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The dynamic karyotype and the complex genetic structure of the obligate parthenogen *Clonopsis gallica* (Insecta: Phasmatodea)

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Abstract
The stick insects of the genus *Clonopsis* embody bisexual, parthenogenetic and androgenetic taxa. *Clonopsis gallica* is an obligate parthenogen with a very wide geographic range, spreading eastwards from Morocco to Tunisia, and across Gibraltar to southern Europe. The body morphology and the egg parameters and pattern are very stable throughout the geographic range, while cytological investigations on follicular mitotic plates and oocyte maturation mechanisms as well as mtDNA findings neatly separated the Moroccan *C. gallica* collections from the remaining African and European samples, thus revealing a complex phylogenetic scenario. Here we investigated, by two-colour fluorescence *in situ* hybridization (FISH) of 28S ribosomal and telomeric $(TTAGG)_n$ sequences, European *C. gallica* specimens from three allopatric Italian sites, namely Sicily, Sardinia and Tuscany. We confirmed the variability of mitotic chromosome number and revealed a shared occurrence of colocalized, highly-amplified ribosomal genes and $(TTAGG)_n$ interstitial telomeric sequences (ITSs). The labelled sites were constant within the same specimen, but variable in number and chromosome location among specimens, even those coming from the same collecting site. Moreover, the telomeric signals also labelled the centromeric/pericentromeric regions of a few chromosomes, thus showing the presence of another ITS class, generally considered to be the trace of chromosome rearrangements. The finding of different classes of ITSs located on several chromosomes of the complement makes the *C. gallica* karyotype highly dynamic; its diffuse chromosome repatterning well accounts for the differences between the Moroccan and non-Moroccan populations.

Key policy highlights
- The stick insect *Clonopsis gallica*, an all-female obligate parthenogen, represents a good study-case of complex relationships among possibly related evolutionary events, such as hybridization, parthenogenesis and polyploidy.
- Our two-colour FISH experiments revealed the occurrence of colocalized highly-amplified ribosomal genes and $(TTAGG)_n$ interstitial telomeric sequences (ITSs) and of centromeric/pericentromeric ITSs. The presence of different classes of ITSs located on several chromosomes of the complement makes the karyotype of European *C. gallica* highly dynamic and reveals the occurrence of a diffuse chromosome repatterning.
- All FISH-investigated stick insects revealed the interesting and shared occurrence of a NOR/telomere colocalization, and its constant presence would also suggest a functional significance. We first found it in the genus *Leptynia* and confirmed it in species of the *Bacillus* genus. We published our results in *The European Zoological Journal* (Scali et al. 2016, 2020), and we herein would like to improve our stick insect cytogenomic study with the genus *Clonopsis*.

Keywords: *Stick insects*, *FISH*, *parthenogenesis*, *ribosomal and telomeric sequences colocalization*, *Interstitial telomeric sequences (ITSs)*

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Introduction

The stick insect *Clonopsis gallica* (Charpentier, 1825), an all-female obligate parthenogen, represents a good study-case of complex relationships among possibly related evolutionary events, such as hybridization, parthenogenesis and polyploidy (Scali 2009). *C. gallica* has a very wide geographic range, spreading eastwards from Morocco to Tunisia and across Gibraltar, to southern Europe. The Moroccan Rif area appears to be the evolutionary hotspot of the genus *Clonopsis,* which comprises three bisexual species: *C. algerica* Pantel, 1890 (2n = 32/31, XX/X0), *C. maroccana* Bullini and Nascetti, 1987 (2n = 22/21, XX/X0) and *C. felicitatis* Scali and Milani, 2009 (2n = 36/35, XX/X0); two telitokous obligate parthenogens: *C. gallica* (Charpentier, 1825) (54–57 chromosomes) and *C. soumiae* Scali and Milani, 2009 (72 chromosomes); and two all-male androgenetic strains: *C. androgenes-35* Scali and Milani, 2009 and *C. androgens-53* Scali and Milani, 2009 with 35 (X0) and 53 (X0) chromosomes, respectively (Milani et al. 2014).

On the whole, the Moroccan *Clonopsis* species realized an intriguing case of "reticulate evolution", by exploiting a variety of reproductive modes and independent cladogenetic events, including hybridization and androgenesis (Mantovani & Scali 1992; Milani et al. 2010). Among them, from the haploid number $n = 18$, *C. felicitatis*, *C. gallica* and *C. soumiae* form a polyploid series, with 36, 54, 72 chromosomes, respectively (Scali & Milani 2009). However, their karyotypes always kept a diploid structure, with chromosomes clearly arranged in pairs (Milani et al. 2008, 2010, 2014).

Within *C. gallica*, cytological investigations have differentiated the Moroccan specimens showing karyotypes with chromosomes arranged in pairs (Milani et al. 2008, 2010, 2014) from the Algerian, Tunisian and European ones that appear to have a variable karyotype (Bullini & Bianchi Bullini 1971; Bullini & Nascetti 1987; Scali & Milani 2009).

Furthermore, from mitochondrial genes analyses, *C. gallica* clearly appeared polyphyletic, being a mix of strains rather than a singly defined parthenogenetic taxon (Milani et al. 2010, 2014).

The polyphyletic origin of *C. gallica* received direct evidential support from the cytological analysis of its distinct oocyte maturation mechanisms, since Algerian, Tunisian and European strains go through the same modified meiotic process with synapsis, providing them a chance for recombination, but without chromosome number reduction, while the Moroccan ones just perform a single mitotic division (Scali et al. 2010).

Recently, the utilization of a cytogenomic approach, i.e. the two-colour fluorescence *in situ* hybridization (FISH) of 28S and $(TTAGG)_n$ telomeric arthropod repeat (Sahara et al. 1999; Vítková et al. 2005) in the genera *Leptynia* and *Bacillus* (Scali et al. 2016, 2020), as well as in five additional unrelated phasmid species (Liehr et al. 2017), revealed the occurrence of colocalized highly-amplified ribosomal genes and $(TTAGG)_n$ interstitial telomeric sequences called ITSs, in the nucleolar organizer region (NOR) (Ocalewicz 2013; Bolzan 2017; Aksenova & Mirkin 2019; Vicari et al. 2022).

In this connection we decided to analyse the same markers in *C. gallica* specimens from the Italian mainland (Tuscany) and from well-separated insular populations (Sardinia, Sicily) to further characterize the European *C. gallica* karyotype.

In all specimens we ascertained the colocalization of highly amplified ribosomal and telomeric sequences as found in all previously studied phasmids. Interestingly, the telomeric signals also labelled the centromeric/pericentromeric regions of some chromosomes, thus showing the presence of another ITS class, generally considered to be the trace of chromosome rearrangements.

Materials and methods

A total of 16 field-collected *C. gallica* specimens were analysed. The collecting sites were bramble bush from Italian mainland Follonica (FOL), 42°55'42"N, 10°45'00"E, Tuscany, from Torre Salinas (TSA) 39°21'54"N, 9°36'05"E and Valledoria (VAL) 40°55'42"N, 8°49'30"E, Sardinia Island, and from Pedara (PED) 37°37'43"N, 15°03'13"E and Massannunziata (MAS) 37°35'30"N, 15°02'18"E, Sicily Island. The sites are reported in the map (Figure 1) and summarized in Table I.

Chromosome preparations

This research followed all applicable international, national, and/or institutional guidelines for the care and use of animals. The chromosomal preparations were obtained from anesthetized specimens by manual dissection of the gonads in Ringer's solution, then a 5–10 min hypotonic shock in 1% sodium citrate solution, followed by a 30 min fixation in Carnoy solution. A gentle teasing of Carnoy-fixed tissue fragments on a slide and their smear in a few drops of 60% acetic acid, followed by cell drying on a hot plate (60°C), were then performed. Finally, a post-fixation treatment with the same fixative was applied (for more detailed information, see Scali et al. 2016).

Figure 1. Collecting sites of the investigated *C. gallica* specimens: Sardinia Island: TSA (Torre Salinas) and VAL (Valledoria); Sicily Island: PED (Pedara) and MAS (Massannunziata); Italy mainland: FOL (Follonica).

Tens of chromosomal mitotic plates were obtained from the follicular cells of ovarioles.

Two-colour FISH

Genomic DNA was extracted using the PureLink Genomic DNA Mini Kit (Invitrogen). The major ribosomal gene unit (45S rDNA, that contains the genes encoding 18S, 5.8S, and 28S rRNAs), was localized using 28S rDNA probes obtained by polymerase chain reaction (PCR) amplification of the DNA of *Bacillus rossius* and *Clonopsis gallica* using the universal primers D1F and D1R (Zardoya & Meyer 1996) according to Scali et al. (2020).

The insect pentameric telomeric probe $(TTAGG)_n$ (Sahara et al. 1999; Vítková et al. 2005) was amplified by non-template PCR following Ijdo et al. (1991) using the primers F: $(TTAGG)_5$ and R: $(CCTAA)_5$.

The 28S rDNA probe had been labelled with biotin-16-dUTP and the telomeric $(TTAGG)_n$ probes with digoxigenin-11-dUTP, using a nick translation kit (Roche Diagnostics), following the manufacturer's instructions. Two-colour FISH with 28S and telomeric $(TTAGG)_n$ probes was performed according to Schwarzacher and Heslop-Harrison (2000). Biotin-labelled probes were detected with extrAvidin - FITC (Sigma-Aldrich) and digoxigenin labelled probes were detected with Anti- Digoxigenin-Rhodamine (Roche) producing, respectively, green and red fluorescence. Chromosomes were counterstained using 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). Two-colour FISH produced a yellow/orange fluorescence on the entire cytological satellites as a result of the overlapping labels. After FISH, the slides were washed in 0.025% Tween 20 (Sigma-Aldrich) in 4×SSC, fixed in 3:1 ethyl alcohol-acetic acid overnight, rehydrated with a descending scale of alcohols, incubated in Mc Ilvaine buffer for 20 min and then rinsed. The slides were, finally, stained with 1:3 Wright's stain in 0.06 M phosphate buffer, pH 6.8, for 8 min.

Karyotyping

A total of 472 mitotic plates were analysed in depth, on average 20 mitotic plates per individual (Table I). A few karyograms of each specimen

Table I. Chromosome number and FISH data of analysed *C. gallica* specimens.

Collecting sites	Specimens	Metaphases per individual	Chromosome number	NOR-ITSs
VALLEDORIA Sardinia	VAL1	40	55	2
	VAL2	40	55	2
	VAL3	37	55	3
	VAL4	13	55	2
	VAL5	38	55	1
	VAL6	21	55	1
TORRE SALINAS Sardinia	TSA1	3	Not countable	3
	TSA3	41	55	3
FOLLONICA	FOL1	28	58	5
Tuscany	FOL2	12	57	3
	FOL3	40	57	3
	FOL4	29	56	3
PEDARA	PED2	37	55	3
Sicily	PED3	4	55	-
	PED4	36	55	3
MASSANNUNZIATA Sicily	MAS1	53	55	1

All metaphases (excepting PED3, only stained with DAPI) have been analysed through a two-colour fluorescence *in situ* hybridization (FISH) of 28SrDNA and $(TTAGG)_n$ telomeric repeats.
NOR-ITS: Interstitial Telomeric Sequences colocalized with 28S rDNA in the Nucleolar Organizer Region.

were prepared after FISH as well as after the subsequent Wright's staining. Since only a few chromosomes would support an arrangement in pairs while for the remaining ones no homology could be assigned with certainty, chromosomes were individually arranged according to their decreasing size. Owing to the varying size of cytological satellites, only the length of the non-satellite chromosome part was considered for the karyotypic ranking.

Image processing

Metaphase plates were observed under a Zeiss Imager M1 fluorescence microscope, captured with a Hamamatsu digital camera C8484 and processed using a karyotyping- and FISH-dedicated image analysis system (Cromowin Plus, TESI Imaging).

Results

Among the 469 countable plates of follicular cell mitoses, chromosome number varied from 55 to 58. The most frequent number found was 55 in Sardinia and Sicily specimens, while in the four Italian mainland individuals the chromosome number varied from 56 to 58 (Table I). In all investigated specimens the two-colour FISH of 28S ribosomal DNA and $(TTAGG)_n$ telomeric repeats revealed the occurrence of colocalized ribosomal and telomeric sequences, which we named NOR-ITSs (interstitial telomeric sequences colocalized with 28S rDNA in the nucleolar organizer region). These regions, often appearing as very large cytological satellites, showed an inter-individual variability in number and location even among specimens collected from the same bramble bush, but were constant within the same individual. Furthermore, the size of the NOR-ITSs regions often showed a considerable intra-individual variability, as shown in the inset of Figure 2(a), where the NOR-ITS-bearing chromosome 53 from two different metaphases of the same individual is shown.

Overall, one, two, three or five NOR-ITSs per individual were detected, and the most frequently encountered condition was three signals. Figure 2 shows karyograms with one (a), two (b), and three (c) colocalized ribosomal and telomeric FISH signals; in the insets, satellite-bearing chromosomes are shown after FISH and Wright's staining to better highlight their size and shape.

The situation found in four Italian mainland specimens from Follonica (Figure 3(a-d)) is to be mentioned: in the same bramble bush one specimen (FOL1) with five signals–58 chromosomes (Figure 3(b)) and three (FOL2, FOL3, FOL4) with three signals–56/57 chromosomes were observed (Figure 3(a,c,d)). In all these specimens three signals were located in a similar position: the short arm of chromosomes 37 and 49, often forming very large satellites, and terminally in the long arm of chromosome 40 (Figure 3). Furthermore, in the FOL1 specimen, two additional elements entirely FISH labelled were present, we put them at the end of the karyotype and numbered them as 57 and 58.

The FOL4 individual may present the likely ancestral situation with three signals: two very large satellites on the short arm of the chromosomes 37 and 49 and a third signal terminally located on the long arm of chromosome 40 (Figure 3(a)). In the five-signal FOL1 specimen (Figure 3(b)) the detachment of the cytological satellites from their generating chromosomes (probably chromosomes 37 and 49, which bear smaller signals), likely brought the chromosome number to 58. Their derivation from previously chromosome-linked satellites, thus becoming independent elements, might also be inferred from FISH features, since their labelling spanned the whole length of the "new chromosomes".

Figure 3(c,d) show the labelled chromosomes of FOL2 and FOL3 specimens with three signals, respectively: in FOL2 two small signals were localized on the short satellites of chromosomes 37 and 49 and the third terminally on the long arm of chromosome 40, while in FOL3 the signals were located in the very large satellite of chromosome 37, on the small satellite of 49 and terminally on the long arm of chromosome 40.

Beside telomeres and NOR-ITSs, telomeric signals also labelled centromeric/pericentromeric regions of a few chromosomes, showing the presence of another class of ITSs: the centromeric/pericentromeric ITSs. These ITSs were present in all specimens examined, but the number of involved chromosomes was variable among individuals as well as among plates. Out of 50 plates examined, we counted a range from 5 to 12 chromosomes with ITSs, with 8–10 being the most frequent condition (arrows in Figure 4). This class of ITSs was mostly found on large metacentric and submetacentric chromosomes (Figures 2, 3 and 4). Moreover, only the Italian mainland specimens (FOL) had centromeric/pericentromeric ITSs in the large submetacentric chromosome 2 (Figure 3(a,b)) while the Sardinian TSA3 specimen and the Sicilian PED4, PED2 (not shown) specimens carried both centromeric/pericentromeric ITSs and NOR-ITSs on the chromosome 30 (Figure 4(a,b)). Moreover, on chromosomes 12 and 17 of PED4 the telomeric signals stretch all over the short arm (Figure 3(a)).

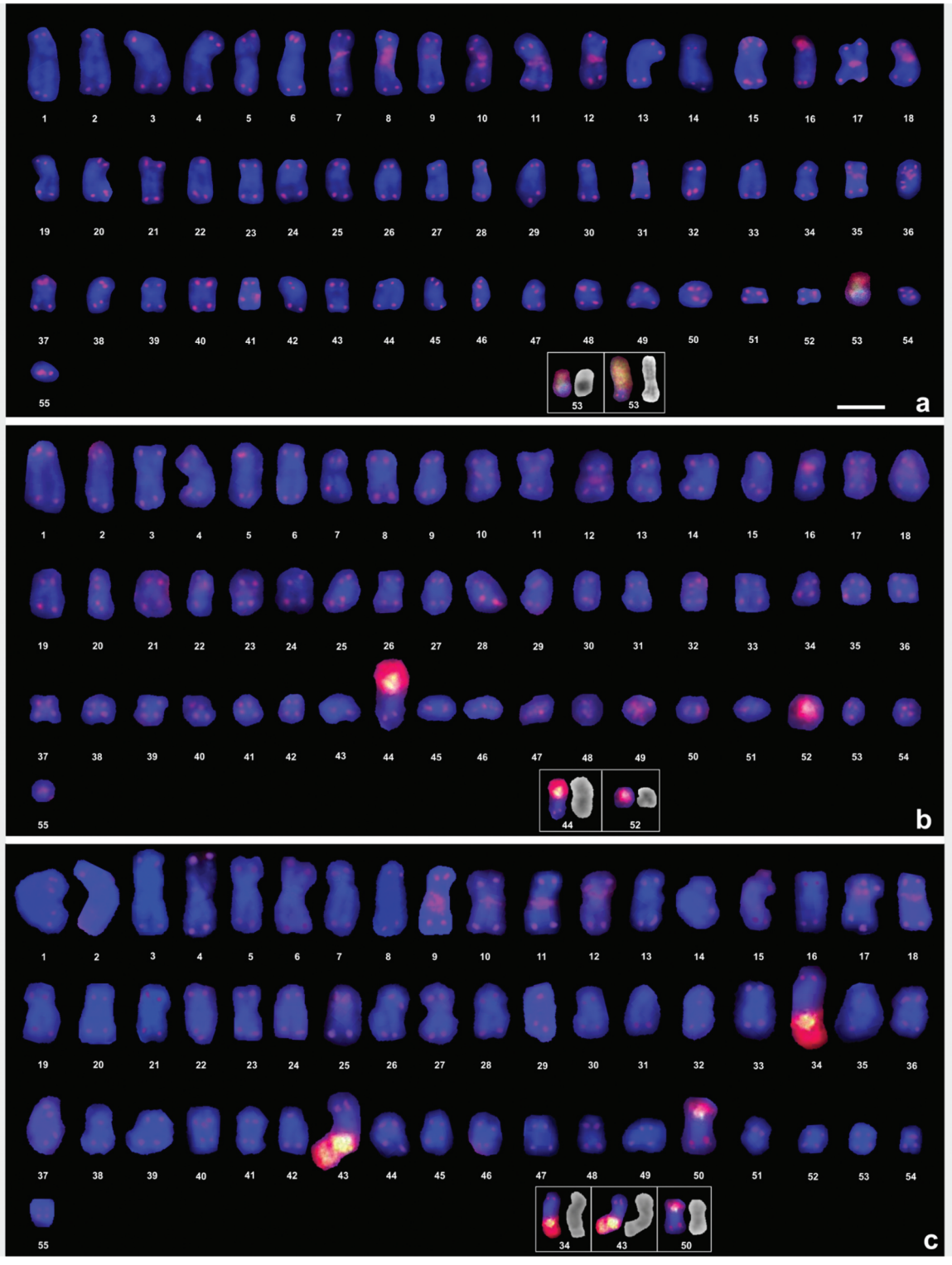

Figure 2. Karyograms of specimens with one, two, and three signals after two-colour *in situ* fluorescence in situ hybridization (FISHs) with 28S (green fluorescence) and telomeric (red fluorescence) probes: the orange-shadowed markings result from the sum of red and green labels. The satellite-bearing chromosomes after FISH and Wright's staining are shown in the insets. (a): MAS specimen with one signal, the inset points out the outstanding size variability of the cytological satellite in two chromosome 53 of different metaphases of the same individual; (b): VAL4 specimen with two signals; (c): VAL3 specimen with three signals.

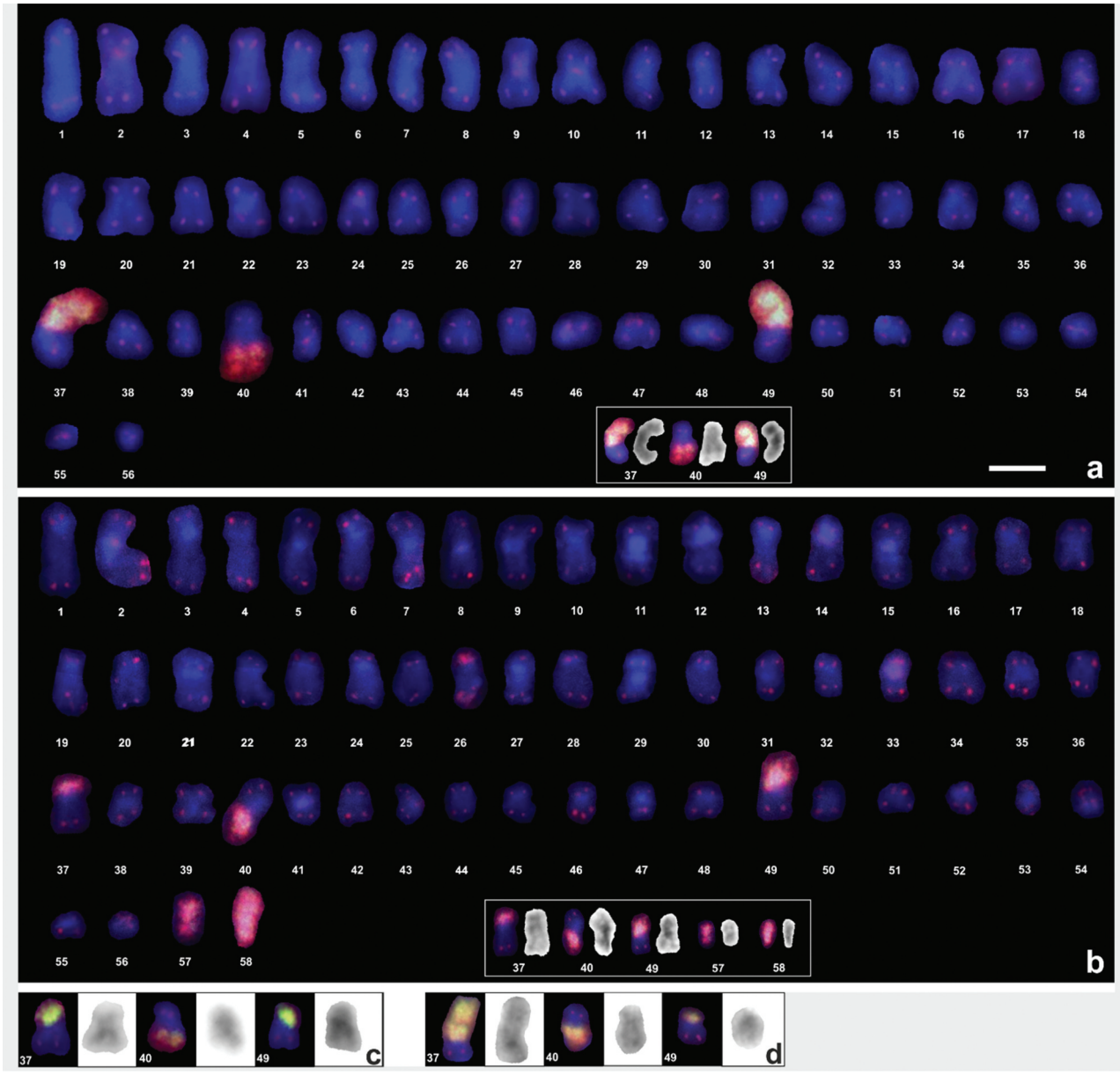

Figure 3. Karyograms of FOL specimens, after two-colour FISH with 28S (green fluorescence) and telomeric (red fluorescence) probes. The satellite-bearing chromosomes after Wright's staining are shown in the insets. (a): FOL4 specimen with three signals: two localized on the large satellites of chromosomes 37 and 49 and the third terminally on the long arm of chromosome 40; (b) FOL1 specimen with five signals: two localized on small satellites of chromosomes 37 and 49, one terminally on the long arm of chromosome 40, and the last two on "chromosomes" completely labelled (nos. 57 and 58). In (c, d) the labelled chromosomes of FOL2 and FOL3 specimens are shown. Here the signals, in addition to those located terminally on the long arm of chromosome 40, are localized in the short arm of chromosomes 37 and 49 in FOL2 (c) and on a very large satellite of chromosome 37 and in the short arm of chromosome 49 in FOL3 (d).

Discussion

The two egg-maturation mechanisms at work in different *Clonopsis gallica* strains, namely a single mitotic division in the Moroccan specimens or a meiotic-like maturation process in the others, led to the production of cytologically and genetically invariant eggs on one side, but chromosomally repatterned and genetically varied eggs on the other. These processes generate karyotypically differentiated lineages from a thelytokous parthenogen and made *C. gallica* a very successful multi-strained unisexual taxon, which from African Mediterranean areas spread through central Europe up to Normandy (Scali et al. 2010).

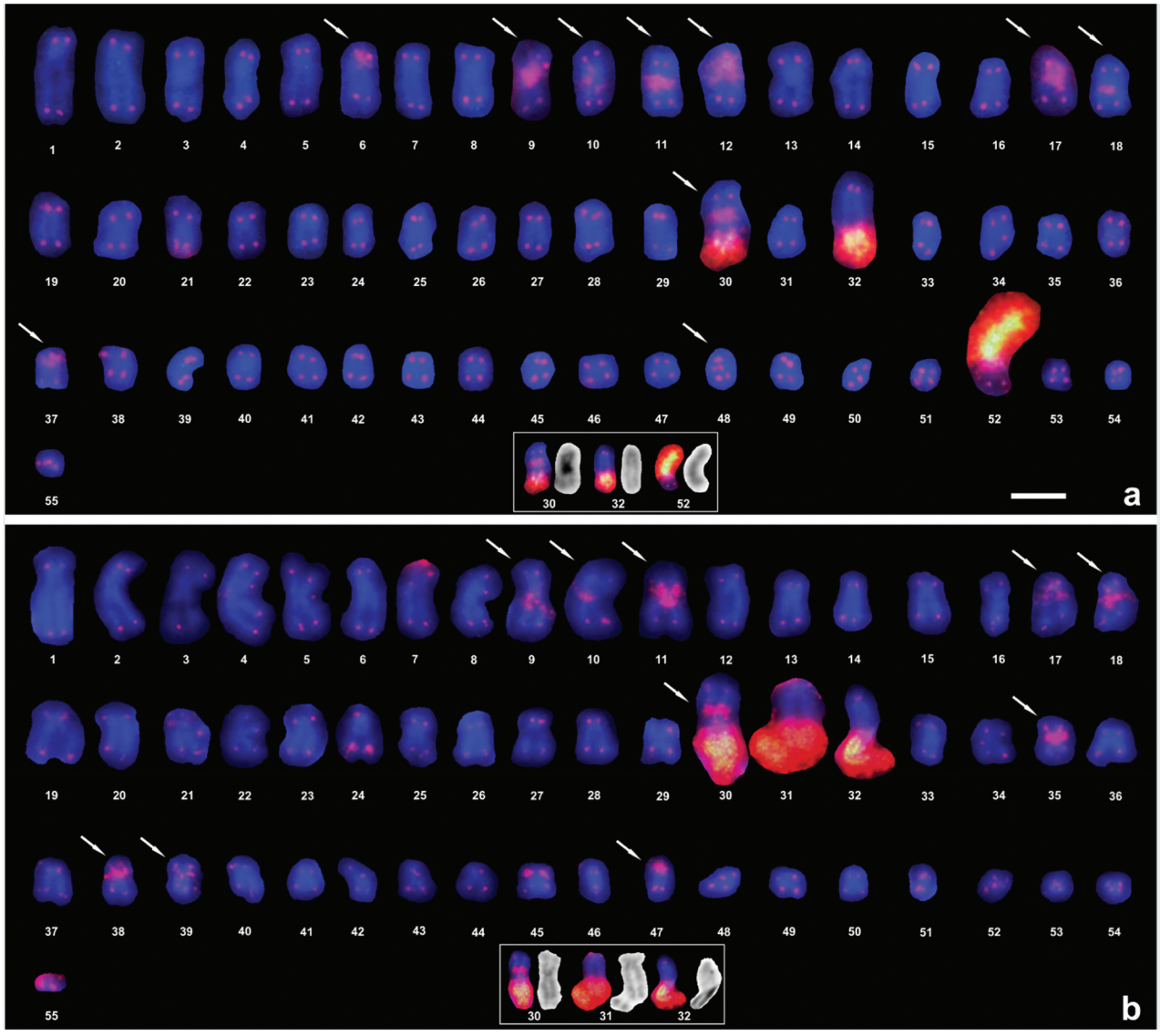

Figure 4. Karyograms of specimens PED4 (a) and TSA3 (b) after 28S (green fluorescence) and telomeric (red fluorescence) two-colour FISH. The satellite-bearing chromosomes after Wright's staining are shown in the insets. In both karyograms, both centromeric/pericentromeric ITSs and NOR-ITSs are present on the chromosome 30 (a, b); in PED4 the telomeric signals span over the whole short arm of chromosomes 12 and 17 (a). The arrows show centromeric/pericentromeric ITSs.

We characterized the chromosomes of some widely separated Italian strains of *C. gallica* using the two-colour FISH of telomeric and ribosomal sequences as probes and we found the presence of two classes of ITSs, NOR-ITSs and centromeric/pericentromeric ITSs, which revealed an intense process of karyotype remodelling taking place in European *C. gallica* strains.

NOR-ITSs

In all analysed specimens of *C. gallica* we found a colocalization of highly amplified ribosomal and $(TTAGG)_n$ telomeric sequences also found in the other studied phasmids (Scali et al. 2016, 2020; Liehr et al. 2017). The NORs often formed very large cytological satellites, always entirely FISH marked. In a single specimen the labelled sites were constantly located, while they varied in number and chromosome location among specimens, even those from the same bush. As shown in Table I, the most frequent number of NOR-ITSs among the 15 individuals studied was three, never located on seemingly homologous chromosomes. Some recurrent locations appear shared by different specimens, i.e. on the short arm of a small submetacentric

chromosome ranking around position 50, as well as in the telomeric region of the long arm of chromosomes around position 30; in particular it is worth noting the remarkable resemblance of the 30th chromosome among the specimen from Torre Salinas (Sardinia) and two from Pedara (Sicily), in which both NOR-ITSs and centromeric/pericentromeric ITSs are present.

A paradigmatic finding of the above-described situation has been fully envisaged in the Italian mainland specimens of Follonica, where in a single bramble bush, one female (FOL1) with five signals and 58 chromosomes and three (FOL2, FOL3, FOL4), with three signals and 56/57 chromosomes were demonstrated (Figure 3(a-d)). In four individuals that likely originated from the same mother, the detachment of the cytological satellites from the originating chromosomes determined a change in the number and shape of the chromosomes, accounting for the ascertained variability in the karyograms.

The finding of NOR-ITSs, deeply modifying the chromosome shape and located on different chromosomes of the complement, confirmed the operative choice of ranking the chromosomes according to their length for karyotype definition.

The colocalization of 28S and $(TTAGG)_n$ telomeric repeats in *C. gallica* would confirm that in stick insects this is not a random event, as it appears to be in other Arthropoda (Salvadori et al. 2012, 2023). Actually, all FISH-investigated stick insects revealed a shared occurrence of a NOR/telomere colocalization (Scali et al. 2016, 2020; Liehr et al. 2017), and its constant presence might also suggest a functional significance.

In *C. gallica* the variability in number, size and chromosome location of NOR-ITSs might be related to the peculiar meiotic mechanism, and the occurrence of unequal crossing-over or DNA amplification in somatic cells could be suggested (Zhdanova et al. 2007; Liehr et al. 2017). Moreover, even the action of transposable elements (TEs) can also be reasonably hypothesized, also in view of the presence in *Bacillus* taxa of a high copy-number of R2 retrotransposons presenting a strict sequence specificity for an insertion target-site in the 28S rRNA gene (Martoni et al. 2015; Satovic et al. 2016; Bonandin et al. 2017; Scavariello et al. 2017).

Even if little is known about the mechanisms, NOR-ITSs, highlighted in several animals and plants, turn out to be a "hot spot" of genomic recombination, enhancing genetic differentiation and karyotype plasticity (Meyne et al. 1990; Salvadori et al. 1995; Zhdanova et al. 2007; Li et al. 2012; Ocalewicz 2013; Dvořáčová et al. 2015; Scali et al. 2016, 2020; Liehr et al. 2017; Aksenova & Mirkin 2019).

Centromeric/pericentromeric ITSs

This class of ITSs, also named heterochromatic ITSs (Het-ITSs), is generally deemed a remnant of chromosomal rearrangements (Zhdanova et al. 2007; Ruiz-Herrera et al. 2008; He et al. 2013; Bolzan 2017; Aksenova & Mirkin 2019) and has been found in other Phasmatodea studied (Liehr et al. 2017). The presence in *C. gallica* of centromeric/pericentromeric ITSs, mostly in large metacentric and submetacentric chromosomes, could therefore be the result of chromosome rearrangements such as translocations and/or inversions.

These ITSs were found in all specimens examined, but the number of chromosomes involved is variable both among individuals and within the same individual, with 8–10 being the most frequent. It cannot be excluded that the intra-individual variability observed could be the result of imprecise counting due to different levels of chromosome contraction of the metaphase plates and/or to the resolution power of the FISH, as well as to the coverage of the plate by cytoplasmic residues, quite likely in chromosomal preparations obtained by direct methods. In some cases, the telomeric signals spread from the centromere to a large pericentromeric area up to the entire short arm of some chromosomes, as observed in the two Pedara specimens (Figure 4(a)).

As already highlighted, the 30th chromosome of specimens from Torre Salinas (Sardinia) and from Pedara (Sicily), presents both NOR-ITSs and centromeric/pericentromeric ITSs. This situation is not present in the specimens from Follonica (Italian mainland), which instead present centromeric/pericentromeric ITSs in the large chromosome 2. These differences among the labelled chromosomes of specimens from different collecting sites reveal the active process of chromosome remodelling at work in the European *C. gallica*.

Conclusions

The non-clonal chromosome constitution and the varied egg-maturation mechanisms of *C. gallica* highlight complex phylogenetic and genetic scenarios even in an obligate all-female parthenogen which would be supposed to be clonal.

The presence of different classes of ITSs located on several chromosomes of the complement makes

the karyotype of European *C. gallica* highly dynamic and reveals the occurrence of a diffuse chromosome repatterning which accounts for the genetic differences between Moroccan and non-Moroccan populations of *C. gallica*.

The presence of ITSs in turn could account for the remarkable karyotype diversity and chromosomal repatterning shown by Phasmatodea (Craddock 1972; John et al. 1987; Passamonti et al. 2004; Milani et al. 2010).

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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