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Comparative cytogenetics in four species of Palinuridae: B chromosomes, ribosomal genes and telomeric sequences

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Abstract The evolutionary pathway of Palinuridae (Crustacea, Decapoda) is still controversial, uncertain and unexplored, expecially from a karyological point of view. Here we describe the South African spiny lobster *Jasus lalandii* karyotype: n and 2n values, heterochromatin distribution, nucleolar organizer region (NOR) location and telomeric repeat structure and location. To compare the genomic and chromosomal organization in Palinuridae we located NORs in *Panulirus regius, Palinurus gilchristi* and *Palinurus mauritanicus*: all species showed multiple NORs. In *J. lalandii* NORs were located on three chromosome pairs, with interindividual polymorphism. In *P. regius* and in the two *Palinurus* species NORs were located on two chromosome pairs. In the two last species 45S ribosomal gene loci were also found on B chromosomes. In addition, the nature and location of telomeric repeats were investigated by FISH in *J. lalandii, P. gilchristi, P. mauritanicus Palinurus elephas,* and *P. regius* (Palinuridae, Achelata), and in *Scyllarus arctus* (Scyllaridae, Achelata): all these Achelata species showed the (TTAGG)n pentameric repeats. Furthermore, in *J. lalandii* these repeats occurred in all the telomeres and in some interstitial chromosomal sites, associated with NORs.

Keywords Decapoda · Palinuridae · B chromosomes · Telomeres · Ribosomal genes

Susanna Salvadori and Elisabetta Coluccia have contributed equally to this work.

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Introduction

Scyllaridae and Palinuridae constitute the Achelata group, considered to be monophyletic and the Palinuridae now includes the Sinaxidae family (Patek et al. 2006; George 2006; Groeneweld et al. 2007; Palero et al. 2009a; Tsang et al. 2009).

Fossil records from North America, Europe and Australia suggest that the Palinuridae arose in the early Mesozoic (George 2006). The family currently comprises approximately ten genera and fifty species (including those of the Synaxidae), which can be subdivided into two groups: the Stridentes, and the Silentes. This family has received much attention and there are data on comparative morphology of living and fossil species, life cycles, and population genetic structure. Nevertheless, there are still controversies and uncertainties regarding the phylogenetic relationships and the evolutionary origin (George and Main 1967; George 2006; Groeneweld et al. 2007; Palero et al. 2009a, b; Tsang et al. 2009, Babbucci et al. 2010).

To date, the available karyological data (Lécher et al. 1995; Tan et al. 2004; Scalici et al. 2010) do not allow a clear interpretation of karyotype evolution in Decapoda. The diploid values range from 2n = 54 in *Liocarcinus vernalis* (Trentini et al. 1989) to 2n = 254 in *Eupagurus ochotensis* (Niiyama 1959). The very high number of 376 chromosomes in *Pacifastacus trowbidgii* reported by Niiyama (1962), has been disputed by Komagata and Komagata (1992). In Palinuridae, there are reports only in the Stridentes group: in *Palinurus elephas, Palinurus mauritanicus* and *Palinurus gilchristi* the numerical variability has been shown to be linked to the presence of B chromosomes, (Salvadori et al. 1995a; Coluccia et al. 2003, 2004, 2005, 2006), which are not found in *Panulirus regius* (Cannas et al. 2004); *Palinurus japonicus* and *Palinurus marginatus* (Nakamura et al. 1988).

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In this work, the karyotype of one Silentes species, the South African spiny lobster *Jasus lalandii* (H. Milne-Edwards, 1837) living on the western and southern coasts of Namibia and South Africa, is described. To compare the genomic and chromosomal organization in Palinuridae we located nucleolar organizer regions (NORs) in *P. regius, P. gilchristi* and *P. mauritanicus*, and investigated the structure and location of telomeric repeats in five Palinuridae species (Achelata), and in *Scyllarus arctus* (Scyllaridae, Achelata).

Materials and methods

Chromosome preparations were obtained from the testicular tissue of ten *J. lalandii* specimens and six *P. gilchristi* specimens from commercial stocks by South Africa, twenthy *P. elephas* and fifteen *P. mauritanicus* specimens captured in the Southern Sardinia (Mediterranean sea), and two *P. regius* specimens of commercial stocks from western Atlantic sea.

For chromosome preparation, tissue fragments were dissociated into a small Petri dish with 1–2 ml of a hypotonic solution (0.075 M KCl) by rubbing tissues against a stainless steel grid (mesh 350 µm) with a curved forceps. The cell suspension was transferred to a tube containing the prewarmed hypotonic solution, kept for 30 min at 32 °C and centrifuged at 1,000 rpm for 6 min. After discarding of the supernatant, 3–5 ml of cold, freshly prepared Carnoy's fixative (3 parts methanol or absolute ethanol: 1 part glacial acetic acid) was added drop by drop, carefully agitated with a Pasteur pipette and centrifuged at 1,200 rpm for 6 min (a total time of at least 30 min of fixation is needed). Slides were stained with 1:3 Wright's stain in 0.06 M phosphate buffer, pH 6.8, for 8 min, briefly rinsed in distilled water and air-dried. For C-banding, slides aged for 6–9 days were treated with 5 % $Ba(OH)_2 8H_2O$ for 5 min at 30 °C, briefly rinsed in 0.1 HCl followed by distilled water, incubated in 2× SSC for 1 h at 60 °C and stained with 1:2 Wright's stain in phosphate buffer for 7–9 min. 45S rDNA FISH was performed with biotinylated probes obtained from a complete major ribosomal unit of *Xenopus laevis*. Telomeric sequences FISH were carried out using a PCR generated pentanucleotide $(TTAGG)_n$ repeat according to Ijdo et al. (2001) and a $(TTAGGG)_n$ Human Pan-telomeric Probe (cambio) both labelled with biotin-16-dUTP using a nick translation kit (Roche).

FISH was performed according to Salvadori et al. (1995b). In particular, chromosomes were pretreated by incubating the slides with RNAse (100 µg ml^{-1}) for 1 h at 37 °C, dehydrated in ethanol series, air dried and immediately used for FISH or stored at −20 °C until use. Chromosome preparations were denaturated in 70 % formamide/2× SSC (pH 7.0) for 2 min, dehydrated in a cold ethanol series and air dried. Hybridization solution containing 200 ng of labeled probe, 20 % dextran sulfate, 50 % formamide, and 2× SSC (pH 7.0) in a final volume of 30 µl was denaturated for 8 min at 70 °C, immediately dropped onto the slides, which were incubated overnight at 37 °C in a moist chamber. Posthybridization washes were performed at 37 °C in 50 % formamide/2xSSC (pH 7.0) for 15 min and in 2× SSC for other 15 min. Signal detection was carried out by incubation of the slides with fluorescein isothiocyanate-avidin (Vector labs.) with two amplification steps. After each step of amplification, slides were washed 3 times each for 5 min with 0.025 % Tween 20 in 4× SSC. Chromosomes were counterstained with propidium iodide (Sigma) (1 µg ml^{-1}) and mounted in Vectashield mounting medium (Vector labs.). After FISH, the slides were destained and stained in Wright's stain or CMA_3, as described by Schweizer (1976). Metaphases were observed using a Zeiss Imager M1 fluorescence microscope, captured with a Hamamatsu digital camera C8484, and were analyzed and processed with the karyotyping and FISH-dedicated image analysis Cromowin Plus system (TESI Imaging). A minimum of 20 metaphases was analysed for a specimen in each treatment.

Results

From a count of 20 mitotic metaphases, the *J. lalandii* modal diploid number was 148 (with a range from 130 to 158) and the modal haploid number (from 29 first and 7 second meiotic metaphases) was 74 (with a range from 68 to 76). A few polyploid mitosis ranging from 200 to 278 chromosomes were also observed.

The complement comprised metacentric, submetacentric and acrocentric chromosomes (Fig. 1a). All chromosomes at the first meiotic metaphase were paired without observable asynaptic chromosomes (Fig. 1b).

The heterochromatic regions were located in the centromeres of several chromosomes and many of them showed large paracentromeric and intercalary bands. Some of the first largest pairs had large heterochromatic blocks in the long arm, and some chromosomes had entirely heterochromatic short arms (Fig. 1c) .

45S rDNA FISH revealed that NOR in *J. lalandii* was located in three chromosome pairs: the fluorescent signals were located on six mitotic chromosomes (Fig. 2a) and on three meiotic cross-shaped diplotene bivalents, two large and one medium sized, clearly identifiable after subsequent Wright's staining (Fig. 2b, and arrows in 2c). Nevertheless, two individuals showed polymorphism of this region, in fact in mitosis the FISH signals were present in 5 chromosomes (Fig. 2d), and in metaphase I the signals were on

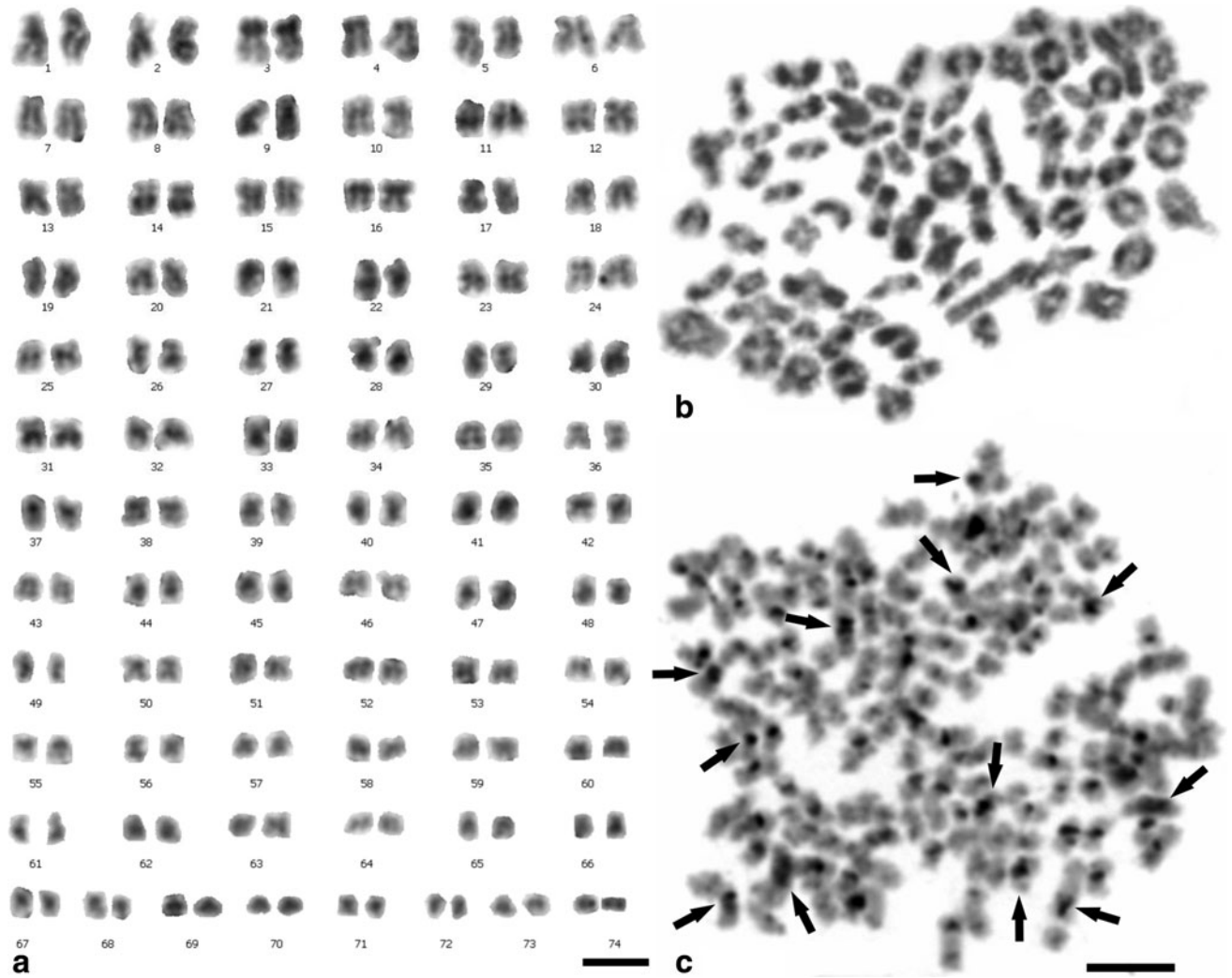


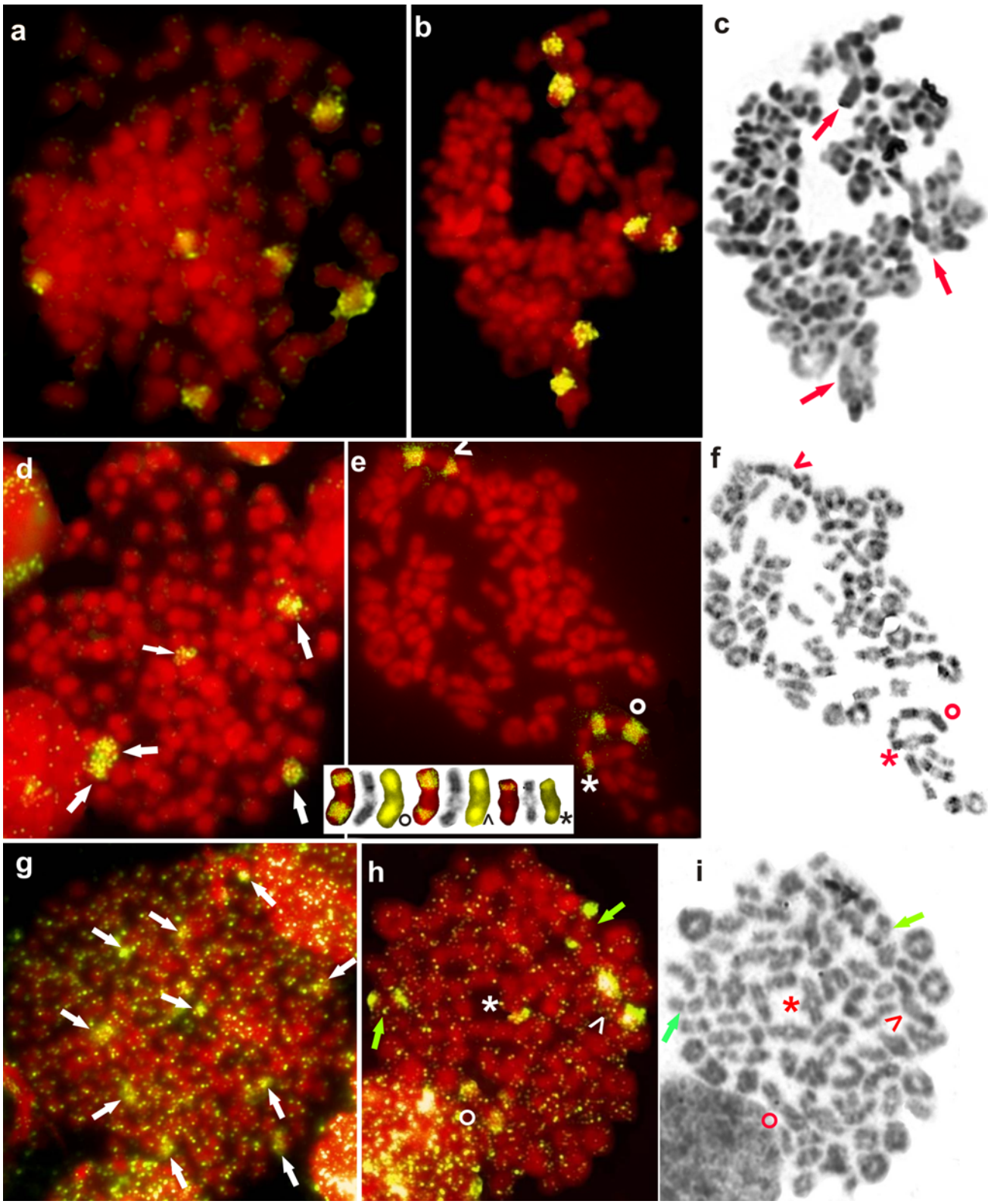
Fig. 1 **a** Karyotype of *J. lalandii*; chromosomes were arranged in decreasing order of length; **b** metaphase of the first meiotic division with ring-, cross- and dumb-bell configurations and no unpaired chromosomes; **c** spermatogonial mitosis after C-banding, beside centromeric bands, paracentromeric and intercalary bands are present (*arrows*). The *bar* represents 5 µm

both the homologous chromosomes of the two large diplotene bivalents, while in the medium-sized bivalent the signal was not detectable in one of the two homologs, clearly identifiable after the subsequent Wright's staining (Fig. 2e, f). FISH signals and evident CMA_3 bands were overlapped in the bivalents; the bivalent with only a FISH signal showed CMA_3 bands in both homologues (the inset in Fig. 2).

In situ hybridization of the $(TTAGGG)_n$ hexanucleotide and the $(TTAGG)_n$ pentanucleotide telomeric sequences was made in *J. lalandii*. No signals were detected with the $(TTAGGG)_n$, while the $(TTAGG)_n$ probe labeled all telomeres as well as large interstitial chromosome regions in about ten chromosomes in mitosis and five diplotene bivalents in the first meiosis (Fig. 2g, h). Three of them coincided with NORs (Fig. 2h, i); in particular the medium-sized cross-shaped diplotene bivalent that showed only one homologous chromosome labelled after 45S FISH, similarly showed only one chromosome labelled by telomeric probe in the same individuals (* in Fig. 2h, i).

Following 45S rDNA FISH, in the first meiotic division of *P. gilchristi* and *P. mauritanicus*, besides two small diplotene bivalents (green arrows in Fig. 3b, d), some unpaired B chromosomes were also labelled (red arrows in Fig. 3b, d). In particular, in *P. gilchristi* two bivalents and 3–6 B chromosomes, and in *P. mauritanicus*, two bivalents and 3–5 B chromosomes were labelled. In *Panulirus regius* two medium sized bivalents were marked (green arrows in Fig. 3f).

Both the $(TTAGGG)_n$ hexanucleotide and the $(TTAGG)_n$ pentanucleotide telomeric repeats were used as probes in FISH experiments in *P. gilchristi* (Fig. 4a),



P. mauritanicus (Fig. 4b), *P. regius* (Fig. 4c), *P. elephas* (Fig. 4d) (Achelata, Palinuridae), and in *S. arctus* (Fig. 4e) (Achelata, Scyllaridae). In all the species, no hybridization signals were detected with the $(TTAGGG)_n$ repeat, whereas clear signals were observed at the end of the chromosomes with the $(TTAGG)_n$ repeat.

◀ **Fig. 2** **a**, **b** *J. lalandii* mitotic and first meiotic metaphases after 45S rDNA FISH. Six chromosomes and three cross-shaped diplotene bivalents are brightly labelled; **c** the sequential Wright's staining shows that the signals are in two large and one medium-sized bivalents; **d**, **e** mitotic and first meiotic metaphases after 45S rDNA FISH in two individuals showing polymorphism of NORs. Five chromosomes in mitotic metaphases and three cross-shaped diplotene bivalents in meiotic metaphases I are *brightly labelled*. These bivalents are identifiable after subsequent Wright's staining (**f**). The two largest are marked by *empty arrowhead* and *empty circle symbols* and the smallest, with only one chromosome marked, by *asterisk*. The *inset* shows the three bivalents after FISH, Wright's staining and CMA_3 staining; **g**, **h** *J. lalandii* mitotic and first meiotic metaphases after pentanucleotide $(TTAGG)_n$ telomeric repeat FISH. The probe labels all telomeres and large interstitial chromosome regions in mitotic metaphases (*arrows* in **g**). In the first meiotic metaphase signals are present in five bivalents clearly identifiable after subsequent Wright's staining: two large cross-shaped indicated by *empty arrowhead* and *empty circle* in **h** and **i**, one medium sized cross-shaped with only one chromosome marked indicated by *asterisk* in **h**, **i**, and two small bivalents (*green arrows* in **h** and **i**)

Discussion

Cytological parameters

To date, the karyological data in decapods are scarce but our analysis clearly indicated a different situation in Scyllaridae and Palinuridae. Palinuridae show a more uniform situation with medium to high haploid Genome Sizes (GS) (3.15–5.33 pg) and high chromosome numbers (always above 100) with the Silentes *Jasus* showing the highest GS (Deiana et al. 1999, 2007) and also the highest chromosome number (present report). In Scyllaridae, there is a clear separation between the Arctidinae and Scyllarinae groups, the Arctidinae having about three times the GS values (6.99–6.8 pg) and double the chromosome number (126–140) compared with the Scyllarinae (1.94–2.01 pg and $2n = 70–76$) (Deiana et al. 1999, 2007). Fossil records demonstrate an earlier emergence of the Palinuridae than the Scyllaridae and of the genus *Scyllarides* (Arctidinae) than the *Scyllarus* (Scyllarinae) within the Scyllaridae (Baisre 1994; Sekiguchi et al. 2007). These data suggest a trend towards a reduction of the karyological parameters within Scyllaridae and from Palinuridae to Scyllaridae (Deiana et al. 2007). The same trend could occur within Palinuridae if the *Jasus, Projasus Sagmariasus* group is considered to be the basal lineage in Palinuridae (Tsang et al. 2009).

45S rDNA FISH

The nucleolus organizer region (NOR) forms a conspicuous chromosomal structure that can be stained differentially with silver as well as with FISH (Sumner 2003). Based on 45S rDNA FISH *Jasus lalandii*, *Panulirus regius, Palinurus gilchristi* and *P. mauritanicus* showed multiple NORs. In *J. lalandii*, the FISH of 45S ribosomal genes highlighted an interindividual polymorphism of this region. The sequential CMA_3 staining of the metaphases produced bright fluorescent signals; with some of them corresponding in localization to the FISH signals, showing the GC richness of NORs.

B-chromosomes

In *Panulirus regius*, two tetrads in metaphase I and a corresponding number of chromosomes in mitotic metaphase were marked. In *Palinurus gilchristi* and *P. mauritanicus* metaphase I, besides two very small diplotene bivalents, some small, unpaired B chromosomes were entirely labelled. We previously found this same situation for 45S rDNA in *P. elephas,* (Coluccia et al. 2006).

B-chromosomes are optional chromosomes found in many eukaryote species, mostly heterochromatic, with irregular inheritance and interindividual variation from a same population (Jones and Rees 1982, Jones 1985; Camacho 2005; Jones et al. 2008).

The accumulation of repeated DNA sequences seems to be a very common pathway in B chromosome differentiation, and the presence of rDNA in B chromosomes of many species might be indicative of a possible role of this kind of DNA in B origins (Dhar et al. 2002; Camacho et al. 2000; Jones et al. 2008). Recently, genic activity of ribosomal genes in some B chromosomes has been shown (Leach et al. 2005; Teruel et al. 2009). In Decapoda, B chromosomes have been found in the Nephropidae *Homarus americanus* (Coluccia et al. 2001), and *Nephrops norvegicus* (Deiana et al. 1996) accounting for the numerical variability that has been observed in mitotic and meiotic chromosome in these species (Roberts 1969; Hughes 1982; Corni et al. 1989).

In Palinuridae, numerical variability is always present, but in *J. lalandii* and *P. regius* (Cannas et al. 2004) B chromosomes have never been found. On the contrary, in all the *Palinurus* studied to date (three out of six living species) the presence of B chromosomes has been demonstrated (Coluccia et al. 2004, 2005). In these species, B chromosomes have the same shape and size, meiotic behaviour and some of them carry 45S ribosomal genes. These data would suggest a homologous origin of B chromosomes and that they could derive from processes of karyotypic evolution and could have persisted through speciation events. According to Palero et al. (2009b), the *Palinurus* speciation was very rapid, with very short branches separating the species in the speciation pattern.

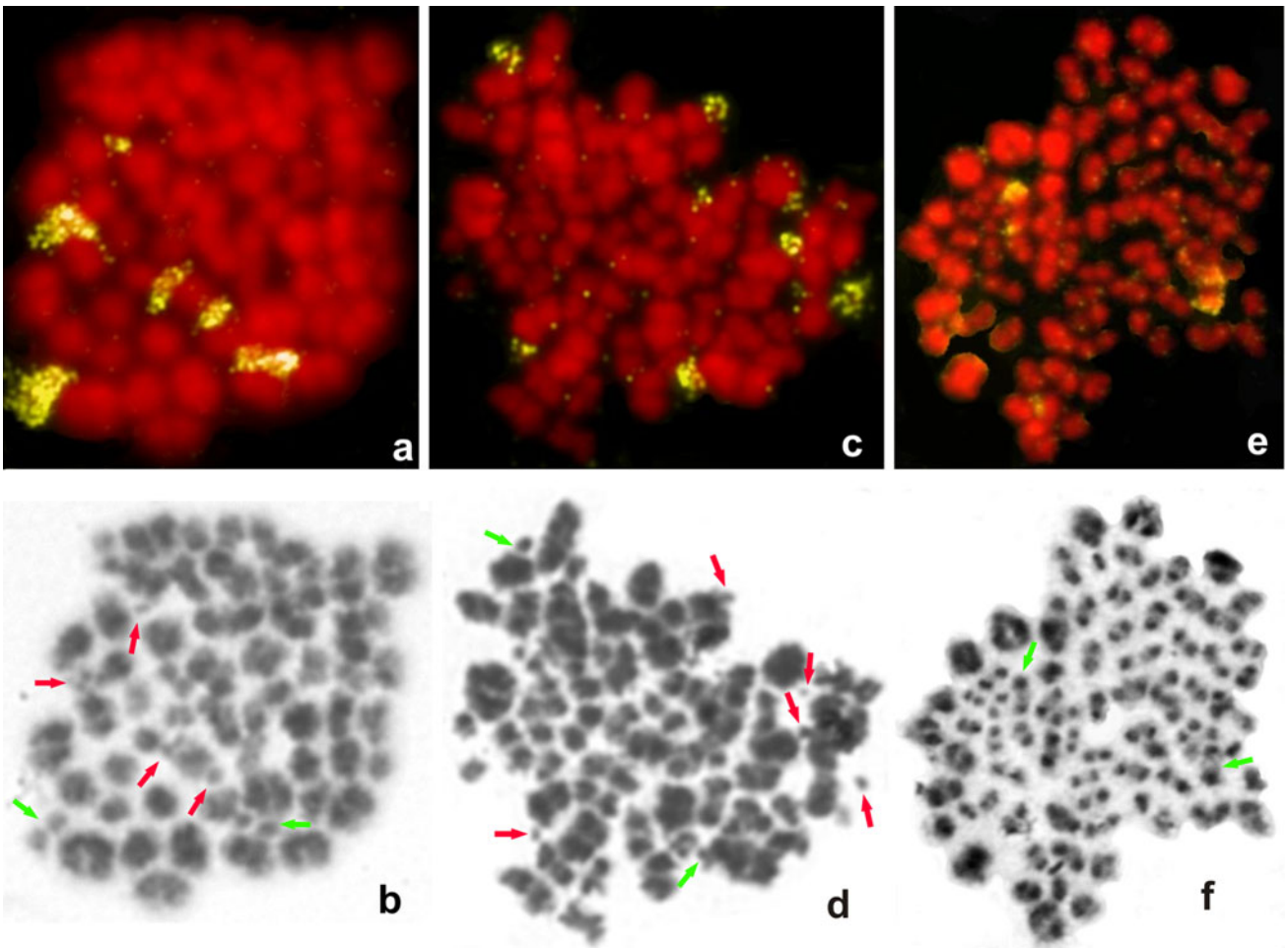


Fig. 3 *Palinurus gilchristi* (**a**, **b**), *P. mauritanicus* (**c**, **d**) and *Panulirus regius* (**e**, **f**) meiotic metaphases I after 45S rDNA FISH and sequential Wright's staining; in the two *Palinurus* species, besides two small diplotene bivalents (*green arrows* in **b**, **d**), some unpaired B chromosomes, clearly visible after Wright's staining of the same plates, were labelled (*red arrows* in **b**, **d**), while in *Panulirus regius* two medium sized bivalents were marked by FISH (green arrows in **f**)

Telomeric sequence

The telomeric sequence has been studied in five Palinuridae species and also in *Scyllarus arctus*. The hexameric sequence $(TTAGGG)_n$ is highly conserved among vertebrates and widespread in animals and it has been hypothesized that it could be the ancestral sequence in Metazoa (Faijkus et al. 2005; Traut et al. 2007). The pentanucleotide $(TTAGG)_n$ motif is widespread among insects (but lost in several orders) and present in other arthropods, including crustaceans, so that it has been proposed as an ancestral telomere motif for arthropods (Sahara et al. 1999; Vitkova et al. 2005). In crustaceans, available data refer to the presence of the $(TTAGG)_n$ motif in species from different orders, including the Decapoda (Vitkova et al. 2005 and references herein). Nevertheless, the ancestral telomere motif was probably lost independently in different groups, as in *Asellus aquaticus* (Isopoda), where the presence of the vertebrate (TTAGGG)*n* repeat has been reported by Pelliccia et al. 1994. In this work, the FISH of the pentameric $(TTAGG)_n$ and of the hexameric $(TTAGGG)_n$ repeats indicates that all the Achelata studied have the pentanucleotidic sequence. In *J. lalandii*, the TTAGG telomeric probe labels not only the chromosome ends but also some large heterochromatic regions, and in particular the same regions labelled by the 45S probe, showing the presence of telomeric sequence at non-telomeric sites, interspersed with ribosomal genes. ITSs (interstitial telomeric sequences) are found in animals and plant chromosomes, often associated with rDNA; the origin of this association could be not casual and be linked to the nucleolus formation or to chromosome rearrangements (Hall and Parker,1995; Liu and Fredga 1999; Rousselet et al. 2000; Dobigny et al. 2003). Interstitial telomeric sequences interspersed within NOR sequences could cause unequal crossing over and contribute to the NOR polymorphism present in *Jasus lalandii*, as we hypothesized in the Anguilliform fish *Anguilla anguilla* and *A. rostrata* (Salvadori et al. 1995b).

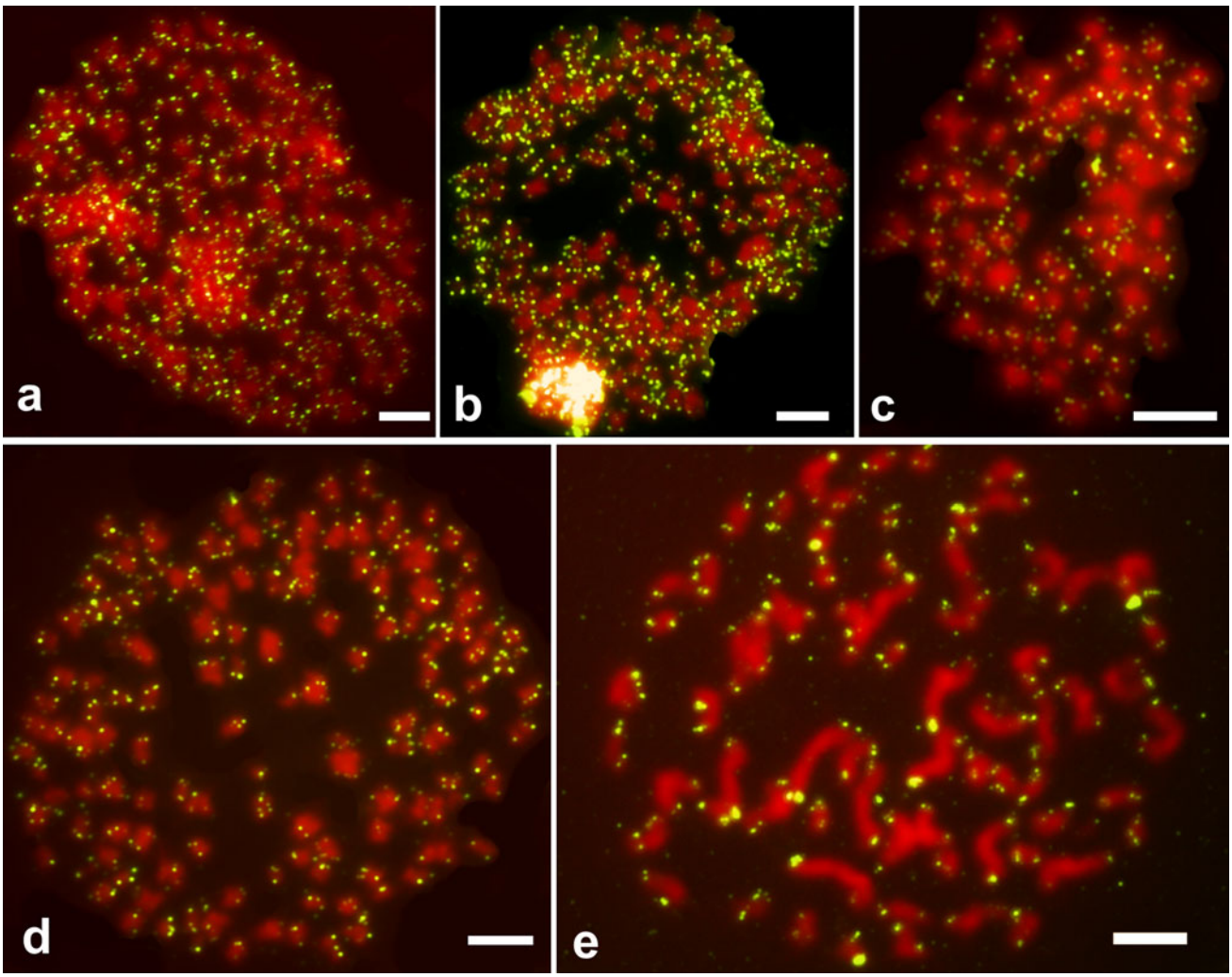


Fig. 4 The $(TTAGG)_n$ telomeric probe FISH clearly marks the end of the chromosomes in *P. gilchristi* mitosis (**a**), *P. mauritanicus* mitosis (**b**), *Panulirus regius* metaphase II (**c**), *Palinurus elephas* mitosis (**d**), and in *Scyllarus arctus* mitosis (**e**). The *bar* represents 5 μm

Our findings give new insights and point to reliable chromosome markers useful for comparative karyologycal analyses in Palinuridae. These markers enable a comparison of karyological data and their study in a larger number of species could give information about karyotype evolution and speciation mechanisms in Palinuridae.

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