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## COMPARATIVE KARYOTYPE ANALYSIS OF SLUGS OF THE GENUS *ARION* (GASTROPODA, PULMONATA, ARIONIDAE)

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**Comparative Karyotype Analysis of Slugs of the Genus *Arion* (Gastropoda, Pulmonata, Arionidae).** Harbar, A. V., Kadlubovska, N. S., Harbar, D. A. — Karyotypes of four species of the genus *Arion* were investigated, namely, *A. distinctus* ( $2n = 48m + 8sm = 56$ ; FN = 112), *A. lusitanicus* s. l. ( $2n = 44m + 6sm + 2st = 52$ ; FN = 104), *A. fuscus* ( $n = 26$ ,  $2n = 52$ , FN = 104) and *A. fasciatus* ( $n = 29$ ,  $2n = 58$ , FN = 116). The karyotype of *A. lusitanicus* s. l. was identical to those of *A. fuscus*, *A. ater* and *A. rufus*. The karyotype of *A. fasciatus* in the haploid number of chromosomes is identical to another close species — *A. circumscriptus* ( $n = 29$ ) from the United Kingdom. The identical number of chromosomes in species of the subgenus *Arion* (*A. lusitanicus* s. l., *A. ater*, *A. rufus*) and species of the subgenus *Mesarion* (*A. fuscus*) ( $n = 26$ ) may be a reason for their merging.

**Key words:** slugs, *Arion*, chromosomes, karyotype.

**Сравнительный анализ кариотипов рода *Arion* (Gastropoda, Pulmonata, Arionidae).** Гарбар А. В., Кадлубовская Н. С., Гарбар Д. А. — Исследованы кариотипы четырех видов слизней рода *Arion*: *A. distinctus* ( $2n = 48m + 8sm = 56$ ; FN = 112), *A. lusitanicus* s. l. ( $2n = 44m + 6sm + 2st = 52$ ; FN = 104), *A. fuscus* ( $n = 26$ ,  $2n = 52$ , FN = 104) и *A. fasciatus* ( $n = 29$ ,  $2n = 58$ , FN = 116). Кариотип *A. lusitanicus* s. l. оказался идентичным *A. fuscus*, *A. ater* и *A. rufus*. Кариотип *A. fasciatus* по гаплоидному числу хромосом идентичен другому близкому виду — *A. circumscriptus* из Великобритании ( $n = 29$ ). Идентичные числа хромосом у представителей подродов *Arion* (*A. lusitanicus* s. l., *A. ater*, *A. rufus*) и *Mesarion* (*A. fuscus*) ( $n = 26$ ) могут быть основанием для их объединения.

**Ключевые слова:** слизни, *Arion*, хромосомы, кариотип.

### Introduction

The genus *Arion* Férussac, 1819 includes from 30 to 50 species, according to the variety of published data, most of which are confined to the Iberian Peninsula (Kozłowski, 2005). Some researchers suggest that this genus was distributed worldwide antrophorously (Wiktor, 1996). Some slugs of this genus are invasive species which have recently distributed in many European countries and are considered as serious pests in agriculture (Wiktor, 1996; Kozłowski, 2007).

Some species of this genus belong to cryptic species complexes. In 1994 two essentially different genetic forms of *A. subfuscus* (Draparnaud, 1805) were allocated. They are identified as F- and S-types as they are different by fast and slow elektromorf of phosphoglucomutase (Pgm) (Backeljau et al., 1994). F-slugs were characterized by small dark gonads, while S-slugs were characterized by larger and lighter gonads. Later these facts were confirmed by the DNA analysis. As a result, the F-slugs were assigned to *A. fuscus* (Müller, 1774), and S-type slugs were named *A. subfuscus* (Pinceel et al., 2004). In 2010, slugs from Romania and Poland were redescribed and assigned to *A. transsylvanus* Simroth 1885, a forgotten nominal taxon from Transylvania. Gonad morphology, allozymes and mtDNA data showed consistently that this slug is a strongly differentiated taxon within the *A. subfuscus* complex. The size of the gonad of *A. transsylvanus* was larger than that of *A. fuscus* and was smaller than that of *A. subfuscus* (Jordaens et al., 2010). Previously it was thought that *A. subfuscus* is spread in Ukraine. Our analysis of the gonads morphology proved that complex *A. subfuscus* s. l. in Polissya and Forest-steppe of Ukraine is represented by the species *A. fuscus*, which is characterized by small dark gonads (Garbar et al., 2014). It is also supported by the geographical distribution of *A. subfuscus* complex representatives.

The genus *Arion* is presented by 6 species in the fauna of Ukraine: *A. fuscus*; *A. circumscriptus* Johnston, 1828; *A. fasciatus* (Nilsson, 1823); *A. silvaticus* Lohmander, 1937; *A. distinctus* Mabilie, 1868 and *A. lusitanicus* (Bank et al., 2007) s. l. (Sverlova, Gural, 2005; Gural-Sverlova, Gural, 2010; Gural-Sverlova, 2011; Garbar et al., 2014).

Invasive species *A. lusitanicus* s. l. has been spread in Ukraine recently. The first colonies were found in the western Ukraine (Gural-Sverlova, Gural, 2010; Gural-Sverlova, 2011). In 2014 we found this species in Rivne.

Slugs of the genus *Arion*, as the majority of other terrestrial gastropods mollusks, are investigated karyologically insufficiently. The first karyotype descriptions of this group of species were made by Beeson (1960). He established haploid chromosome numbers (n) for 6 species of the genus *Arion*. Their haploid chromosome numbers varied from  $n = 25$  to  $n = 29$  (Beeson, 1960). Chromosome numbers are known for only two species from Ukrainian populations: *A. fuscus* ( $n = 26$ ,  $2n = 52$ ), and *A. fasciatus* ( $n = 29$ ,  $2n = 58$ ) (Garbar et al., 2010).

The purpose of this study is the description and the comparative analysis of karyotypes of slugs of the genus *Arion*. Their chromosome number may be informative, since Backeljau and De Bruyn (1990) suggested that in arionids, chromosome numbers might correlate with the subgeneric division of the genus.

## Material and methods

Slugs were collected in the spring-autumn period of 2012-2014 (table 1). Slugs were identified in the fauna of Ukraine (Gural-Sverlova, Gural, 2012).

Chromosome slides were prepared from gonad tissues using methods successfully used to study karyotypes of other molluscs (Garbar, Garbar 2007). The animals were injected with 0.02 % colchicine 18 h before the dissection. Gonad fragments were minced and subjected to the hypotonic treatment for 40 min in the distilled water. The material was placed in the mixture of 96 % ethanol and glacial acetic acid in a 3 : 1 ratio. The cell suspension was prepared by the maceration of the material in the mixture of the glacial acetic acid and 60 % lactic acid (30 : 1); then it was dropped onto dry slides warmed to 50 °C, using the capillary pipette. The dried slides were stained for 10 min in the 10 % Romanovskii solution of azure-eosin in 0.01 M phosphate buffer (pH 6.8). The slides were examined with the ULAB XY-B2 microscope (at 10×100 magnification).

Metaphase plates (2n) with the satisfactory scatter of chromosomes and the equal degree of spiralisation were selected for the subsequent photography and measurements. Based on the measurements, total complement length (TCL), relative length of chromosomes ( $Lr$  — total length of chromosomal pair/TCL100 %) and centromeric index ( $Ci$ , length of short arm/chromosome length) were calculated. The morphological type of chromosomes was determined according to the Levan's classification (Levan et al., 1964). The fundamental number (FN) was determined as the number of chromosome arms in a diploid set. The linear parameters of chromosomes were processed using methods of variation statistics (STATISTICA 6.0).

## Results and discussion

*Arion distinctus*. The diploid set (2n) of this species consists of 56 chromosomes (fig. 1, 1). In haploid set (n) 28 chromosomes are found (fig. 2, 1). The  $TCL = 105.29 \pm 15.02 \mu\text{m}$ . The chromosomes gradually decrease in size from the 1st to the 28th pair. Their relative length varies from 1.51 (the 1st pair) to 0.36 % (28th pair) (table 2).

The morphological characteristics of the karyotype are as follows: the 3rd, 5th, 6th, 7th, 9th, 10th and 23rd pairs of chromosomes are represented by submetacentrics (sm); the remaining pairs are metacentric (m) (fig. 1, 1). The chromosomal formula is  $2n = 48m + 8sm = 56$ . The fundamental number  $FN = 112$ .

*A. lusitanicus* s. l. The diploid set (2n) of *A. lusitanicus* s. l. consists of 52 chromosomes (fig. 1, 2). The  $TCL = 79.01 \pm 2.58 \mu\text{m}$ . The chromosomes gradually decrease in size from the 1st to the 26th pair. Their relative length varies from 8.56 (the 1st pair) to 1.28 % (26th pair).

The morphological characteristics of the karyotype are as follows: the 2nd pair of chromosomes is represented by subtelocentrics (st); the 6th, 7th, and 8th pairs of

**Table 1.** Material used for karyological studies

specimens	Studied*		Geographic coordinates (DD)		Locality	Species
	plates (n)	(2n)	latitude	longitude		
10	9	5	48.6208	22.2727	Uzhgorod	<i>A. distinctus</i>
20	-	28	49.8248	24.0305	Lviv	<i>A. lusitanicus</i> s. l.
30	18	27	50.1527	27.9299	Romaniv (Zhytomyr Reg.)	<i>A. fuscus</i>
25	15	4	49.5484	27.9501	Khmilnyk (Vinnitsa Reg.)	<i>A. fasciatus</i>

\* No metaphase plates were obtained from some specimens.

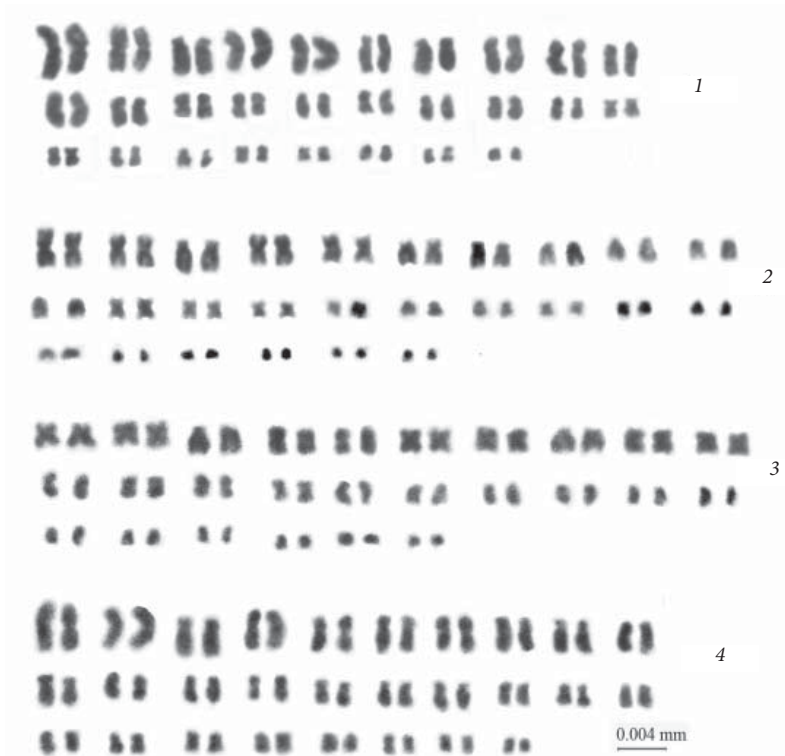


Fig. 1. Chromosomes of *Arion* spp. in the mitotic metaphase (2n): 1 — *A. distinctus*; 2 — *A. lusitanicus* s. l.; 3 — *A. fuscus*, 4 — *A. fasciatus*.



Fig. 2. Chromosomes of *Arion* spp. in the diakinesis (meiosis) (n): 1 — *A. distinctus*; 2 — *A. fasciatus*; 3 — *A. fuscus*.

chromosomes are represented by submetacentrics (sm); the remaining pairs are metacentric (m) (fig. 1, 2). The chromosomal formula is  $2n = 44m + 6sm + 2st = 52$ . The fundamental number is  $FN = 104$  (Garbar, Kadlubovska, 2014).

*Arion fuscus*. The metaphase plate of *A. fuscus* from Ukrainian populations contains 52 chromosomes (fig. 1, 3). In haploid set (n) 26 chromosomes are found (fig. 2, 3). The fundamental number  $FN = 104$ . The chromosome number ( $n = 26$ ,  $2n = 52$ ) is identical with that of *A. lusitanicus* s. l., *A. ater* (Linnaeus, 1758) and *A. rufus* (Linnaeus, 1758) which may indicate their close relationship. These results contradict G. Beeson's data (Beeson, 1960) who considered this species haploid number to be  $n = 50$ .

*Arion fasciatus*. According to the karyogram containing 29 paired elements ( $2n = 58$ ) all members of the species *A. fasciatus* were diploid (fig. 1, 4). In the full compliance of the diploid number on the stage of meiosis diakinesis was observed with 29 bivalent (fig. 2, 2). The fundamental number  $FN = 116$ . The karyotype of *A. fasciatus* in the haploid chromosome number turned out to be identical to another close species — *A. circumscriptus* from Great Britain (Beeson, 1960).

**Table 2.** Measurements (Lr — relative length, Ci — centromeric index, SE — standard error) and classification of chromosomes (m — metacentric, sm — submetacentric chromosome) of *A. distinctus*

Pair no.	Ci,%		Lr,%		Type
	Mean	SE	Mean	SE	
1	49.38	0.51	1.51	0.32	m
2	48.60	1.62	1.39	0.24	m
3	29.23	3.01	1.11	0.23	sm
4	47.96	0.66	1.09	0.23	m
5	30.08	4.90	0.99	0.16	sm
6	30.57	5.23	0.95	0.18	sm
7	29.32	3.88	0.90	0.17	sm
8	48.41	0.98	0.87	0.16	m
9	37.33	2.26	0.83	0.15	sm
10	31.92	1.58	0.79	0.12	sm
11	47.51	0.87	0.75	0.12	m
12	38.40	1.84	0.72	0.11	m
13	47.68	0.64	0.69	0.11	m
14	47.02	1.89	0.64	0.08	m
15	38.25	3.75	0.62	0.09	m
16	46.99	1.48	0.59	0.10	m
17	37.53	3.53	0.57	0.09	m
18	42.66	1.56	0.55	0.09	m
19	39.92	0.78	0.54	0.09	m
20	47.21	1.80	0.52	0.09	m
21	45.22	2.43	0.51	0.09	m
22	49.06	0.46	0.49	0.08	m
23	33.75	2.21	0.47	0.07	sm
24	47.99	1.96	0.46	0.07	m
25	48.23	1.56	0.04	0.01	m
26	48.10	1.46	0.43	0.07	m
27	48.64	1.80	0.37	0.04	m
28	48.49	1.14	0.36	0.03	m

In recent years, cytogenetic methods play an important role in solving problems of taxonomy, clarifying the taxonomic status of organisms and phylogenetic relationships between certain groups of animals. Karyotype can be considered as one of the most stable of regular features, even taking into account cases of chromosomal polymorphism. That is why for the past few decades the systematics of many groups of animals is not possible without the use of karyological methods.

The obtained results indicate that the baseline chromosomes number of *Arion* genus is heterogeneous. The most common basic number is  $x = 26$  (*A. fuscus*, *A. lusitanicus* s. l., *A. ater* and *A. rufus*) (table 3). The haploid set of *A. fasciatus* and *A. circumscriptus* contains 29 chromosomes ( $n = 29$ ). *A. distinctus* from Ukraine has a basic chromosome number  $x = 28$ , which corresponds to the chromosome number of *A. hortensis* Férussac, 1819 and *A. intermedius* Normand, 1852 (Beeson, 1960) (table 3).

According to some authors (Hagnell et al., 2003) the hybridization between *A. subfuscus* s. l., *A. ater* and *A. rufus* is possible. However, a serious obstacle for the hybridization of these species is connected with different numbers of chromosomes. Our data, however, indicate the nature of chromosomes numbers of these species. Therefore, hybridization of them is quite likely.

**Table 3. Chromosome number and karyotype structure of slugs of the genus *Arion***

Species	Region	n	2n	Reference
<i>A. ater</i>	Britain	26	–	Beeson, 1960
<i>A. subfuscus</i> s.l.	Britain	25	–	Beeson, 1960
<i>A. fuscus</i>	Ukraine	26	52	Present study
<i>A. rufus</i>	France	26	–	Beeson, 1960
<i>A. lusitanicus</i> s. l.	Ukraine	26	52	Garbar, Kadlubovska, 2014
<i>A. distinctus</i>	Ukraine	28	56	Present study
<i>A. hortensis</i>	Britain	28	–	Beeson, 1960
<i>A. intermedius</i>	Britain	28	–	Beeson, 1960
<i>A. circumscriptus</i>	Britain	29	–	Beeson, 1960
<i>A. fasciatus</i>	Ukraine	29	58	Present study

The previous chromosome studies of slugs (Beeson, 1960) and euthyneuran gastropods in general (Burch, 1965) suggested that karyological data might be useful to distinguish taxa of (sub)generic level in limacid and arionid slugs. So the correspondence between the haploid number of chromosomes and subgenus groups in the genus *Arion* is established:  $n = 25$  in *Mesarion* Hesse, 1926,  $n = 26$  in *Arion* s. str.,  $n = 28$  in *Kobeltia* Seibert, 1873 and *Microarion* Hesse, 1926 and  $n = 29$  in *Carinarion* Hesse, 1926 (Beeson 1960). These data were the basis for inclusion *Kobeltia* in the subgenus *Microarion* (Bacheljau, De Bruyn, 1990). Identical diploid chromosome numbers of the subgenera *Mesarion* and *Arion* representatives ( $n = 26$ ) may be also the reason for their association.

If the correspondence between chromosome numbers and species groupings is confirmed, and if it is accepted that in euthyneuran molluscs higher chromosome numbers represent phylogenetically more advanced states (Burch, 1965; Patterson, Burch, 1978), then it follows that *Carinarion* ( $n = 29$ ) is the most recent species-group, whereas *Mesarion* ( $n = 25$ ) should be the oldest one (Bacheljau, De Winter, 1987). On the other hand, it has been suggested that in eukaryotes chromosome fusions (the reduction of the chromosome number) are more common than chromosome fissions (increasing chromosome numbers) (Dobzhansky et al., 1977). However, the interpretation of chromosome numbers as an indicator of the evolutionary relationship must be viewed with caution. According to some authors (Patterson, Burch, 1978), molluscan chromosome numbers have the tendency to increase, but others (Butot, Kiauta, 1969; Ahmed, 1976) suggest that the chromosomal evolution leads to lower numbers.

The analysis of the literature data and the results of our investigation indicate the substantial variability in chromosome numbers of slugs at different taxonomic levels. Giving the fact that the taxonomy of this group has been recently revised, karyological data may be useful to clarify the systematic position of some taxa.

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