line dividing cells is another unusual trait of phasmids and suggests that centrosomal γ-tubulin could be involved, since asters are the most direct descents of γ-tubulin MT-nucleating activity (Riparbelli *et al.* 1997; Callaini *et al.* 1999; Cowan & Hyman 2006). Investigations with immunoblots on the expression and molecular characteristics of γ-tubulin in *Bacillus rossius* reveal that *B. rossius* γ-tubulin is lighter than in *Drosophila* and other animals (Megraw & Kaufman 2000). Furthermore, among different subfamilies of phasmids several mobility patterns have been recognized and even at the intrageneric level the γ-tubulins of *Bacillus* taxa have different MWs (Marescalchi *et al.* 2002b). The finding of γ-tubulin variability between species of the same genus and within the same class is quite surprising, if we consider that γ-tubulin is a very conserved protein, very similar from yeast to man (Joshi *et al.* 1992).

Concluding remarks

Investigations on metasexual animals, particularly parthenogens, are appealing owing to the questions they raise on sexual, reproductive, ecological and evolutionary issues. Parthenogenesis certainly reduces genetic variability but does not wholly suppress it. Would all parthenogenetic organisms behave as bdelloid rotifers, then it would seem adequate to identify parthenogenetic reproduction with the loss of sex (*sensu* Bell 1982), but the complex scenario of interactions which has gradually emerged from the study of phasmids, rather appears to open unusual and yet additional pathways to sexual reproduction. The occurrence of recombination even in apomicts, parthenogen backcrossing with repeated genome incorporation, hybridogenesis and androgenesis do not fit within the assumption of asexuality. It seems also undisputable that many other parthenogenetic complexes, even in distantly related animal groups, share most features we know from phasmids.

To avoid flaws in interpreting parthenogenetic animals, the widest array of approaches should be utilised. Every effort should be made to obtain as many as

possible genotypic and cytological data, since the nature of egg maturation processes is central to understanding the genetic features of the ensuing metasexuals, in order to avoid simplistic, unsupported generalizations. Furthermore molecular and statistical analyses will provide reliable insights into their history and relationships to their ancestors.

In circum-Mediterranean stick insects, such joint investigations were carried out to a good extent. On the whole, it has been possible to demonstrate the occurrence in these phasmids of different kinds of parthenogenesis: in *B. rossius* automictic thycoparthenogenesis arose many times at several different locations, while the present-day automictic *B. atticus* can be interpreted as a diploid or triploid interracial hybrid (Bullini 1994; Marescalchi & Scali 2003). In turn, apomictic parthenogenesis exploiting an intrameiotic DNA extra-synthesis still allowing some recombination, has been evidenced in the species hybrids *B. withei* and *B. lynceorum* (Marescalchi *et al.* 1991). The low but real rate of unsuspected recombination, as well as the hybrid and polyploid structure, help understanding the relatively long persistence of the *Bacillus* and *Pijnackeria* parthenogens. Corresponding investigations suggest that features promoting genetic variability are likely present in other ancient, well differentiated genera of parthenogenetic stick insects such as the New Zealand *Acanthoxyla* (Morgan-Richard & Trewick 2005; Buckley *et al.* 2008) and the north American *Timema* (Sandoval *et al.* 1998).

The polyploid hybrid structure of phasmids appears to have mainly followed the pathway of repeated backcrosses of the diploid hybrids to the paternal species with the incorporation of genomes, as observed in *Bacillus, Pijnackeria* and *Clonopsis* polyploids (Ghiselli *et al.* 2007; Scali 2009a, b; Scali & Milani 2009).

In a wider perspective, the accumulation of conspecific or heterospecific paternal genomes in diploid hybrid matasexuals appears a very common pattern in the route to polyclonal polyploids, such as the fresh water planarian complex *Schmidtea polychroa* (Pongratz *et al.* 2003), several weevils (Stenberg *et al.* 2003), *Calligrapha* leaf-beetles (Gomez-Zurita *et al.* 2006), and also *Cobitis*, *Poecilia*, *Poeciliopsis*, and *Squalius alburnoides* among the fishes (Vrijenhoek 1998; Evans *et al.* 2004), as well as the urodele *Ambystoma* complex, the African clawed frogs *Xenopus* and the teiid lizards (Dawley & Bogart 1989).

It seems also sound to consider as significant additional sources of genetic diversification in diploid or polyploid hybrid parthenogens the different composition and expression of rDNA cistrons (Manaresi *et al.* 1993; Marescalchi & Scali, 1997). Even differences in sequences and copy-number of either coding or non coding DNA, such as satDNAs, including micro-satellites (Mantovani 1998; Scali & Tinti 1999; Luchetti *et al.* 2003; Andersen *et al.* 2005) cannot be completely disregarded as possible adaptive responses at organism and cell levels.

In *Bacillus* stick insects, comparative analyses made also possible to reveal the occurrence of the hybridogenetic and androgenetic reproductive modes, which otherwise would have escaped attention. Hybridogenetic strains of phasmids

represent up to now the only instance of hemiclonal land animals, the other known hybridogens belonging to fish and amphibian complexes (review in Dawley & Bogart 1989; Carmona *et al.* 1997). Furthermore, a most interesting genetic and evolutionary novelty is produced when a switch from hybridogenesis to androgenesis occurs. It does not only accomplish a reversion from hybridity, but it provides also a new genetic subject with enhanced evolutionary prospectives, since heterologous mitochondrial and nuclear genomes are suddenly confronted within one cell (or organism). Natural androgens also provide an opportunity for new cladogenesis to occur by fixing and spreading homozygous underdominant chromosome constitutions (Passamonti *et al.* 2004). Actually, it is likely that a diploid androgen escaping a former hybrid structure has been an intermediate step to the *L. hispanica* tetraploids (Ghiselli *et al.* 2007). Quite independently, a similar origin had been suggested for some *Daphnia pulex* hybrid parthenogens (Dufresne & Hebert 1994).

The widespread occurrence of repeated and complex interactions among eu-Mendelian and related metasexuals, even allowing complete reversion from thelytoky to amphimixis and further hybridization events, is further blurring the boundaries between what would be defined as either sexual or asexual organisms according to the popular but improperly accepted terminology. In face of the jigsaw-puzzle pattern of interactions emerging from the *Bacillus, Leptynia* and *Clonopsis* metasexuals, the term 'asexual' should be abandoned for them, to be limited to organisms such as cnidarians which only reproduce mitotically and do not differentiate germ cells (agametic reproducers; Hughes 1989). Furthermore it should be noticed that phasmids are not unique in having genetic connections between sexual and metasexual taxa. Tangled interactions have been also ascertained in salamander hybrids (Bogart 1989) and in the polyphyletic tetraploid metasexual complex *Hyla versicolor* (Holloway *et al.* 2006).

Another reason for not defining as asexual the asymmetrically reproducing systems is to be found in the widespread presence among them of sex differentiating chromosomes. This is quite clearly observed in polyploid parthenogenetic phasmids, where all deviations from the 1:1 X/A ratio entrains the production of intersexes (Bergerard 1962; Pijnacker 1964; Pijnacker & Ferwerda 1980, 1986; Tinti & Scali 1995). Furthermore, the dosage compensation of sex-linked genes finds a wider corresponding condition in parent specific gene expression: genome imprinting appears to operate in many instances of thelytokous system origin and maintenance (Normark 2006). A meiotic program is at work even in apomictic phasmids, since a short chromosome pairing and the formation of synaptonemal complexes can occur (Koch *et al.* 1972; Scali *et al.* 2009): all these features are clear remnants of sexual significance. In conclusion, it seems undisputable that unisexual animals are far from being dead-end evolutionary pathways, as it has been simply accepted by many students of metasexual animals.

Turning now to centrosome dynamics and the role of γ-tubulin in stick insects it can be pointed out that the ability of direct MT nucleation around egg chromatin is, together with chromosome number regulation, the most outstanding trait of virgin egg development. In turn, in stick insects this trait could be somehow related to the unusual γ-tubulin molecule, also reflected into the lack of aster formation all throughout the life cycle (Marescalchi *et al.* 2002a, b).

The alternative occurrence of parthenogenesis and gynogenesis among animal groups is possibly related to the ability of their eggs to quickly build spindles at the onset of embryonic development: generally speaking, the parthenogenetic eggs of rotifers, crustaceans, insects, reptiles should be able to quickly organize the centrosome, while turbellarians, nematods, annelids, fish and amphibians would lack this ability and therefore be gynogenetic (pseudogamous), needing centrosomal sperm contribution. Sequencing of the phasmid γ-tubulin gene(s) and checking the centrosome cycle in parthenogens *versus* gynogens will provide a deeper insight into the mechanics of reproduction in unisexual animals.

Acknowledgements

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