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Cytogenetic and Morphological Characterization of *Corbicula fluminalis* (O. F. Müller, 1774) (Bivalvia: Veneroida: Corbiculidae): Taxonomic Status Assessment of a Freshwater Clam

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Chromosomes of *Corbicula fluminalis* were characterized by karyotype analysis and nucleolar organizer region (NORs) localization. The triploid chromosome number was confirmed as 54; the karyotype is composed of 3 metacentric, 15 submetacentric and 36 subtelo-acrocentic chromosomes. Silver staining revealed nucleolar organizers on the telomeric regions of three subtelo-acrocentic chromosomes. This is the first study on chromosomes of *C. fluminalis*. The results are discussed with regards to *Corbicula* species as well as its relationships to other mollusc species based on cytogenetic characters and morphometric of the shells.

Key words: AgNOR, chromosomes, cooling water, *Corbicula fluminalis*, invasive species, karyotype, triploid.

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Taxonomy of Corbiculidae is not well resolved. A revision of the 50 species described by HEUDE (1880) has shown that as many as 43 of them are synonyms of *Corbicula fluminea* (O. F. Müller, 1774) (PRASHAD 1929). From among many *Corbicula* species earlier described from Asia, MORTON (1982) distinguished only two: *C. fluminea* (freshwater) and *Corbicula fluminalis* (O. F. Müller, 1774) (estuarine). In the latest report on the malacofauna of Germany, *C. fluminea* and *C. fluminalis* are also mentioned as two species (GLÖER & ZETTLER 2005). They are distinguished on the basis of the structure of the shell, environmental preferences and mode of reproduction (MORTON 1982; BIJ DE VAATE & GREIJDANUS-KLAAS 1990; RAJAGOPAL *et al*. 2000). However, the distinction of species in the genus of *Corbicula* remains uncertain and questionable. The problem is the atypical sympatric appearance of the invasive *C. fluminalis* accompanied by *C. fluminea* in Europe (CSÁNYI 1998-1999; REUMER 1993; SWINNEN *et al.* 1998; NGUYEN & DE PAUW 2002; DOMAGAŁA *et al.* 2004; ŁABĘCKA *et al.* 2005; PAUNOVIĆ *et al.* 2007). TITTIZER & TAXACHER (1997) in a description of the invasion of this clam in the Danube River, in Germany, used the name *Corbicula fluminea/fluminalis*. HAESLOOP (1992), but also MORTON (1982) finally described *C. fluminalis* as *Corbicula* cf. *fluminalis*, pointing to its uncertain systematic classification.

A molecular investigation of the *Corbicula* populations sympatrically occurring in the Rhine River has revealed the co-existence of two evolutionarily different lineages producing cryptic hybrids (PFENNINGER *et al.* 2002). Taking into regard their potential incomplete reproductive isolation, PFENNINGER *et al.* (2002) have proposed that different lineages of *Corbicula* can be treated rather as the initial stage of a group of species than as a few well-defined species.

In view of the above issues, it is desirable to search for new features that could be used in the taxonomy of *Corbicula.* Chromosomal knowledge is increasingly recognized as an important force in animal evolution and therefore the aim of this study was to describe the karyotype and identify the active organizer regions, NORs, in *C. fluminalis* chromosomes.

Material and Methods

The *C. fluminalis* specimens studied were collected from a channel discharging cooling water from the Power Plant Dolna Odra (N 53°12' E 14°27', Western Pomerania Region, N-W Poland). The clams were collected manually by free diving, at a depth of 0.5-2.0 m between May and July 2007. The grain size of the bottom substrate and particle sorting was measured by a laser Malvern Mastersizer Micro Ver. 2.19 in order to determine the type of river bottom in which the clams lived. Water parameters such as temperature (°C), dissolved oxygen (O_2 dm^{-3}), pH and electrolytic conductivity ($\mu S\ cm^{-1}$) were measured by an Elmetron measuring device.

After transportation to the laboratory, the clams were placed in aerated aquariums. The shell dimensions (length L, height H and width W) were measured by an electronic Etalon slide-calliper to an accuracy of 0.1 mm (Figs 1A, B). The elongation index (L/H) × 100, the height index (H/L) × 100 and the width index (W/H) × 100 were calculated. Detailed shell morphology was observed under a Carl Zeiss Stemi 2000-C microscope, under a magnification of 50 ×. The systematic position of the *C. fluminalis* specimens was established on the basis of the key for identification of freshwater molluscs (GLÖER & MEIER-BROOK 1998). Voucher specimens of the clams studied and their shells are stored at the Department of General Zoology, University of Szczecin (acronym KZOUSZ, Katedra Zoologii Ogólnej, Uniwersytet Szczeciński).

Cytogenetic investigation was performed on the basis of analysis of 26 metaphase plates isolated from 20 specimens of *C. fluminalis*. Because of a low level of the mitotic index, *in vivo* cell division was stimulated by a 0.4% solution of cobalt chloride was applied in the amount of 0.05 ml, injected directly into the body of each specimen. Cobalt chloride blocks two major steps of cellular respiration, inducing tissue hypoxia, which leads to cell proliferation (WO NICKI 2004). After 60 hours the clams were injected *in vivo* with 0.1% solution of colchicine in the amount of 0.25 ml per individual per 6 hours. After this time the gills were dissected and hypotonized in a 0.01% NaCl solution for 1 hour. The suspension was centrifuged (1000 rpm, 10 min) in a MPW-350R centrifuge (MPW Med. Instrument), the precipitate was fixed in a 3:1 solution of methanol and glacial acetic acid and centrifuged again at 1000 rpm for 10 minutes. The supernatant was replaced by fresh fixative solution. The centrifugation was repeated twice and then the cellular suspension was suspended in a 50% acetic acid solution. This cell suspension was dropped onto a microscope slide, heated to 40-45°C and after a few seconds the suspension drop was withdrawn back into the Pasteur pipette (BOROŃ *et al.* 2004).

The slides were stained with a 2% orcein solution at 46°C for 30 minutes. Observations were conducted under a Nicon Eclipse E600 microscope with an immersion objective of 100 × magnification. The photographs were stored in computer memory using an Applied Imaging ER-3339 camera (Applied Imaging International Ltd.). In order to detect the active NORs, the AgNOR chromosomes were stained according to the method described by HOWELL & BLACK (1980).

Morphometric measurements of the chromosomes were made using the Micromeasure Program Ver. 3.3 (www.colostate.edu/Depts/Biology/MicroMeasure). The lengths of the chromosome arms were measured (short arm, p; long arm, q). The relative chromosome length (RL) , the ratio of the lengths of the arms (q/p) and the centromeric index (CI) were calculated:

$$RL = 100 \times [(p+q)/ \text{ total haploid length})]$$

$$CI = 100 \times [p/(p+q)]$$

The chromosomes were classified according to the criteria proposed by LEVAN *et al.* (1964). The karyogram was made with the help of the Genus Program Ver. 3.1 (Applied Imaging International Ltd.).

Results

The clams lived on a sandy and muddy river bottom of moderately well sorted grains. The size of grains and their percent contributions encompassed: medium sand 19.4%, fine sand 64.5%, very find sand 11.9%, very coarse silt 0.9%, coarse silt 3.0%, medium silt 0.3%.

The mean water temperature during the period of study was 25.3°C (SD = 4.3), pH 8.8 (SD = 0.18), dissolved oxygen 11.76 mg O_2 dm^{-3} (SD = 0.9), and electrolytic conductivity 863.7 $\mu S\ cm^{-1}$ (SD = 95.7).

The shells of *C. fluminalis* were oval-triangular, strongly convex and of clear asymmetry (Figs 1A, B). The shell umbo was above the ligament, slightly rotated and directed to the front of the shell. The *periostracum* was glossy, olive-green coloured and covered with ribs. The number of ribs per 10 mm of the shell surface was 12-16. The *endostracum* was intensely violet in the ventral, posterior and anterior parts, while in the middle part and under the umbo it was white-violet with orange spots. The pallial line was clearly marked, the adductor muscle impressions were well visible.

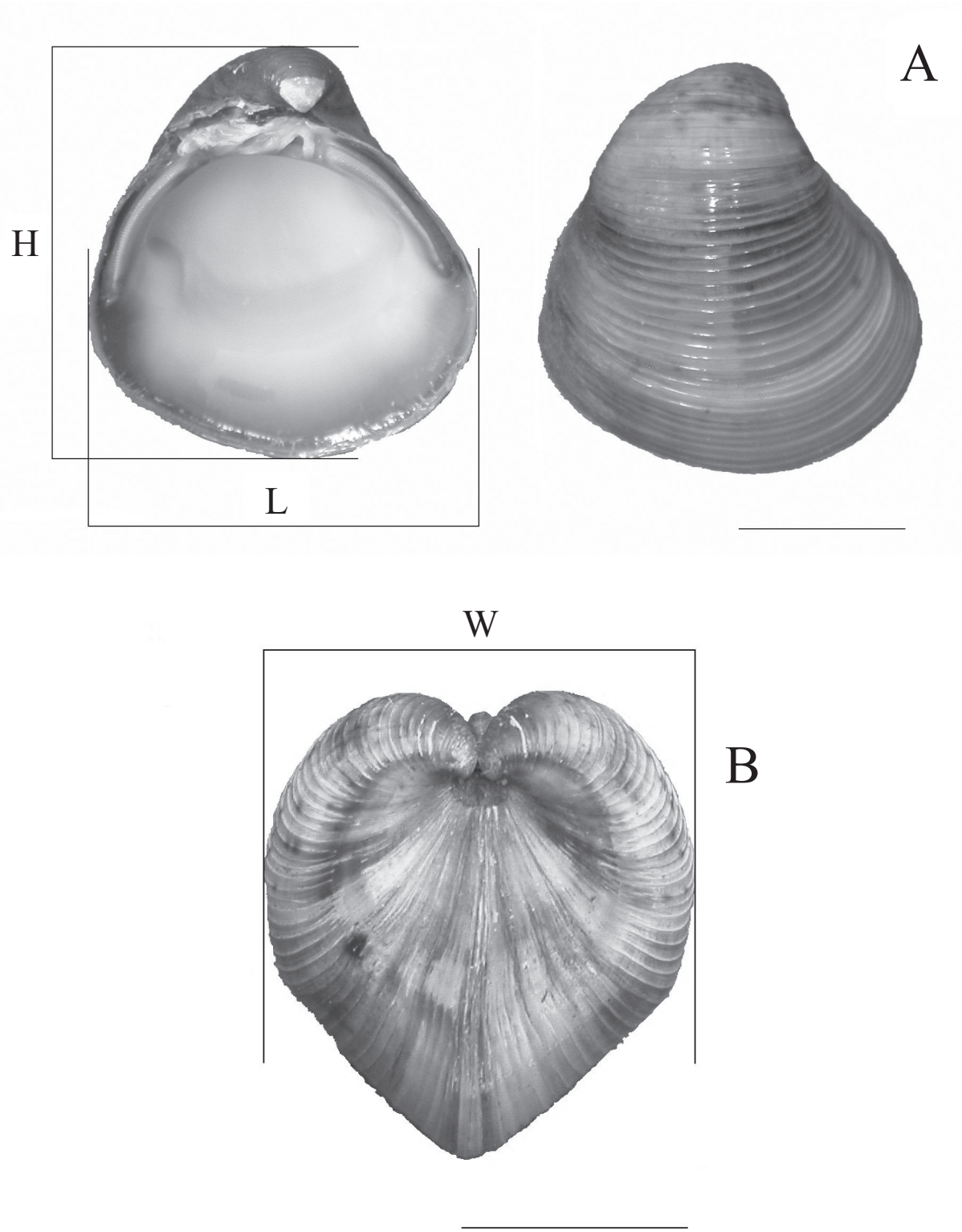

Fig. 1. The shells of *C. fluminalis*. (A) Shell shape, *endostracum* and *periostracum*. (B) Umbo view. Abbreviations: L – length, H – height, W – width. Bar = 1 cm.

The morphometric data concerning the shells of the individuals used in the cytogenetic studies are presented in Table 1. Analysis of the number of chromosomes in 26 metaphase plates revealed that the karyotype of the studied species is composed of 54 chromosomes, (Fig. 2A) that can be divided into 18 groups of 3 phenotypically similar chromosomes (Fig. 2B). Analysis of the centromeric index (Table 2) has proved that the karyotype is composed of 3 metacentric, 15 submetacentric and 36 subtelo-acrocentric chromosomes (Fig. 2B).

Table 1

Morphometric data of shells of *C. fluminalis* from the Odra River, Poland

	L (mm)	W (mm)	H (mm)	(L/H)×100	(H/L)×100	(W/H)×100
mean	19.0	15.9	19.1	100.1	99.9	82.8
SD	3.6	3.4	3.9	3.6	3.5	2.3
min	10.3	7.6	9.8	95.5	91.2	77.6
max	22.6	19.3	22.8	109.5	104.6	87.9

Abbreviations: L – shell length, H – shell height, W – shell width.

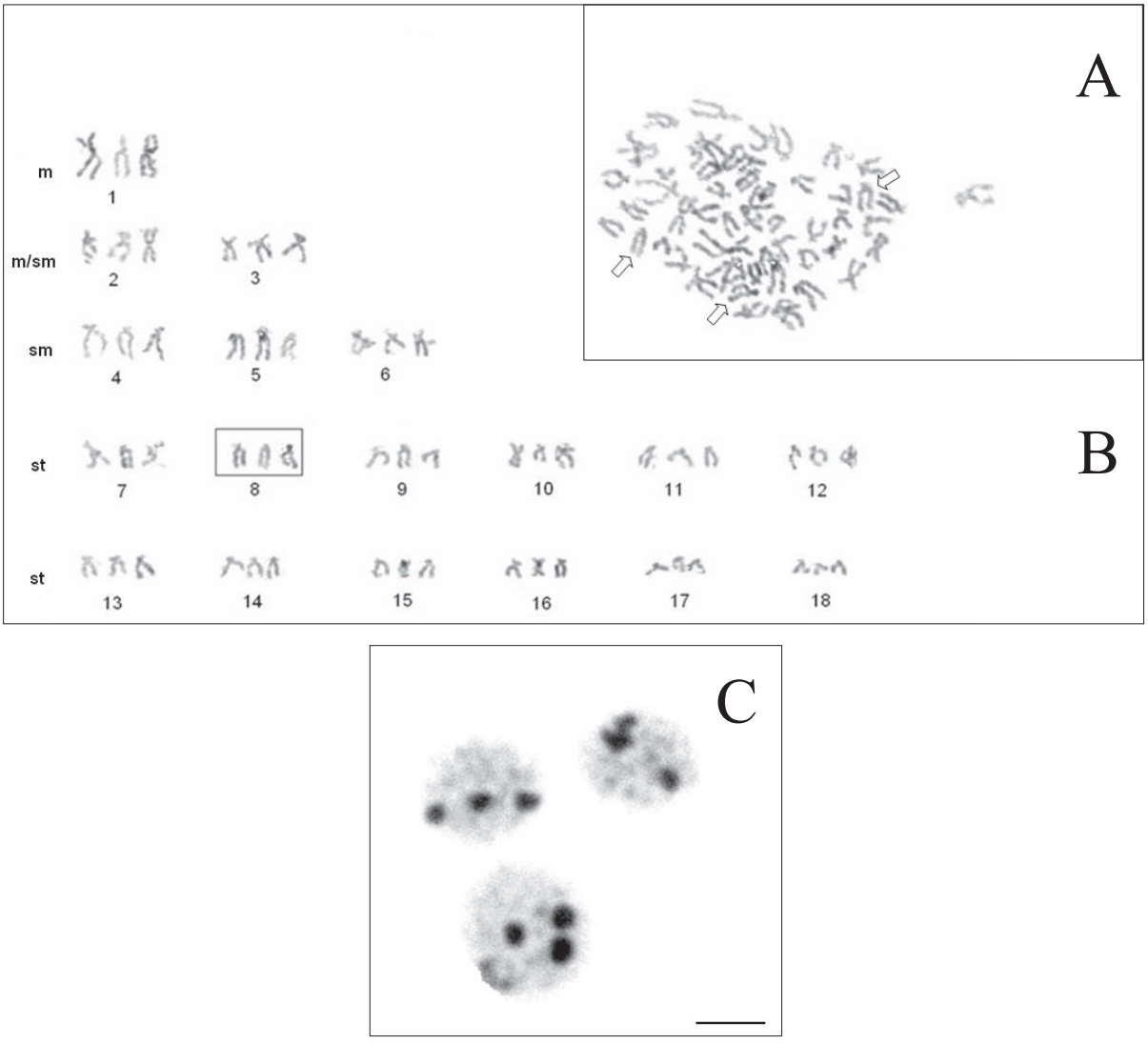

Fig. 2. (A) The metaphase chromosomes of *C. fluminalis* after Ag-staining. Arrows indicate NOR chromosomes. (B) Karyotype. AgNOR chromosomes are in the frame. Abbreviations: m – metacentric, sm – submetacentric and st – subtelocentric chromosomes. (C) Interphase nuclei stained with AgNOR with the three active NORs. Bar = 5 μm.

AgNOR staining of 11 metaphase plates revealed 3 active NORs, localised on the shorter arms of the three subtelo-acrocentric chromosomes (Fig. 2A). The number of AgNOR sites of 3 was confirmed by silver staining of interphase nuclei (Fig. 2B).

Table 2

Measurements of the chromosomes of *Corbicula fluminalis*

Chromosome triplet number	Relative length		Arm ratio (q/p)		Centromeric index		Classification
	mean	SD	mean	SD	mean	SD	
1	14.23	1.8	1.20	0.11	45.54	0.02	m
2	9.22	0.27	2.13	1.44	35.96	0.13	sm/m
3	8.72	0.02	2.02	0.91	34.91	0.09	sm/m
4	5.40	0.34	2.39	0.60	30.2	0.05	sm
5	11.68	0.89	2.56	1.03	30.14	0.10	sm
6	7.24	0.04	2.96	1.66	28.46	0.11	sm
7	7.91	0.02	3.37	0.65	23.28	0.03	st
8	7.67	0.03	3.82	2.03	23.11	0.08	st
9	6.35	0.12	3.83	1.67	22.9	0.09	st
10	10.14	0.27	3.59	0.78	22.26	0.04	st
11	8.45	0.08	4.09	2.14	21.89	0.08	st
12	6.61	0.08	4.07	1.18	20.6	0.05	st
13	7.53	0.03	4.07	1.02	20.24	0.03	st
14	7.07	0.15	4.40	1.97	20.03	0.06	st
15	5.99	0.28	4.56	1.98	19.52	0.06	st
16	8.05	0.09	4.78	1.17	17.74	0.03	st
17	10.68	0.17	4.92	0.72	17.06	0.02	st
18	6.80	0.08	5.21	0.43	16.14	0.01	st

Abbreviations: m – metacentric, sm – submetacentric, st – subtelocentric.

Discussion

C. fluminalis was described for the first time by O. F. MÜLLER as *Tellina fluminalis* (O. F. MÜLLER 1774). It originates from the Euphrates River and is one of the three species from the *Tellina* genus, besides *T. fluminea* and *T. fluviatilis*, distinguished by this author.

C. fluminalis is one of the best known interglacial bivalves identified in north-western Europe (MEIJER & PREECE 2000). It has also been found in the Pleistocene fauna of molluscs in Poland (SKOMPSKI 1991, 2000).

At present we can compare mainly the data on morphology and ecophysiology of *C. fluminalis* and related species. The similarity between *C. fluminalis* and *Corbicula japonica* (Prime, 1864) was indicated by KADO & MURATA (1974). They compared the distributions of the clams and their ecological preferences, including tolerance of salinity. The two taxa have been described in the literature as estuarine and *C. japonica* has been assumed as a younger synonym of *C. fluminalis* (KADO & MURATA 1974; MORTON 1982; KORNIUSHIN 2004). In Poland *C. fluminalis* occurs (with *C. fluminea*) only in the lower section of the Odra River (DOMAGAŁA *et al*. 2004; ŁABĘCKA *et al*. 2005) and although in Europe it occurs usually sympatrically with *C. fluminea*, since 2005 this second species has been noted as the only one inhabiting the upper and middle sections of the Odra River (WAWRZYNIAK-WYDROWSKA 2007).

Shell analysis has shown that the morphometric indices of the museum specimens of *C. fluminalis* coincide with the values of the indices obtained for the *C. fluminalis* individuals found in Poland (Table 3). A comparison of the lectotype of *C. fluminalis* (acronym UZMC, Universitetets Zoologisk Museum of Copenhagen) with the paratype *Corbicula sandai* Reinhardt, 1878 (acronym SMF 6008, Senckenbergsmuseum in Frankfurt/ Main) reveals that they are morphologically indistinguishable (MORTON 1986; ARAUJO *et al.* 1993). It should also be mentioned that *C. sandai* had been originally described by REINHARD as a variety of *C. japonica* (REINHARD 1878). It has been shown that *C. sandai* is not an endemic species restricted only to Lake Biwa, as indicated by ITASAKA *et al.* (1980), who reported it also from the Seta River. Therefore the question is whether *C. fluminalis*, *C.*

Table 3

Shell measurements and morphometric indices

Species and lot	n	L	H	W	H/L	2W/H
C. fluminalis, UZMC	1	29.9	29.7	11.1	0.99	0.75
C. fluminalis, ZIN, 32	1	22.4	22.2	8.3	0.99	0.75
C. fluminalis, ZMB 50930	3	20.6	19.2	7.4	0.94 ± 0.05	0.81 ± 0.09
C. fluminalis, KZOUSZ	20	19.0 ± 3.6	19.1 ± 3.9	15.9 ± 3.4*	0.99 ± 0.03	0.82 ± 0.02

Abbreviations: L – shell length, H – shell height, W – width of one valve (* the widths of the shells were measured in the position of exact match of the two halves of the shell), UZMC – Universitetets Zoologisk Museum of Copenhagen (Denmark), ZIN – Zoological Institute in St. Petersburg (Russia), ZMB – Zoologisches Museum Berlin (Germany), KZOUSZ – Katedra Zoologii Ogólnej, Uniwersytet Szczeciński (Poland).

Table 4

Karyotypes of *Corbicula* species distributed in Asia and Poland

Taxa	Geographic origin	Number of chromosomes	Haploidal karyotype			References
			m	m-sm, sm, sm-st	st-t/st	
C. fluminea	Korea, Lake Uiam	54 (3n)	1	5	12	PARK *et al.* 2000
	China, Anyue County	54 (3n)	2	13 (3+4+6)	3	QIU *et al.* 2001
	China, Anyue County	72 (4n)	2	13 (3+4+8)	1	QIU *et al.* 2001
	Japan, creek Shishigatani	2n				ISHIBASHI *et al.* 2003
	Taiwan, Keelung River	2n, 3n				KOMARU & KONISHI 1999
C. leana	Japan, Lake Biwa	54 (3n)	1	4	13	OKAMOTO & ARIMOTO 1986
C. papyracea	Korea, Lake Uiam	54 (3n)	1	5	12	PARK *et al.* 2000
C. papyracea colorata	Korea, Changpyung Dam reservoir	38 (2n)	1	–	18	PARK *et al.* 2000
C. japonica	Japan, Yodo River	38 (2n)	1	1	17	OKAMOTO & ARIMOTO 1986
C. sandai	Japan, Lake Biwa	36 (2n)	1	1	16	OKAMOTO & ARIMOTO 1986
C. fluminalis	Poland, Odra River	54 (3n)	1	5	12	present study

Abbreviations: m – metacentric, m-sm – meta-submetacentric, sm – submetacentric, sm-st – submeta-subtelocentric, st-t – subtelo-telocentric, st – subtelocentric.

japonica and *C. sandai* are the same species or only three sibling species.

The relationship between *C. japonica* and *C. sandai* was tested by OKAMOTO and ARIMOTO (1986) who studied the number and morphological type of the chromosomes. According to their interpretation, *C. japonica* (2n = 38) may be an ancestor of *C. sandai* (2n = 36). The latter species may have appeared as a result of the centric fusion of a metacentric chromosome with a submetacentric one or an acrocentric with a subtelocentric. Both in *C. sandai* and in *C. fluminalis* there are large metacentric chromosomes that may have appeared in this way. An interesting observation involved the significant differences in the number of chromosomes in particular groups of submetacentric and subtelo-acrocentric chromosomes: 5 and 12 in *C. fluminalis* (3n = 54) and 1 and 16 in *C. sandai* (2n = 36), respectively. The number of chromosomes in particular groups in *C. fluminalis* from Poland is comparable with those established for *C. fluminea* and *Corbicula papyracea* Heude, 1880 from Korea but not with *C. sandai*. However, a cytogenetic study within the *C. fluminea* species has revealed a great variety in this respect (Table 4). According to some researchers from Asia, besides the diploid species of 2n = 36 and 38 chromosomes, some *Corbicula* species are triploid or even tetraploid.

Cytogenetic studies conducted on bivalves have revealed significant variation in the number of chromosomes. In a review of chromosomes in molluscs, NAKAMURA (1985) reports that the Veneroida have from 24 to 48 diploid chromosomes, and the most common number is 2n = 38 (n = 19). However, deviations from this pattern appear within individual families. In the Sphaeriidae high variation in the number of chromosomes is observed, both within the species and genera, e.g. *Sphaerium corneum* (Linnaeus, 1758), 2n = 30 and 36 (KEYL 1956; PETKEVIČIŪTĖ *et al.* 2006); *Sphaerium striatinum* (Lamarck, 1818), 2n ~152 (LEE 1999); *Pisidium* (*Cyclocalyx*) *adamsi* Prime, 1852, 2n>100 (LEE & Ó FOIGHIL 2002); *Pisidium* (*Cyclocalyx*) *casertanum* (Poli, 1795), 2n ~150, ~180, ~190 (BARŠIENĖ *et al.* 1996; BURCH *et al.* 1998); *Pisidium dubium* (Say, 1816), 2n>200 (LEE & Ó FOIGHIL 2002). The question is if the number and type of chromosomes are a correct determinant of the species classification.

It is known that habitat is of great significance in the development of a different number and morphology of chromosomes. The occurrence of a centric fusion can also explain the appearance of the two chromosome forms of the snail *Nucella lapillus* (Linnaeus, 1758), living on the Atlantic coast of Europe. One of its forms has 2n = 26, while the other 2n = 36 chromosomes. The form with the lower number of chromosomes lives in the zone of waves and tides, while the other form lives in quieter water. In the intermediate zone, snails with an intermediate number of chromosomes can be found (BANTOCK & COCKAYNE 1975). CROTHERS (1973) did not study the cytogenetic aspects of *N. lapillus*, but he analysed the shapes of the shells of the population living on the coast of South Wales. He showed that snails exposed to waves had a shell with a wider aperture permitting stronger attachment to the surface on which they lived. The snails living in quieter zones had longer shells well correlated with a small aperture.

Centric fusions and chromosome arms losses have also been recorded in marine mussels, oysters Ostreidae and scallops Pectinidae. In diploid (2n = 20) *Crassostrea gigas* (Thunberg, 1793) the karyotype consisted of 16 metacentric and 4 submetacentric chromosomes (AHMED & SPARKS 1967) as well as 20 metacentric chromosomes (THIRIOT-QUIÉVREUX 1984). In *Crassostrea rhizophorae* (Guilding, 1828) (2n = 20) the karyotype consisted of 10 metacentric and 10 submetacentric chromosomes (RODRIGUEZ-ROMERO *et al*. 1979) as well as 16 metacentric and 4 submetacentric chromosomes (MARQUEZ 1992). *Argopecten purpuratus* (Lamarck, 1819) (2n = 32) has 10 subtelocentric and 22 telocentric chromosomes (GAJARDO *et al*. 2002) as well as 4 metacentric, 14 m/sm, 6 subtelocentric and 8 telocentric chromosomes (VON BRAND *et al*. 1990). *Chlamys farreri* (Jones *et* Preston, 1904) is another example of two karyotypic patterns (2n = 38): 6 m + 8 sm + 14 sm/st + 8 st + 2 st/t (YONGPING & XIMING 2004) and 6 m + 2 sm + 12 sm/st + 14 st + 4 st/t (KOMARU & WADA 1985).

The silver-stained nucleolar organizer regions in triploid *C. fluminalis* were only found at the terminals of the short arms of subtelo-acrocentric chromosomes, i.e. a single locus for the rRNA genes was identified. This result can be considered as the ancestral state within taxa and suggests that this karyotype is plesiomorphic. For the family Veneridae, the species studied show small terminal NORs (INSUA & THIRIOT-QUIÉVREUX 1992). For the family Mytilidae, two and three NOR-bearing chromosome pairs have been described (TORREIRO *et al*. 1999). In diploid *Ostrea angasi* Sowerby, 1871 (2n = 20) three heteromorphic Ag-NORs were identified on the long arms of metacentric pair 3, subtelocentric pair 9 and submetacentric pair 10 (LI & HAVENHAND 1997).

Why is *C. fluminalis* polyploid? Such organisms can appear as a result of disturbances to meiosis leading to formation of gametes of unreduced chromosome number. Changes in chromosome number are favoured by a life cycle that is longer than one season. Polyploidy is easier to find in organisms living for many years than in annual ones, and *C. fluminalis* individuals live up to 10 years (MORTON 1982). The multiplication of the chromosome number is also favoured by partheno-, andro- and gynogenetic reproduction (MOGIE 1986; WALLACE 1992). Among *Corbicula* clams, androgenesis (KOMARU *et al.* 1998; ISHIBASHI *et al.* 2003) and production of spermatozoon of unreduced number of chromosomes have been reported (KOMARU *et al.* 1997; KOMARU & KONISHI 1999). In the process of androgenesis in *Corbicula*, the genetic material of the mother is eliminated in the form of polar bodies already at the first meiosis and the female pronucleus does not develop. However, KOMARU *et al.* (2001) noted that some cells of the female line can undergo a typical meiosis with proper segregation of chromosomes and formation of the female pronucleus. In such a situation, if the parents were diploid, the haploid egg cell and the diploid spermatozoon (of an unreduced number of chromosomes and unreduced amount of DNA) are produced. After fertilization the cells form a triploid embryo. Therefore, the process of androgenesis favours the appearance of changes in the number of chromosomes in these clams.

The *Corbicula* are also unique because they can be either dioecious or hermaphroditic, capable of

self-fertilization (OKAMOTO & ARIMOTO 1986; KOMARU *et al.* 1997; BYRNE *et al.* 2000). This genus comprises oviparous, ovoviviparous and viviparous species brooding their offspring in the inner demibranchs (most often), in the outer demibranchs, or in both (tetragenous) (BYRNE *et al.* 2000; KORNIUSHIN & GLAUBRECHT 2003; KORNIUSHIN 2004; GLAUBRECHT *et al.* 2006). Thanks to a diversity of reproductive strategies, the *Corbicula* are highly adaptable, which probably explains their expansion and successful colonisation of new areas in North and South America and Europe.

An external factor than can favour polyploidy is the availability of new ecological niches. Polyploids are usually characterised by greater expansiveness than their diploid ancestors. For these reasons, the analysis of the number of chromosomes in bivalves in newly colonised areas of Europe is important. Such investigations are expected to detect polyploid populations and bring information on the role of polyploidy in adaptation of animals to new habitats.

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