CHROMOSOME AND CYTOLOGICAL ANALYSIS OF THE ENDANGERED LIMPET *PATELLA FERRUGINEA* GMELIN, 1791 (GASTROPODA: PATELLIDAE): TAXONOMICAL AND MONITORING IMPLICATIONS

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Abstract *Cytogenetic data are increasingly being used in taxonomic analyses and can also be used to investigate phylogenetic patterns. Additionally, studies on genotoxicity using cytogenetic analyses have been demonstrated as an useful tool for biomonitoring. The endangered limpet* Patella ferruginea *was investigated under karyo and cytological analyses in order to explore the taxonomic status of the two different forms of the species, the phylogenetic relationships and to show up its suitability for future biomonitoring programs. The haploid complement was of n = 9 (5 metacentric, 1 meta-submetacentric, 1 submetacentric, 1 subtelocentric, 1 telocentric), and the ideograms of the two forms failed to establish taxonomic separation between them.* P. ferruginea *is close to* P. rustica *according to the karyotypes and recent molecular studies. Finally, several nuclear abnormalities have been detected in the species that could be useful for monitoring the cytotoxic effects on the endangered populations of the species.*

Key words Patella ferruginea*, chromosome, cytological, rouxi, lamarcki*

INTRODUCTION

Presently, in parallel with morphological and molecular studies, cytogenetic data are increasingly being used in taxonomic analyses, at all hierarchical levels (Thiriot-Quiévreux & Seapy, 1997), and can also be used to investigate phylogenetic patterns (Thiriot-Quiévreux, 1988). In this sense, Vitturi, Catalano & Macaluso (1986) noted that cytogenetic studies are a favored method for clarifying phylogenetic relationships and mechanisms of chromosomal evolution relevant to speciation in closely related species. Moreover, despite numerous studies on the taxonomy of limpets, evolutionary relationships among them are poorly known (Nakano & Ozawa, 2004). In fact, to date, research papers on the comparative cytotaxonomy of this group are still very scarce, (Nishikawa, 1962; Nakamura, 1982 a, b; Cervella, Robotti & Sella, 1988), and only the latter study was conducted on the north atlantic-mediterranean genus *Patella*.

The limpet *Patella ferruginea* Gmelin, 1791, endemic to the Mediterranean, is the most endangered marine species on the list of the European Council Directive 92/43/EEC on the conservation of Natural Habitats and of Wild Fauna and Flora, 1992 (Ramos, 1998), and it is, presently, under serious risk of extinction (Laborel-Deguen & Laborel, 1991; Templado & Moreno, 1997). Nevertheless, its biology and ecology is presently little known (Casu, Casu, Lai, Pala, Gazalle, Zanello, Castelli & Curini-Galletti, 2004; Guerra-García, Corzo, Espinosa & García-Gómez, 2004). *P. ferruginea* was the first species of Mediterranean limpets from genus *Patella* that diverged from others, in the early Miocene, about 18 Ma (Nakano & Ozawa, 2004), for this reason, its cytogenetic study could reveal what kind of karyological pattern would be the primitive on the genus *Patella* and would allow the determination of clear phylogenetic relationships. Additionally, two different morphological types of *P. ferruginea* have been described by Payraudeau in 1826: lamarcki and rouxi forms. They can be morphologically differentiated since lamarcki shows a depressed shell with few and thick ribs, while rouxi has a conical shell with numerous thin ribs. Some authors (Porcheddu & Milella, 1991) have suggested the hypothesis that the two forms could be two different species, whereas Grandfils (1982) attributed the morphological differences to ecological features.

The Mediterranean Sea serves as an example of an over-exploited sea disturbed by heavy effluent loads (Fishelson, 1973; Herut, Hornung, Krom, Kress & Cohen, 1993; Herut, Hornung & Kress, 1994, Herut, Hornung, Kress, Krom & Shirav, 1995), intense maritime transportation

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and diverse human activities along the coasts, all of which pose a continuing threat to the marine environment (Bresler, Abelson, Fishelson, Feldstein, Rosenfeld & Mokay, 2003). In this context gastropod molluscs are among the most useful organisms for environmental biomonitoring (Phillips & Rainbow, 1994; Boening, 1999). The use of cytogenetic assays in fish and some molluscs has proven to be useful in studying genotoxicity. The correlation between exposure to genotoxic substances and micronuclei (MN) frequency in fish cells has been shown under both laboratory and field conditions in organisms (Al-Sabti & Metcalfe, 1995). Several types of nuclear abnormalities (NA) have been described and classified in fish as blebbed, notched and lobed nuclei by Carrasco, Tilbury & Mayers (1990), and these abnormalities have been used by several authors as indicators of genotoxic damage (Bombail, Aw, Gordon & Batty, 2001; Pacheco & Santos, 1998; Ayllon & García-Vazquez, 2000). Gill cells and haemocytes from mussels have been used to analyse both the basal and chemical induced frequencies of MN and other nuclear abnormalities (Venier, Maron & Canova, 1997). However, the sensitivity of limpets under genotoxicity studies has been less studied, only Bresler *et al*. (2003) on *P. caerulea* Linnaeus, 1758, carried out micronuclei studies, whereas no nuclear abnormalities have been described in this group.

The aim of the present study was to describe the karyotype of *P. ferruginea*, in both forms to elucidate their taxonomic status and the phylogenetic relationships among *Patella* species, taking into account the previous studies. Additionally, morphological different types of cells and abnormalities were also investigated in order to establish the suitability of limpets for biomonitoring programs, since they have specific localization and do not venture across the water to other areas according to Bresler *et al*. (2003).

MATERIAL AND METHODS

KARYOLOGICAL STUDY

Specimens of *Patella ferruginea* were collected in April 2005 from a breakwater outside of the harbour of Ceuta, where one of the more dense populations in the world occurs (Guerra-García *et al*, 2004). Following Porcheddu & Milella (1991), we have considered rouxi form when height / length ratio (H/L) of the shell exceeds 0.3, whereas lamarcki form would be below 0.3, together with the morphological characters. Metaphases were obtained following a modified protocol described by Thiriot-Quiévreux (1988), Foighil & Thiriot-Quiévreux (1999) and Bonnaud, Ozouf-Costaz & Boucher-Rodoni (2004). Briefly, specimens from both, *P. ferruginea* rouxi and lamarcki, were immediately taken to the laboratory and then incubated for 24 hours in aerated seawater containing 0,005% colchicine at 19ºC for metaphase arrest. Small fragments of gill-mantle from each individual were exposed to 0,55% KCl at 20ºC for 10 min and mechanically disgregated. After centrifugation, cells were fixed with three baths (10, 20, 30 min) of freshly prepared solution of methanol-acetic acid (3:1) at 4ºC, and centrifuged after each step. The pellet from the last centrifugation was resuspended in 2 ml fixative, and the cell suspension was then dropped onto clean slides and stained for 10 minutes with Giemsa 4%. Although this protocol was conducted, obtaining adequate metaphase plates was extremely difficult, and is described by Cervella *et al*. (1988) on three species of *Patella.* Photographs of well-spread metaphases were taken with an Olympus BX61 photomicroscope. For karyotyping, chromosomes were cut out of photomicrographs and were paired on the basis of size and centromere position. Measurements of chromosomes were made with a ImageJ 1.33u program. Chromosome numbers and measurements were made from three well-spread metaphases for each form of the species, and the mean and standard deviation (S.D.) for each parameter were obtained, according to Foighil & Thiriot-Quiévreux (1999) that used three metaphases for measurements and Cervella *et al*. (1988) that used six metaphases for each species analysed. The relative lengths for each chromosome were obtained from the mean of the two homologous. Relative length or percent total complement length was expressed as 100 (absolute chromosome pair length / total length of haploid complement). Centromeric indices (Ci) were calculated as 100 (length of short arm / total length of chromosome). Arm ratios were calculated as length of short arm / length of long arm. Both the centromeric index and the arm ratio are given because each expresses centromeric position and allows comparisons with previous studies. Terminology relating to centro-

Table 1 Chromosome measurements in rouxi form of *Patella ferruginea*.

Chromosome pair No.	Relative length		Arm ratio		Centromeric index		
	Mean	SD	Mean	SD	Mean	SD	Classification
1	13,51	0,130	0,833	0,140	45,33	4,088	m
2	13,46	0,352	0,47	0,056	31,94	2,436	sm
3	12,89	0,887	0,66	0,066	39,82	2,320	m-sm
4	11,92	0,098	0,897	0,032	47,29	0,873	m
5	11,15	0,773	0,975	0,021	49,45	0,396	m
6	10,34	1,544	0,753	0,150	42,61	5,092	m
7	10,33	1,074	0,77	0,035	43,41	1,302	m
8	8,433	0,254	0,253	0,040	20,29	2,378	st
9	7,18	0,439	0,117	0,015	10,62	1,332	t

Table 2 Chromosome measurements in lamarcki form of *Patella ferruginea*.

Chromosome pair No.	Relative length		Arm ratio		Centromeric index		
	Mean	SD	Mean	SD	Mean	SD	Classification
1	13,44	0,282	0,95	0,026	48,66	0,840	m
2	13,37	0,323	0,45	0,017	30,90	0,921	sm
3	12,13	0,806	0,513	0,006	33,61	4,400	m-sm
4	12,02	0,881	0,893	0,040	47,1	1,126	m
5	12	0,534	0,907	0,045	47,39	1,434	m
6	10,81	0,217	0,753	0,127	42,78	4,160	m
7	10,347	0,66	0,703	0,117	40,91	3,220	m
8	8,533	1,304	0,273	0,075	21,22	4,352	st
9	7,347	1,018	0,113	0,032	9,733	2,230	t

meric position follows that of Levan, Fredga & Sanders (1964). A chromosome is metacentric (m) if Ci falls in the range 37,5-50, submetacentric (sm) if Ci is between 25 and 37,5, subtelocentric (st) if Ci is in the range 12,5-25, and telocentric (t) if Ci is between 0-12,5. When a centromere position was found to be on the borderline between two different categories, taking into account the S.D., the two chromosome categories are listed.

CYTOLOGICAL STUDY

In this study detection of NA in gill-mantle cells and the analysis of morphologically different types of cells between the external major tissues was carried out.

According to Hayashi, Ueda, Uyeno, Wada, Kinae, Soatome, Tanaka, Takai, Sasaki, Asano, Sofuni & Ojima (1998) and Manna & Sadhukhan (1986), gill cells are more sensitive to genotoxic exposure than other tissues, for this reason we used the gill-mantle tissue of limpets to observe NA. Classification of NA was made following Carrasco, Tilbury & Mayers (1990). Briefly, cells with two nuclei are considered as binucleates. Blebbed nuclei present a relatively small evagination of the nuclear membrane, which contains euchromatin. Evaginations larger than the blebbed nuclei which could have several lobes, are classified as lobed nuclei. Nuclei with vacuoles and appreciable depth into a nucleus that does not contain nuclear material are recorded as notched nuclei. The criteria for MN identification

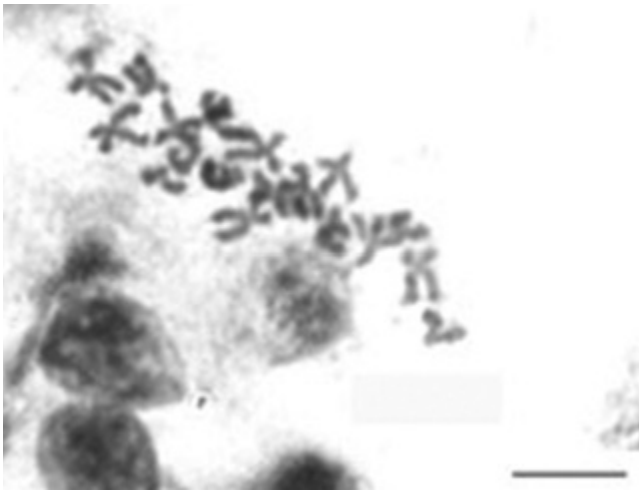

Figure 1 Microphotograph of karyotype of rouxi form of *Patella ferruginea*. Scale bar = 5 µm.

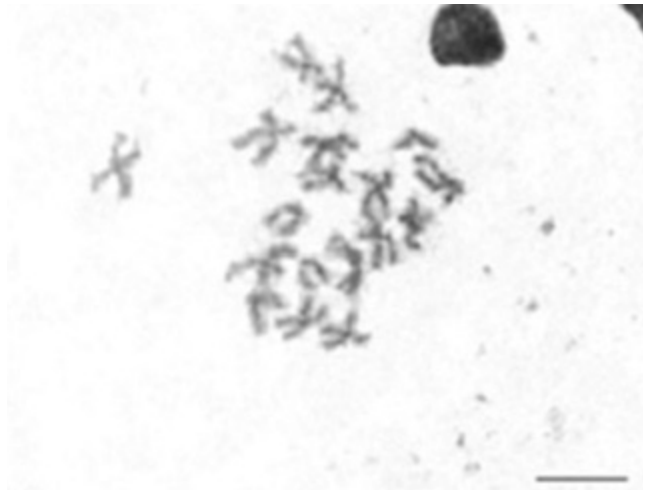

Figure 2 Microphotograph of karyotype of lamarcki form of *Patella ferruginea*. Scale bar = 5 µm.

were from Cavas, Garanko & Arkhipchuk (2005): (a) MN must be smaller than ⅓ of the main nuclei, (b) MN must be clearly separated from the main nuclei, (c) MN must be on the same plane of focus and have the same colour. Three limpets were used for the cytological study. The limpets were collected from the outside of the harbour of Ceuta, where good conditions are present, both for physico-chemical parameters and for diversity of organisms (Guerra-García & García-Gómez, 2005).

Samples from gill-mantle and foot tissue of the three specimens were processed as described by Cavas & Ergene-Gözükara (2003). Briefly, fragments from each tissue were removed and treated with 20% acetic acid solution. Cells were then gently scraped off using mechanical disgregation. Free cells were collected by centrifugation and then fixed in three successive changes of freshly prepared methanol-acetic acid (3:1) solution. Fixed cells were spread on clean slides and stained with 4% Giemsa solution for 10 minutes. For NA analysis, three slides with gill-mantle cells from each individual were prepared, and 1500 cells from each slide were scored under 1000x magnification. Frequencies of NA were expressed per 1000 cells (‰), MN and binucleate cells were only registered.

In order to detect cytological differences between the external main tissues, three slides with foot cells, one from each individual, were prepared. The frequencies of the different types of cells were calculated by scoring 2000 cells in each of the three slides, and the percentage of different cellular types was expressed per 100 cells (%). We established the following criteria to separate the different cellular types: cells with major axis > 4 times minor axis were considered as muscle cells (elongated), whereas the other cells were considered as connective tissue cells. Both microphotographs (different nuclear abnormalities and cellular types) were taken with a Olympus BX61 photomicroscope.

RESULTS

KARYOLOGICAL STUDY

The diploid complement of both forms (rouxi and lamarcki) of *P. ferruginea* is 2n = 18. Microphotographs of karyotypes of the two forms are given in Figures 1, 2, whereas the karyotype of the species are summarised in Figure 3. Mean values ± standard deviation of relative lengths, arm ratio and centromeric indices of each pair of the two forms are reported in Tables 1, 2. For a better exposition of the different morphological types of chromosome pairs, ideograms from the two forms (Figs 4, 5) were constructed using centromeric index and relative length values of chromosome measurements. Both rouxi and lamarcki forms have chromosome pairs with a terminal centromeric position, from ideograms constructed, it can be seen that chromosome sizes are heterogeneous in the two forms, with two shorter chromosomes.

Chromosome numbers and chromosome morphological classification of chromosomes of the haploid karyotypes of the Superfamily Patellacea are listed in Table 3 from different previous stud-

Table 3 Chromosome numbers and morphological classification in Patelloidea.

	Species	Haploid chromosome number	Morphological classification of chromosomes	Reference
Family	**Lottiidae**			
	Collisella (= Lottia) digitalis	10	4 m, 5 sm, 1 st	Chapin & Roberts (1980)
	C. pelta	10	3 m, 6 sm, 1 st	Chapin & Roberts (1980)
	C. strigatella	10	5 m, 3 sm, 3 st	Chapin & Roberts (1980)
	C. heroldi	10	3 m, 5m-sm, 2 sm	Nakamura (1982b)
	C. langfordi	10	4 m, 4m-sm, 1 sm, 1sm-st	Nakamura (1982b)
	Notoacmea scutum	10	4 m, 4 sm, 2 st	Chapin & Roberts (1980)
	N. fenestrata	10	4 m, 5 sm, 1 st	Chapin & Roberts (1980)
	N. persona	10	4 m, 6 sm	Chapin & Roberts (1980)
	N. concinna	10	4 m, 1m-sm, 2 sm, 1sm-st, 1st-t, 1 t	Nakamura (1982a)
	N. schrenkii	10	3 m, 1m-sm, 2 sm, 2sm-st, 1 st, 1 t	Nakamura (1982a)
	N. fuscoviridis	10	3 m, 3 sm, 2sm-st, 1st-t, 1 t	Nakamura (1982a)
	Patelloidea saccharina	10	10 m	Nakamura (1986)
	P. striata	10	7 m, 1 sm, 1 st, 1 t	Nakamura (1986)
Family	**Nacellidae**			
	Cellana toreuma	9	7 m, 2m-sm	Nakamura (1982b)
	C. eucosomia	9	5 m, 1 sm, 3 st	Ebied (2000)
	C. grata	9	9 m	Nakamura (1986)
	C. nigrolineata	9	9 m	Nakamura (1986)
Family	**Patellidae**			
	Patella flexuosa (=Scutellastra flexuosa)	9	8 m, 1 t	Nakamura (1986)
	Patella ulyssiponensis	8	7 m, 1 sm	Cervella *et al*. (1988)
	P. rustica	9	6 m, 1 sm, 1 st, 1 t	Cervella *et al*. (1988)
	P. vulgata	9	6 m, 3 t	Vitturi *et al*. (1982)
	P. caerulea	9	6 m, 1 sm, 2 t	Cervella *et al*. (1988)
	P. ferruginea	9	5 m, 1m-sm, 1 sm, 1 st, 1 t	present study

ies together with the present work.

CYTOLOGICAL STUDY

The total NA observed in gill-mantle cells was 24,6 ± 7,05 ‰. The percentage of muscle cells in the foot tissue was 26,73 ± 8,98 %, whereas no muscle cells were present in the gill-mantle tissue. Measurements of MN and binucleated cells were not possible due to the impossibility of cytoplasm preservation with the protocol used, and only one photograph was taken. Examples of micronuclei, binucleated cells, cells with heterochromatine and nuclear abnormalities are presented in Figure 6.

Figure 3 Karyotype of *Patella ferruginea*. Scale bar = 5 µm.

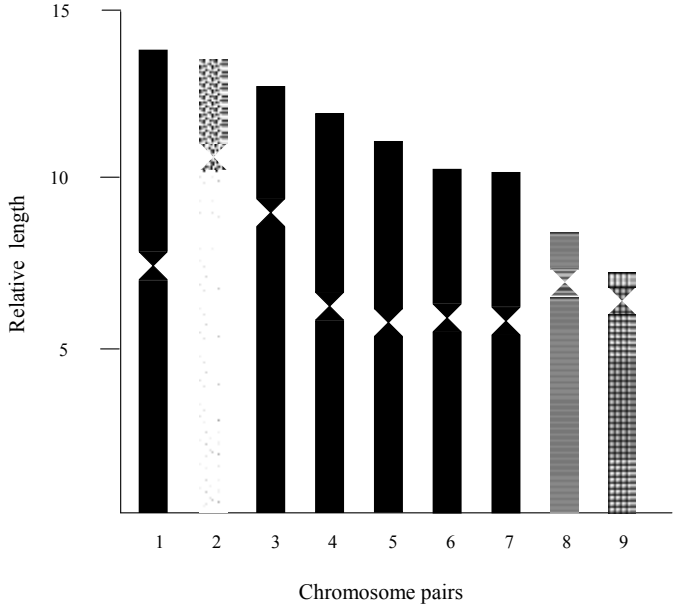

Figure 4 Ideogram of haploid complement of rouxi form of *Patella ferruginea*. Black: metacentric; dotted: submetacentric; bars: subtelocentric; reticulated: telocentric.

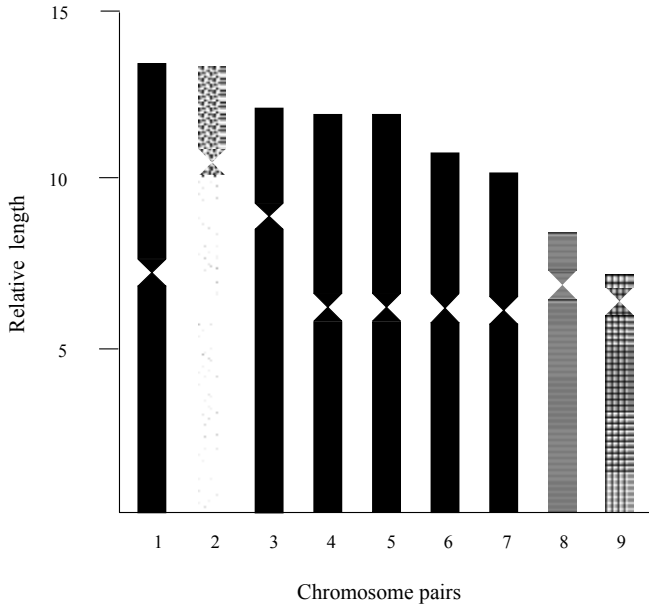

Figure 5. Ideogram of haploid complement of lamarcki form of *Patella ferruginea*. Black: metacentric; dotted: submetacentric; bars: subtelocentric; reticulated: telocentric.

DISCUSSION

Morphological significant differences have been found between lamarcki and rouxi forms, according to the results of Porcheddu & Milella (1991). Lamarcki form would have the characteristics of the Linné holotype and in order to diminish the synonymy, attributed *Patella lamarcki* to *Patella ferruginea*, whereas rouxi form would be a different species, named *Patella rouxi*. These authors concluded that karyological studies were necessary to check this hypothesis, and considered the two different forms as *incertae sedis*. No other morphological differences have been observed in the two forms except shell shape, since Porcheddu & Milella (1991) did not find radular differences. Grandfils (1982) suggested that ecological factors such as position on the shore, could be responsible for different shell shapes in *P. ferruginea*. This hypothesis has been explained as a response to dessication stress at higher levels (Branch, 1975) or to water turbulence (Simpson, 1985). Intraspecific morphological variation has also been reported to occur in several species of Atlantic and Mediterranean limpets in relation to changes in environmental conditions, suggesting that morphological differences in shell shape could be environmentally determined (Corte-Real, Hawkins & Thorpe, 1996; Beaumont & Wei, 1991; Nolan, 1991). Recent genetic approach by Espinosa & Ozawa (2006) based on mitochondrial DNA sequences has failed to detect genetic differences between the two forms. The results of the present study are in agreement with those,

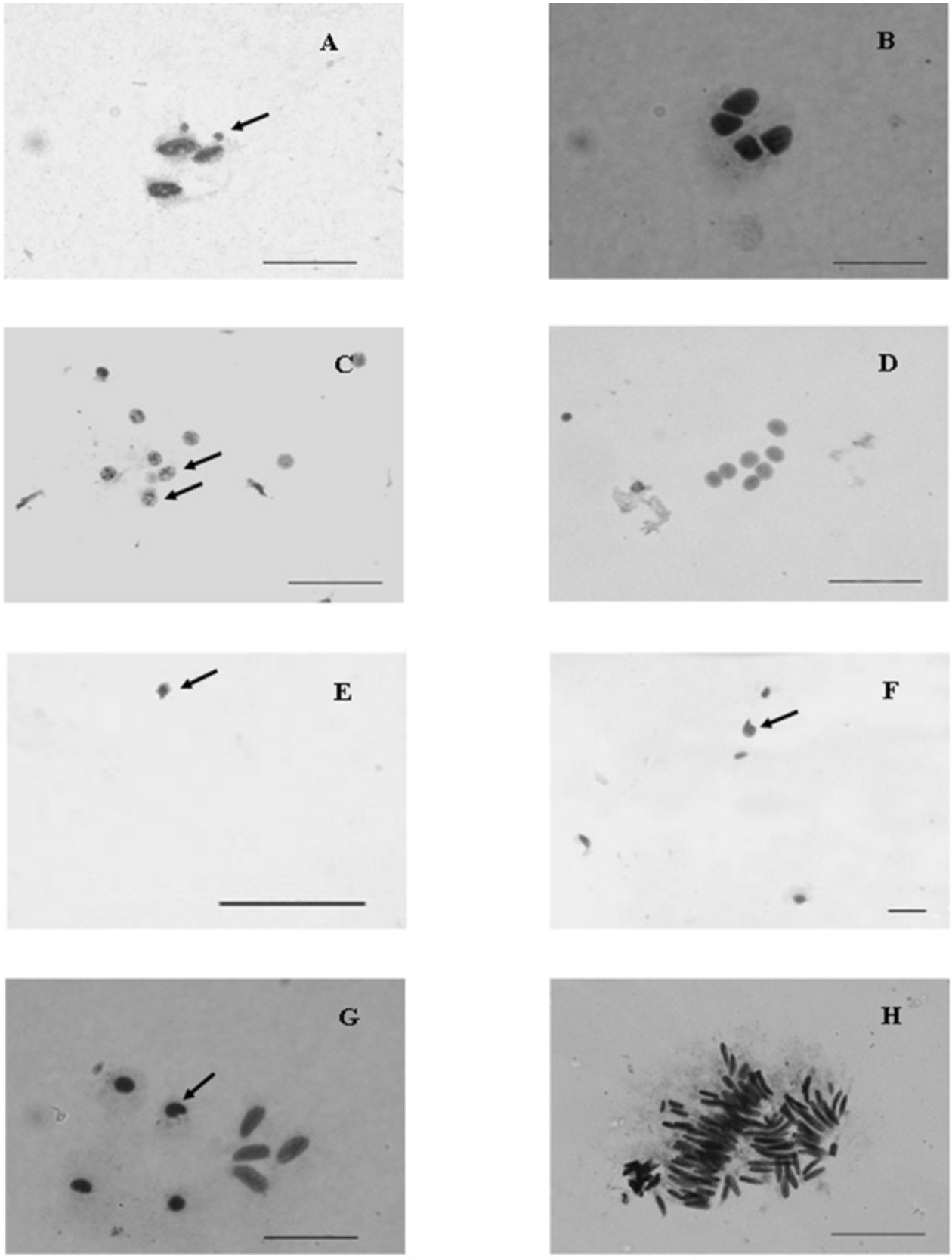

Figure 6 A-G: gill-mantle tissue cells. H: foot tissue cells. **A** Micronuclei. **B** Binucleate cells. **C** Nuclei with heterochromatine. **D** Normal nuclei. **E** Irregular or lobed nuclei. **F** Blebbed nuclei. **G** Notched nuclei. **H** Nuclei of muscle cells of the foot tissue. Scale bars: **A, B,C, D, F, G, H** = 20 µm; **E** = 50 µm.

since the same ideogram has been observed for rouxi and lamarcki forms. Hence, both molecular and karyological evidences show definitively that these two forms are ecotypes instead of different species or subspecies.

Reported chromosome numbers of the Superfamily Patellacea range from 8 to 10, and reliable information on karyotype analysis for comparative purposes is available for only 16 species which are listed in Table 3. According to Patterson (1969), a general conservativeness with regard to chromosomal change is evident in most mollusc groups, whereas this author pointed out that we may expect to find more primitive molluscs to have lower chromosome numbers and species derived from them too often have a greater number of chromosomes, although several exceptions can be detected and care must be taken in assessing which groups are morphologically primitive or advanced. In this sense, the haploid complement of n = 9 appears as the primitive character in the family Patellidae, since *P. ferruginea* is the more ancient species of the genus *Patella* (Nakano & Ozawa, 2004) attending to genetic analyses of mitochondrial DNA and moreover all the species of the family present this number. The family Acmaeidae presents one chromosome more than Patellidae, and after Patterson (1969) it could be proposed that the species of Acmaeidae analysed would have appeared later than species of the genus *Patella*. However, in absence of genetic evidences for *Collisella* and *Notoacmea* species this contention must be taken with caution. *Patella ulyssiponensis* Gmelin, 1791 (named with its synonym *P. aspera* by Cervella *et al*., 1988), appears as a distinct lineage according to chromosomal number. Its karyotype could have originated from a basic *Patella* karyotype by means of structural chromosome rearrangements such as a centric fusion (Cervella *et al*., 1988). This is supported by recent genetic phylogeny of the genus *Patella*: *P. ulyssiponensis* appears as a distinct clade, whereas *P. rustica* Linnaeus, 1758 and *P. ferruginea* appear closely grouped in the same clade (Sá-Pinto, Branco, Harris & Alexandrino, 2005). Our results are in agreement with this since the karyotype of both species shows very high similarities, and only the position of pairs 3 and 4 appears interchanged, probably due to slight manual differences in the measurements, since the relative length in both pairs are similar, whereas the rest of the karyotype is greatly similar.

Several nuclear abnormalities have been detected in *Patella ferruginea*, corresponding with the classification made by Carrasco *et al*. (1990), and similar to those found in the gills of fishes by Cavas & Ergene-Gözükara (2003). However, the values observed, came from populations of an unpolluted site (Guerra-García & García-Gómez, 2005) and are higher than those obtained in control conditions for fishes (24,6 ‰ against 1,84-1,93 ‰), whereas the values for fishes in severe polluted conditions were in the range 11,87-12,83 ‰. This can be due to the more effective cellular repair mechanism of the vertebrates compared to those of invertebrates. Furthermore, the examination of nuclear abnormalities in the endangered limpet *P. ferruginea* could be an easy and effective method for monitoring the level of stress in the relic populations of this endangered mollusc, taking into account that sampling programs can be carried out over scarce individuals (virtually one) in each populations and only a small fragment of mantle would be required, without causing death to the individuals.

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