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THE THICK-CLAWED CRAYFISH, *ASTACUS PACHYPUS* (CRUSTACEA, DECAPODA, ASTACIDAE), IN UKRAINE: KARYOTYPE, ALLOZYMES AND MORPHOLOGICAL PARAMETERS

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The Thick-clawed Crayfish, *Astacus pachypus* (Crustacea, Decapoda, Astacidae), in Ukraine: Karyotype, Allozymes and Morphological Parameters. Mezhzherin, S. V., Kostyuk, V. S., Garbar, A. V., Zhalai, E. I., Kutishchev, P. S. — Allozymic, karyological and morphological analyzes confirmed the presence of the Thick-clawed crayfish, *Astacus pachypus* (Rathke, 1837), in Ukraine. This rare species in Ukraine is found only in two small adjacent localities restricted to the Lower Dnieper. It is characterized by a unique set of chromosomes (2n = 116) and a distinct pool of allozymes, allowing, together with morphological features, to clearly distinguish it from the other three species of the Ukrainian fauna.

K e y w o r d s: crayfishes, *Astacus*, allozymes, karyotypes, morphometry.

Толстопалый рак, *Astacus pachypus* (Crustacea, Decapoda, Astacidae), в Украине: кариотип, аллозимы и морфологические параметры. Межжерин С. В., Костюк В. С., Гарбар А. В., Жалай Е. И., Кутищев П. С. — Аллозимный, кариологический и морфологический анализы подтверждают присутствие толстопалого рака, *Astacus pachypus* (Rathke, 1837), в Украине. Этот редкий вид найден только в двух небольших соседних локалитетах на Нижнем Днепре. Он характеризуется уникальным набором хромосм (2n = 116) и отличающимся пулом аллозимов, что вместе с морфологическими особенностями позволяет чётко отличить этот вид от трёх остальных видов раков фауны Украины.

К л ю ч е в ы е с л о в а: речные раки, *Astacus*, аллозимы, кариотип, морфометрия.

Introduction

Issues of species composition and systematics of European crayfishes up to now remains questionable. According to various sources (Brodskiy, 1981; Brodskiy, 1983; Starobogatov, 1995; Holdich, 2002) the number of species within the genus *Astacus* Fabricius, 1775 in Central Europe varies from 3 to 7. The main reason of ambiguities is the lack of genetic studies of putative species. The number of chromosomes is controversial and for some species remains vague, there is no information on the genetic structure of populations and on the possibility of hybridization of species in the wild, as well there is no data on the genetic differentiation of geographic forms of the group of the long-clawed crayfishes.

Obviously, the most eastern representative of the genus, the thick-clawed crayfish *Astacus pachypus* (Rathke, 1837), turned out to be the least studied species. It is traditionally considered that this species mainly inhabits shallow waters of the South and Middle Caspian Sea (Birshtein, Vinogradov, 1934). It is represented to much lesser extent in brackish waters of the Azov Sea and North-Western part of the Black Sea. There is a view that its current natural home range may even include Continental and Eastern regions of Ukraine (Holdich, 2002). However, drastic changes of the Azov and Black Sea environment caused by the damming of the largest rivers (Dnieper, Don, Kuban, etc.) have led to the increased salinity of previously freshwater areas, causing a sharp decline in the numbers of crayfishes (Mezhzherin, 2008). Therefore, of current interest would be to confirm the presence of species in the Azov Sea and Black Sea which even before has been rather rare. Information about the number of chromosomes and the allozyme pool of *A. pachypus* is of interest too. This would not only unambiguously prove a status of the species, but also would help to clear the pattern of kinship within the subfamily Astacinae. There is also a need to establish a set of morphometric parameters that would readily allow identifying the thick-clawed crayfish in relationship to other species of the genus *Astacus.*

Material and methods

Samples of crayfish were collected from various places in Ukraine (see map, fig.1) during the years 2010–2013. The presence of four species of this genus has previously been established on the basis of morphometry, allozyme data (Mezhzherin et al., 2012) and karyotypes (Kostyuk et al., 2013). In total 10 specimens of *Astacus pachypus* (Rathke, 1837), 42 of *A. astacus*, 267 of *A. angulosus* and 227 of *A. leptodactylus* were studied. The set of morphometric traits were measured (according to Sint et al., 2007) using all specimens: TL — total body length, CLL — the length of claw, CFL — the length of the movable part of the claw, CPL — the length of the fixed part of the claw, CLH — the depth of the claw, CLW — the width of the claw, ROL — the length of the rostrum, ROW — the width of the rostrum, HEL — the length of the principal part of the carapace, ARL — the length of the thoracic part of the carapace, CPH — the height of the carapace, CPW — the width of the rear edges of the carapace, CEW — the width of the rear edges of the carapace, HEW — width of the frontal edge of the carapace, CGW — the width of the carapace on cervical fissure, ARW — the width between heart fissures, ABL — the length of the abdomen, ABW — the width of the abdomen, ABH — the height of the abdomen, TEL — the length of the telson, TEW — the width of the telson.

Multi-locus allozymic analysis was accomplished by applying electrophoresis in a 7.5 % polyacrylamide gel using a continuous buffer system (Peacock et al., 1965).

For the karyological studies males taken within their reproduction period (April–September) were used. Preparations were made out of the gonadal tissue by using a method, which had previously been successful for studying the karyotype of other crustaceans (Silver, Tsukerzis 1964; Sharma, Sharma, 1972; Mlinarec et al., 2011; Tan, et al., 2004; Scalici et al., 2010).

A 0.05 % solution of colchicines was used as a mitotic inhibitor. The injection was performed into the abdominal cavity in the area of the base of the first pair of pleopods, 1 ml per 50 g of body weight. After 5.5–6 hours following the injection, the gonads of males were excised and cut into very small pieces of 1–2 mm and then placed for hypotension into a 0.1 M KCl solution at 25 °C for 45 min. Tissues were fixed in a solution consisting of 3 volumes of methanol and 1 volume of acetic acid for 60 min with the replacement of the fixative every 20 min. Thereafter, tissues were placed into a solution of methanol and acetic acid mixed in the ratio of 1 : 1 and held for 24 hours at 4 °C.

Chromosome preparations were made by using the squashing technique. Dried slide mounts were stained in a 4 % solution of azure-eosin according to Romanovsky using a phosphate buffer (pH = 6.8).

Statistical analysis of the data was performed by using STATISTICA 6.0 software.

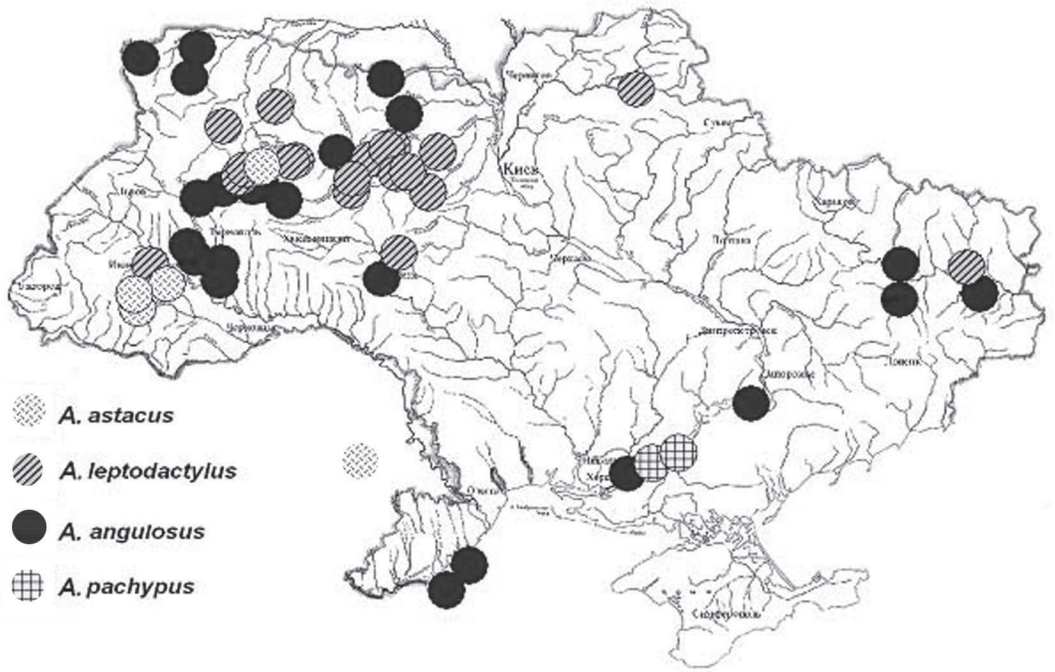

Fig. 1. Locations of samples of four crayfish species in Ukraine.

Рис. 1. Места сбора выборок речных раков в пределах Украины.

Results and discussion

Allozyme analysis. The electrophoretic analysis of a number of enzymes and structural proteins of muscles, which are presumably encoded by 17 loci, has confirmed the presence of fixed genetic differences for each of the four putative species (table 1). The fixation of common alleles in loci *Ldh*-A, *Ldh*-B of the specimens of *A. astacus* and *A. pachypus* clearly distinguishes these two types from *A. leptodactylus* and *A. angulosus*. The obtained genetic distances (table 2) basically corresponded to the values previously obtained for species of this genus (Attard, Pasteur, 1984; Fevolden et al., 1994). The smallest genetic distance has been revealed between *A. leptodactylus* and *A. angulosus* (D_{Nei} = 0.101). Such a level of genetic difference indicates that the divergence of these two species has occurred fairly recently, perhaps not later then 500 thousand years ago.

Fig. 2. Metaphase plate of thick-clawed crayfish, *A. pachypus.*

Рис. 2. Метафазная пластинка толстопалого рака, *A. pachypus.*

Karyotyping. Karyotyping of eight individuals of *A. pachypus* yielded 12 metaphase plates of satisfactory quality. The modal number of chromosomes in these plates was 116 (fig. 2), which obviously corresponds to a diploid set. Thus, the thick-clawed crayfish clearly differs from other species by features the number of chromosomes as well (fig. 3). This species

Table 1. Allozyme pools of four species of the genus *Astacus* of the Ukrainian fauna

Таблица 1. Аллозимные пулы видов рода *Astacus* фауны Украины

Locus	*Allele*	*A. astacus*	*A. pachypus*	*A. leptodactylus*	*A. angulosus*
Aat-1	90			0.04	0.45
	100	1	1	0.96	0.55
Aat-2	100			0.55	
	100	1	1	0.45	1
Ldh-A	100			1	1
	110	1	1		
Ldh-B	100			1	1
	110	1	1		
Es-1	100		1	0.41	0.99
	102	1		0.59	0.01
Es-2	null-allele		1		
	100			1	
	115	1			1
Es-3	98	1			
	100			1	1
	110		1		
Alb	100		1	1	1
	105	1			

Note. Loci: *Ldh-A, Ldh-B, Mdh*-1, *Mdh*-2, *Me*-1, *Pgdh*, *Pgm*-1, *Pt*-1, *Pt*-2 turned out to be monomorphic.

Table 2. Matrix of genetic distances by (Nei, 1975) between the four species of the genus *Astacus,* represented in the fauna of Ukraine

Таблица 2. Матрица генетических дистанций (Nei, 1975) между четырьмя видами рода *Astacus,* представляющих фауну Украины

Species	*A. astacus*	*A. pachypus*	*A. leptodactylus*
A. pachypus	0.269		
A. leptodactylus	0.399	0.327	
A. angulosus	0.348	0.270	0.101

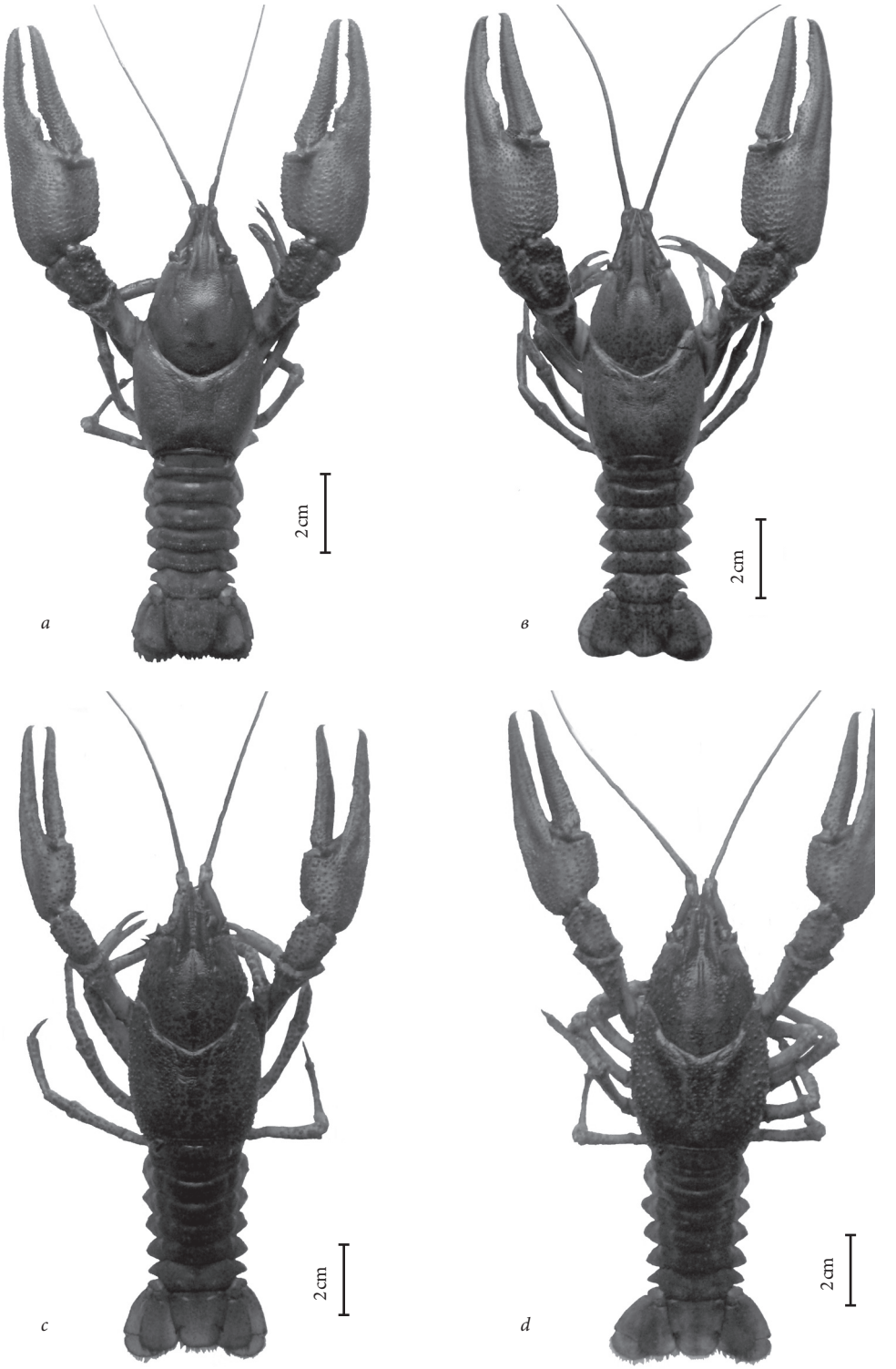

Fig. 3. General view of the male: *a* — broad-clawed crayfish (*A. astacus*); *b* — thick-clawed crayfish (*A. pachypus*); *c* — long-clawed crayfish (*A. leptodactylus*); *d* — *A. angulosus.*

Рис. 3. Общий вид самцов: *a* — широкопалого рака (*A. astacus*); *b* — толстопалого рака (*A. pachypus*); *c* — длиннопалого рака (*A. leptodactus*); *d* — угловатого рака (*A. angulosus*).

Table 3. Diploid number of chromosomes (2n) of crayfishes of the genus *Astacus* of the Ukrainian fauna

Таблица 3. Диплоидные числа хромосом (2n) речных раков рода *Astacus* фауны Украины

Species	2n	References
A. pachypus	112	Present data
A. astacus	90	(Kostyuk, Garbar, 2011)
A. leptodactylus	172	(Kostyuk et al., 2013)
A. angulosus	186	(Kostyuk et al., 2013)

appears to be closer genetically to *A. astacus*, whereas *A. angulosus* and *A. leptodactylus* have an almost doubled chromosome number (table 3). In previous time (Silver, Tsukerzis 1964) this has given the reason to consider the latter as a digenomic species.

External traits and morphometry. A comparison of the features of the external structure, first of all of claw shapes and sizes, with the classical descriptions of diagnostic features of four crayfish species (Birshtein, Vinogradov, 1934; Brodsky, 1980) (fig. 3) agrees with our conclusions.

Morphometric analysis demonstrates significant differences between these species concerning both the body size and the body proportions (table 4). The latter primarily concerns the relative length of claws, the length of head portion of the carapace, the width and height of the abdomen, the width of the rostrum.

Table 4. Loads on the three canonical variables (%) and traits (body measurements) making largest contributions to variability within the four species of the genus *Astacus*

Таблица 4. Нагрузки на три канонических переменных (%) и признаки (промеры тела), вносящие наибольший вклад в изменчивость в пределах четырёх видов рода *Astacus*

Body measurements	Axis 1 64.38 %	Axis 2 26.81 %	Axis 3 8.8 %
CLL	–0.97184	–0.86313	5.3886
CFL	–0.77828	–0.22609	3.7674
TL	–1.1436	–1.1073	5.2317
ROL	–0.77994	–0.24038	3.7285
TEL	–2.4817	0.94006	4.9535

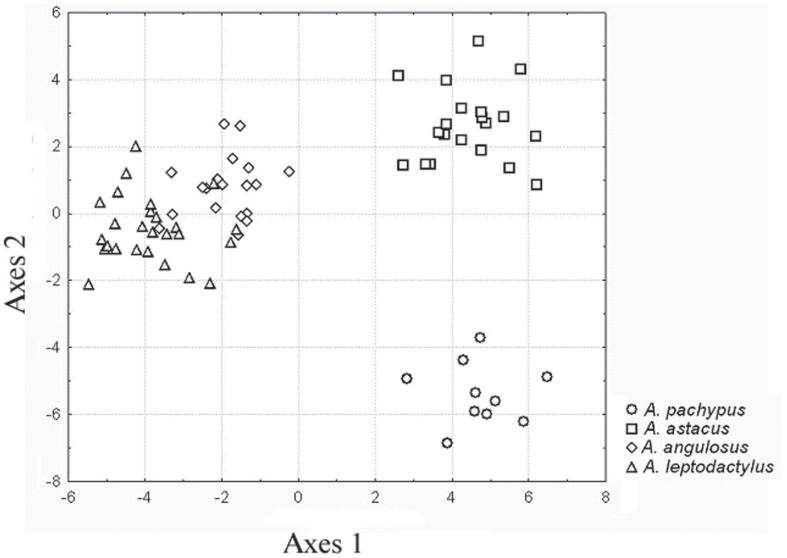

Fig. 4. The distribution of specimens of four species of the genus *Astacus* in the dimensions of the first and the second canonical variables (analysis of absolute body measurements).

Рис. 4. Распределение особей четырёх видов рода *Astacus* в пространстве первой и второй главных компонент (анализ абсолютных промеров тела).

A principal components analysis of crayfishes involving a complex of 21 morphometric characters demonstrated that the first canonical variable was responsible for the greater part of the variability (64.4 %), values for the second and third variables were significantly lesser (26.9 % and 8.8 %, respectively) (table 4). This may be quite expected as far as correlated characteristics were involved. Whatsoever, the most contributing traits are the following: the length of the claws, the length of the movable part of the claws, total body length, the length of the rostrum and the length of the telson.

By taking the first and the second canonical variables, a 2-dimensional graphical representation of the interrelationship of the species reveals the closeness of *A. leptodactylus* and *A. angulosus*, particularly in comparison to their expected distance from *A. astacus* and *A. pachypus*. In addition, the position of the two last types on the graph shows they are clearly separated from each other. A similar analysis involving the first and the third variables (fig. 5) also confirms the reality of the two morphological groups of crayfishes. In this case the kinship of *A. astacus* and *A. pachypus* is observed with greater differentiation from *A. leptodactylus* and *A. angulosus.*

Discriminant analysis confirmed in general the high degree of differentiation of the four kinds of crayfishes according to morphological traits (table 5). The reliability of discrimination was very high and reached an average of 98.7 %. Such species as the broad-clawed, thick-clawed and long-clawed crayfishes could be distinguished by 100 %, whereas *A. angulosus* by 95 %. However such a high degree of discrimination could be due to the fact that geographically distant populations of the four species were studied.

Geographical distribution and ecological preferences. Currently in Ukraine *A. astacus* is found only in water bodies of the Carpathians and in some places in the Podolsk Upland (fig. 1). Over the past 50 years, it has disappeared from the rivers in Central Ukraine. The depleting state of its populations in lowland rivers is quite expected, since areas of suitable habitat for the species are constantly decreasing.

A. angulosus is the most common and apparently most abundant species. It was discovered in all studied regions of Ukraine (fig. 1), with the exception of Transcarpathia. Ecological preferences of the species are difficult to understand. It is a ubiquitous species that occurs in lowland river systems and reservoirs of different types with varying degrees of silting and oxygen saturation.

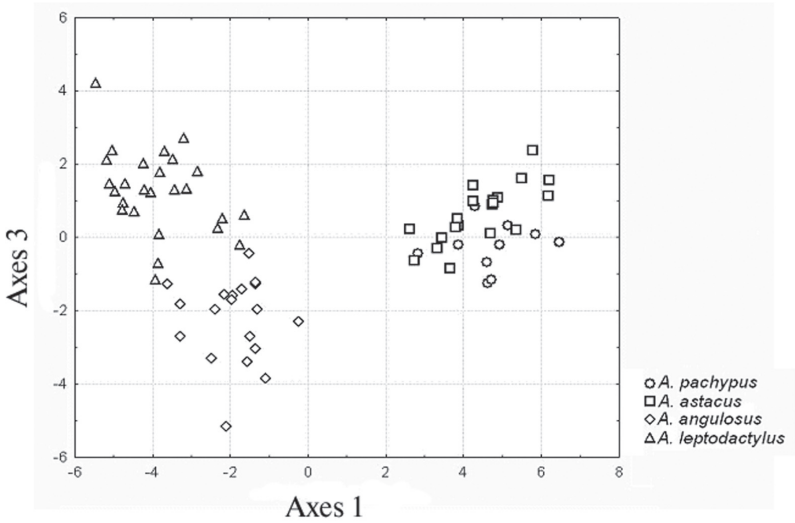

Fig. 5. Distribution of specimens of four species of the genus *Astacus* in the dimensions of the first and the third canonical variables (analysis of absolute body measurements).

Рис. 5. Распределение особей четырёх видов рода *Astacus* в пространстве первой и третьей главных компонент (анализ абсолютных промеров тела).

Table 5. Matrix of the discriminant analysis of the four species of the genus *Astacus* by employing 20 body measurements

Таблица 5. Матрица дискриминантного анализа четырёх видов рода *Astacus,* полученная на основе 20 промеров тела

Species	N	1	2	3	4	%
A. pachypus	1	10	0	0	0	100
A. astacus	2	0	20	0	0	100
A. angulosus	3	0	0	18	1	94.7
A. leptodactylus	4	0	0	0	26	100
In total		10	20	18	27	98.7

Note. *A. pachypus* — lower reaches of Dnieper (n = 10); *A. astacus* — Lake Synevyr, Transcarpathia (n = 20): *A. angulosus* — Kakhovskii Channel (n = 27) ; *A. leptodactylus* — basin of the Teterev River (n = 18).

A. leptodactylus also does not show any explicit preference for a particular type of aquatic ecosystem, as far as occurs both in different river systems and in various reservoirs with stagnant water. However, joint populations including *A. angulosus* have not been found and this is likely due to the competitive relations between these species. Long-clawed crayfish mainly occurs in the north-east of Ukraine (fig. 1).

A. pachypus was discovered only in the Lower Dnieper in two localities. The main number of individuals was found near the village Ponyatovka (46°44'51" N, 32°54'15" E), fewer specimens were also met nearby the village Novotyaginka (46°44'59" N, 32°59'45" E). Apparently this is a species of a narrow ecological niche. The most suitable habitats are those of 4–5 m depth and having a solid bottom covered with shells of dead mollusks and pebbles. Silted areas of the Lower Dnieper and Dnieper estuary are avoided. Nearby the village Ponyatovka a dense population is represented mainly by young individuals. The population here remains stable from year to year.

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