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IMPORTANCE OF GENETIC CHARACTERISTICS IN THE CONSERVATION AND MANAGEMENT OF CRAYFISH IN SERBIA AND MONTENEGRO

A. PETROVIC[1], M. RAJKOVIC[2], S. SIMIC[1], I. MAGUIRE[3] and V. SIMIC[1]
[1]*University of Kragujevac, Faculty of Sciences, Institute of Biology and Ecology, 34 000 Kragujevac, Serbia*
[2]*Institute for the Protection of Nature, 81 000 Podgorica, Montenegro*
[3]*University of Zagreb, Faculty of Science, Department of Biology, 10 000 Zagreb, Croatia*

Abstract

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Research of the population of crayfish of the family Astacidae on the territory of Montenegro and Serbia by using COI gene mDNA as a genetic marker, have shown that all populations of the species *Austropotamobius torrentium* are homogenous and belonging to the haplogroup "Southern Balkan". Population of *Austropotamobius italicus,* recorded only in Montenegro in river Zeta's upper flow, belongs to the (haplogroup), subspecies *meridionalis.* The findings of *Austropotamobius italicus meridionalis* are new and an expansion of the southern range border of this subspecies in the Balkan peninsula. For the populations of *Astacus astacus*, two haplogroups have been detected; the one from Serbia belongs to a new haplotype and is considered to be evolutionally older than the ones from Montenegro and Croatia. Using COI gene analysis, the highest value of nucleotide diversity (π) was noted for the species *Austropotamobius torrentium,* $\pi = 4.6\%$ (0.046±0.006); the lowest one was for the species *Astacus astacus,* $\pi = 3.2\%$ (0.032±0.006). The achieved results implicate urgent measures for conservation of populations *Astacus astacus* on the territory of Serbia and *Austropotamobius italicus meridionalis* on the territory of Montenegro, accompanied by the use of stricter measures in the management of populations of *Astacus astacus* in Montenegro.

Key words: Astacidae, phylogeny, COI gene, Balkan peninsula, conservation, management

Introduction

The first more detailed research of distribution, taxonomy and phylogeny of decapod crayfish in the territory of Balkan peninsula based on morphological and meristic characters has been conducted during the 1960's, by Karaman (Karaman, 1961, 1963). The research was mostly focused on the populations from Macedonia and Croatia (Dalmatia), while the areas of Serbia and Montenegro were considerably less covered by it. In these works according to the high presence of crayfish in the waters of the Balkan Peninsula and that, there was a possibility of their hunting and exploitation.

Simic et al. (2008) gave the first more detailed review of the distribution, ecology and the degree of threat of Astacidae on the territory of Serbia and Montenegro. The main result of this review was information on severe endangerment of the species *A. astacus* and a somewhat better status of populations of *A. torrentium*, compared to the areas of Middle and Eastern Europe. The researches on genetic diversity of the populations of Astacidae on the territory of Serbia and Montenegro, which include taxonomy, phylogeny and phylogeography, have not yet been conducted in this area. The aim of this work was to provide a more thorough view of taxonomy, phylogeny and phylogeography of the crabs from the family Astacidae on the territory of Serbia and Montenegro, by using COI gene from mDNA as a genetic marker. This way, the results are added to the research conducted by (Trontelj et al., 2005), which use the COI gene as a genetic marker to give insight into the phylogeny and phylogeography of the genus *Austropotamobius* on the territory of the Alps, Slovenia, Istria and Dalmatia, as well as to research from the territory of Croatia (Maguire and Gottstein-Matocec, 2004; Marn, 2009).

Other than providing an insight into the phylogeny and phylogeography of Astacidae in the central Balkan, the purpose of this work is to point out genetic characteristics of

E-mail: anapetrovic@kg.ac.rs

populations which can be of importance for the conservation and management of crayfish in this area and further.

Material and Methods

The crayfish from the family Astacidae have been researched on the territory of Serbia, Montenegro and Croatia from March 2003 to June 2010. LiNi traps, by hand using LPG lamps and hand nets, as well as a machine for electric fishing, captured the crayfish. The crayfishhunt was executed by the most suitable combination of the mentioned methods, on the river profile length of 50m, and test areas of 50 m^2 on lakes. The hunt was exectude by the principle of maximum possible total catch. The captured specimens were determined by the keys to determine Astacidae (Bott, 1950, 1972; Karaman, 1961, 1963; Froglia, 1978; Holdich, 1992, 2002; Füreder and Machino, 2002). In total, 134 localities were researched and 1156 crayfish specimens were analyzed.

The captured crayfish were measured for total body length (TL) and weight (W). The sex structure was shown as percentual share of males and females, and the age structure by sorting the units into seven age classes: 0-40, 41-60, 61-80, 81-100,101-120 and 121-140 mm. Total biomass was estimated based on the average weight of units and total number of populations. The annual rate of exploitation was estimated based on official annual reports of concessionaires for *A. astacus* in Montenegro, as well as the report of national inspection on illegal hunt for crayfish by modified traps (weirs) for *A. astacus* in Serbia and *A. pallipes* in Montenegro.

Genetic analyses

Genetic analyses used parapodia (i.e. one extremity at a time), which were kept in 96% ethanol at 20^0C until DNA isolation. After weighing and taking one parapodium (which was to regenerate as time went by), crayfish were taken back to the water.

DNA extraction, amplification and sequencing

Genomic DNA was used as isolated from 0.5 g of mature specimens of the species *Austropotamobius torrentium, Austropotamobius pallipes and Astacus astacus.* DNA was isolated by using the reagents from ″DNeasy Tissue Kit″. The resulting final elution of the isolated DNA was done my means of 100 μL and 200 μL elution buffer, generating thereby two different DNA concentrations used during the experiment, for the preparation of DNA electrophoresis standard. After the settling of genomic DNA of the insect *Tenebrio molitor*, it was approached to the cutting of DNA into smaller fragments using DNA restriction endonuclease enzyme EcoRI.

High Pure PCR Product Purification Kit or QIAquick® PCR Purification Kit purified DNA molecule fragments. The purified DNA fragments from the column were eluted finally by adding 25 μL elution buffer.

After the polymerase chain reaction, the purified DNA molecule fragments were sent to Macrogen Inc. Company based in South Korea, for the purposes of identifying the primary structure of a DNA molecule.

The nucleotide sequences of the DNA molecule fragments were loaded from the Website of Macrogen Inc. as the FASTA, PDF and SCF format chromatogram files. For loading SCF files, the Chromas LITE 2.0 computer software was downloaded from the Web Page at www.technelysium.com.au/chromas.html - was used.

Phylogenetic analysis

The research of phylogenetic relations in freshwater crayfish of the phylum Decapoda, Fam. Astacidae in three species of *Austropotamobius pallipes*, *Austropotamobius torrentium* and *Astacus astacus* was conducted based on mDNA by 167 sequences of COI gene. From the afore mentioned number of sequences, 39 sequences of COI gene were taken from the sampled specimens (18 sequences from Serbia and Montenegro and 21 from Croatia), and the remaining 128 sequences of COI gene were taken from the internet GenBank data base by NCBI (National Center for Biotechnology Information) (Tables 1 and 2).

The results of a molecular phylogenetic analysis are dependant directly on the quality of multiple sequences alignment. The results of the determination of the primary layout of sequences of the COI gene from different populations of the species *A. pallipes, A. torrentium* and *A. astacus,* as well as the species *Orconectes limosus* which was used as the outer group, were alignment multiple times with the use of program CLUSTALX 1.83 (Thompson et al., 1997), after which, they were organized in the program BIOEDIT 7.0.5.2 (Hall, 1999) and re-alignment by CLUSTALX 1.83 (Thompson et al., 1997). Organizing them was done by „manually cutting“ the begginings and the ends of the sequences which were considerably longer than the rest of the sequences. After the alignment, they had relatively big areas insertions and deletions at the ends of sequences. That way, only the places that were phylogenetically informative were analyzed. Multiple alignment of sequences for COI gene was started with a total number of 39 sequences for the COI gene with the length of 397 pb. Nucleotid staructure was calculated with the program MEGA 4.0.1. (Kumar et al., 2007).

Phylogenetic analysis was done by *Bayesian* method (BA method). This is a relatively new method in phylogenetic analysis, which is used in most of the latest phylogentic re-

search (Mallatt et al., 2004; Utevsky and Trontelj, 2004; Waters and Roy, 2004; Wahlberg et al., 2005; Verovnik et al., 2005). Bayesian analysis (Rannala and Yang, 1996; Mau and Newton, 1997; Mau et al., 1999) is based on the knowledge of probabilities which were evaluated by a certain model, the so called posterior probabilities.

Table 1
Simples of the population crayfish used for the analysis of COI gene (Serbia, Croatia, Montenegro)

No.	Haplotype	Locality[1]
1	Aa413Buser1	Lake Buser, SRB
2	Aa414Buser2	Lake Buser, SRB
3	Aa412Liverovici1	Liverovici, MNE
4	Aa419Liverovici2	Liverovici, MNE
5	Aa422Liverovici3JarugaMrez	Liverovici, MNE
6	Aa427Liverovici4	Liverovici, MNE
7	Aa422Liverovici3JarugaMrez	River Mreznica, Dobrenici, CRO
8	Aa443Krapina	River Krapina, CRO
9	AtToplodol	River Toplodolska, SRB
10	AtZlatibor	Zlatibor, SRB
11	AtZlatibor	Zlatibor, SRB
12	AtCrnojevica	River Crnojevica, MNE
13	AtCrnojevica	River Crnojevica, MNE
14	AtCrnojevica	River Crnojevica, MNE
15	AtCrnojevica	River Crnojevica, MNE
16	AtZlatibor	Zlatibor, SRB
17	AtZlatibor	Zlatibor, SRB
18	AtGrosnicka	River Grosnicka, SRB
19	AtCrnojevica	River Crnojevica, MNE
20	AtDunIvanKralj	Ivanecka Zeljeznica, Ivanec, CRO
21	AtDunIvanKralj	River Lonja, CRO
22	AtDunIvanKralj	Stream Kraljevec, Zagreb, CRO
23	AtDunPoz1	Stream Vrhovci, Pozega, CRO
24	AtDunDolje	Stream Dolje, Zagreb, CRO
25	AtDunDolje	Stream Dolje, Zagreb, CRO
26	AtDunLogGailRak	Stream Dubravica, Zagreb, CRO
27	AtDunGrac	Stream Gracani, Zagreb, CRO
28	AtDunPoz1	Stream Vrhovci, Pozega CRO
29	AtBreisgau	Breisgau, A
30	AtDunPoz2	Stream Bukovica, Pozega, CRO
31	AtDunPoz1	Stream Vucjak, Pozega, CRO
32	AtDunPoz1	Stream Vucjak, Pozega, CRO
33	AtPlitvice	Stream Plitvice CRO
34	Ap_317_Ap42_Mirna	River Mirna, CRO
35	Ap_Rasa	River Rasa, CRO
36	Ap_DalmatiaZeta	Prolosko Blato, Prolozac, CRO
37	Ap_DalmatiaZeta	River Zeta, CRO
38	Ap333_ApCG7_Zeta1	River Zeta MNE
39	Ap_DalmatiaZeta	River Zeta CRO

[1]SRB- Serbia, CRO – Croatia, MNE – Montenegro

Table 2
Gene sequences for the COI gene taken from GenBank's

No.	Haplotype	GenBank Accession	Samples[1]
1	Aa422Liverovici3JarugaMrez	GU727619	Stream Jaruga, Stajnicko polje, CRO
2	Aa_AY667146_Trontelj	AY667146	Weißensee, Greifenburg, A
3	Aa_NorwayPoland	AF517104	Norway
4	Aa_NorwayPoland	AF517103	Poland
5	AtBatania	AY667138	Batania, Koúpa, Polikastro, GR
6	AtKefalari	AY667132	Ano Kefalari, Drama, GR
7	AtKoursovitStruma	AY667134	Koursovit, Karidohóri, Sidirókastro, GR
8	AtMaras	AY667133	Maras, Pige, Drama, GR
9	AtMilliRamna	AY667135	Milli, Angikastro, Sidirókastro, GR
10	AtMilliRamna	AY667137	Milli, Angikastro, Sidirókastro, GR
11	AtMilliRamna	AY667136	Ramna, Akritohóri, Sidirókastro, GR
12	AtRCrnojevica	AY667139	River Crnojevica, Cetinje, MNE
13	AtKoursovitStruma	AM180948	Struma tributary, Sandanska Bistrica, BG
14	AtBohinj	AY667124	Bohinj SLO
15	AtBreisgauRouder	AM180942	Rhine & Danube systems, DE and CH
16	AtBreisgauRouder	AM180943	Algäu, Haldensee, A
17	AtBreisgauRouder	AY667141	Schlierbach, Bliesbruck, Sarreguemines, F
18	AtBreisgauRouder	AY667141	Gailbach, Obergailb., Sarreguemines, F
19	AtBreisgauRouder	AY667141	Freiburg im Breisgau, DE
20	AtDunLogGailRak	AY667127	Rakitna, Ljubljana, SLO
21	AtDunLogGailRak	AY667127	River Iska, Ljubljana, SLO
22	AtDunLogGailRak	AY667127	Hotenjka Creek, Logatec, SLO
23	AtDunLogGailRak	AY667127	Stream Jazbinski, Zerjav, SLO
24	AtDunLogGailRak	AY667127	Piano di Fusine, Tarvisio, I
25	AtCerkno	AY667122	River Cerknica, Cerkno, SLO
26	AtDovje	AY667142	Dovje, Jesenice, SLO
27	AtDunLogGailRak	AY667130	Tributary of the Slizza, Tarvisio, I
28	AtDunLogGailRak	AY667130	Schinzengraben, Pressegger See, A
29	AtDunLogGailRak	AY667130	Zainer Bach, Arnoldstein, A
30	AtGLazi	AY667144	Gorenji Lazi, Ribnica, SLO
31	AtGlinscica	AY667128	Glinscica Creek, Ljubljana, SLO
32	AtGrapca	AY667121	Baskagrapa, Tolmin, SLO
33	AtDunLogGailRak	AY667126	Rakitna, Ljubljana, SLO
34	AtBreisgauRouder	AM180945	Algäu, Auerberg, Bavaria, DE
35	AtBreisgauRouder	AM180944	Algäu, Dachssee, Bavaria, DE
36	AtBreisgauRouder	AY667143	Rouderbaach, Grevenmacher, LU
37	AtVelika	AY667131	Velika, Demirköy, Kirklareli, TR
38	AtWienerwald	AM180946	Wienerwald, eastern A
39	AtZala	AY667123	Zala Creek, Godovic, Idrija, SLO
40	AtZaplana	AY667129	Zaplana, Logatec, SLO
41	AtGrivackiP	AY667145	Stream Grivacki, Grivac, Kocevje, SLO
42	AtGKKupa	AY667140	Kolpa River Dolenja Zaga, Kocevje, SLO
43	AtOsilnica	AY667125	Belica Creek, Kocevje, SLO
44	Ap_France	AF526891	Castelbianco, Imperia, I

Table 2 (continued)

No.	Haplotype	GenBank Accession	Samples[1]
45	Ap_France	AF526891	Le Vigan, Lodève, F
46	Ap_France	AF526891	Miagliano, Biella, I
47	Ap_France	AF526891	Norfolk District, GB
48	Ap_REC4_RjecinaKozinaIstria	AY121110	Rijecina, Rijeka, CRO
49	Ap_CST14_Caserta2	AY121111	Las Illas, Perpignan, F
50	Ap_CST14_Caserta2	AY121111	Grognardo, Acqui Terme, I
51	Ap_CST14_Caserta2	AY121111	Valle Castellana, Norcia, I
52	Ap_CST14_Caserta2	AY121111	Torricella in Sabina, Rieti, I
53	Ap_BAT2_Potenza	AY121112	Castelluccio, Potenza, I
54	Ap_GUB4_Perrugia	AY121113	Gubbio, Perugia, I
55	Ap_CUG2_ITCH	AY121114	Claro, Bellinzona, CH
56	Ap_CUG2_ITCH	AY121114	Cugnasco, Lugano, CH
57	Ap_CUG2_ITCH	AY121114	Meride, Lugano, CH
58	Ap_CUG2_ITCH	AY121114	Montecrestese, Domodossola, I
59	Ap_CUG2_ITCH	AY121114	Casalzuigno, Varese, I
60	Ap_CUG2_ITCH	AY121114	Sagliano, Voghera, I
61	Ap_CUG2_ITCH	AY121114	Ottone, Piacenza, I
62	Ap_Iberic	AY121115	Redipollos, Riano, León, EST
63	Ap_Iberic	AY121115	Orozco, Tolosa, EST
64	Ap_Iberic	AY121115	Roncesvalles, Pamplona, EST
65	Ap_Iberic	AY121115	Beceite, Valderrobres, EST
66	Ap_Iberic	AY121115	Arroyo, Granada, EST
67	Ap_Iberic	AY121115	Nirano, Sassuolo, I
68	Ap_Iberic	AY121115	Papiano, Sita, Arezzo, I
69	Ap_BRA5_Verbania	AY121116	Bracchio, Verbania, I
70	Ap_AlpsCres	AY121117	Gitschtal, Carinthia, A
71	Ap_Soca	AY121118	Idrija Creek, Kobarid, SLO
72	Ap_Soca	AY121118	Breginj, Kobarid, SLO
73	Ap_Soca	AY121118	Cósizza, Clódig, Cividade del Friuli, I
74	Ap_MIR9_Buzet	AY121119	Buzet, CRO
75	Ap_DalmatiaZeta	AY121120	Vrba Creek, Donje Postinje, Drnis, CRO
76	Ap_REC4_RjecinaKozinaIstria	AY121121	Botazzo, San Dorligo della Valle, Trieste, I
77	Ap_REC4_RjecinaKozinaIstria	AY121121	Odolina, Kozina, SLO
78	Ap_REC4_RjecinaKozinaIstria	AY121121	Rjecina, Rijeka, CRO
79	Ap_VIM2_Bergamo	AY121122	Santuario, Imagna, Bergamo, I
80	Ap_VED15_Tarcento	AY121123	Vedronza, Tarcento, I
81	Ap_VED15_Tarcento	AY121123	Stella, Sterpo, Codroipo, I
82	Ap_GIT3	AY121124	Leale Avasinis, Gemona del Friuli, I
83	Ap_STE4_Codroipo	AY121125	Stella, Sterpo, Codroipo, I
84	Ap_VIP7_Stanjel	AY121126	Stanjel, Vipava, SLO
85	Ap_BEL2	AY121127	n/a
86	Ap_DalmatiaZeta	AY667106	Lake Modro Oko, Ploce, CRO
87	Ap_DalmatiaZeta	AY667106	Konavoski Dvori, Dubrovnik, CRO
88	Ap_Dragonja	AY667107	Dragonja River, Piran, SLO
89	Ap_Soca	AY667108	Mlake, Vipava, SLO

Table 2 (continued)

No.	Haplotype	GenBank Accession	Samples[1]
90	Ap_Bracana	AY667109	Stream Bracana, Buzet, CRO
91	Ap_REC4_RjecinaKozinaIstria	AY667110	Glinscica Creek, Kozina, SLO
92	Ap_AlpsCres	AY667111	Lake Vransko, Island Cres, CRO
93	Ap_AlpsCres	AY667112	Borenitze Bach, Weißbriach, Hermagor, A
94	Ap_AlpsCres	AY667112	Podovia Bach, SanLorenzen, Hermagor, A
95	Ap_AlpsCres	AY667112	Waldbach, Jaders.-Grünburg, Hermagor, A
96	Ap_AlpsCres	AY667112	Krebsbach, Sanke Daniel, Hermagor, A
97	Ap_AlpsCres	AY667112	Reisach, Hermagor, A
98	Ap_AlpsCres	AY667112	Kriebenbachl, Treßdorf, Hermagor, A
99	Ap_AlpsCres	AY667112	Staudlerbachl, Greifenburg, A
100	Ap_Moosbachl	AY667113	Moosbachl, Sankt Georgen, Bruneck, I
101	Ap_Iberic	AY667114	Tortulhas, Miranda do Douro, PT
102	Ap_Iberic	AY667114	Sant-Genís, Espolla, Figueres, EST
103	Ap_Iberic	AY667114	Arroyo, Guadix, Granada, EST
104	Ap_France	AY667115	Longeau River, Fresnes-en-Woëvres, F
105	Ap_France	AY667115	Opfingen, Freiburg im Breisgau, DE
106	Ap_France	AY667115	Moulin des Adrets Creek, Brignoud, F
107	Ap_DalmatiaZeta	AY667116	Lake Modro Oko, Ploče, CRO
108	Ap_Vipava	AY667117	Mlake, Vipava, SLO
109	Ap_AlpsCres	AY667118	Wiesenbach. Jadersdorf-Grünburg, He., A
110	Ap_Sopotnica	AY667119	Sopotnica Creek, Tolmin, SLO
111	Ap_Osapska	AY667120	Osapska reka, Koper, SLO
112	Ap_Caserta1	AB443447	Regio di Caserta, I
113	Ap_CST14_Caserta2	AB443450	Regio di Caserta, I
114	Ap_CST14_Caserta2	AB443449	Regio di Caserta, I
115	Ap_CST14_Caserta2	AB443446	Regio di Caserta, I
116	Ap_CST14_Caserta2	AB443451	Regio di Caserta, I
117	Ap_CST14_Caserta2	AB443445	Regio di Caserta, I
118	Ap_CST14_Caserta2	AB443448	Regio di Caserta, I
119	Ap_Iberic	EF485041	Navarra, ESP
120	Ap_Iberic	FJ897840	Ebro, Villacantid, ESP
121	Ap_Iberic	FJ897841	Turia, Valencia, ESP
122	Ap_Iberic	FJ897842	Asturias, ESP
123	Ap_Iberic	FJ897843	Burgos, ESP
124	Ap_Iberic	FJ897844	Llobregat, Barcelona, ESP
125	Ap_Iberic	FJ897845	Castellon, ESP
126	Pleptodactylus	AF525228	
127	Cshufeldtii	EU921149	
128	Cdauricus	AY667147	Levaya Creek, Korsakovo, Khabarovsk, RU

[1] I- Italy, SLO- Slovenia, A- Austria, F- France, CRO- Croatia, GR- Grecee, EST- Estonia, ESP- Spain, PT- Portugal, RU- Russia

Bayesian analysis was performed by a program MR. BAYES 3.1.1 (Ronquist and Huelsenbeck, 2003), using the method „*Metropolis-coupled Markov Chain Monte Carlo*“, which is based on a series of independent research for a group of best trunks with ocassional changes of information between researches (Mau et al., 1999).

Bayesian analysis was pefrormed on 5 000 000 generations, trunks were collected on each 1000 generation, which

means that, in total, 5000 trunks were collected. 500 trunks were disposed as the burnin, because there was no convergence in the analysis. In the end, out of the analyzed trunk sequences a concencus trunk of the 4500 best trunks was constructed. Model used for analysis was HKY+G+ I obtained with the use of program Modeltest, with the appointed criterion Corected Akaike Information Criterion (AICc). For the non-coding sequence of COI gene command sets were used according to (Franjević, 2006).

Genetic distance (p) and the average nucleotide diversity (π) in this research was calculated by using the program MEGA 3.1. (Kumar et al., 2007) but, since that program doesn't have HKY model with gamma distribution, the Tamura-Nei model with gamma distribution was used for calculation, being most similar to model HKY (Nei and Kumar, 2000).

Results

Phylogenetic analysis

The result of *Bayesian* analisys is the rooted phylograms of sequence relations for COI genes (Figure 1) with the values of posteriror probabilities in branching nodes, which present and alternative to self-reading values.

By a detailed analysis of the obtained phylograms, it has been determined that the haplotypes of the analyzed gene sequences can be grouped at the COI gene marker into 10 smaller or three larger phylogeographic groups, the so-called haplogroups. Within each haplogroup, there are several haplotypes (Table 3). Each haplotype has a characteristic and unique nucleotide arrangement. Between the sequences within the same haplotype there is no pairwise distance.

In the case of COI gene, BA phylogram has shown the separation of haplotypes for the species *A. astacus* into two haplogroups, called «Buser» and «Evropa», for the species *A. torrentium* four haplogroups, called «Dunav», «juzni Balkan», «Kupa» and «Plitvice». For the complex *A. pallipes / italicus* five haplogroups, or subspecies: *A. i. meridionalis, A .i. carinthiacus* + *italicus*, *A. i. carsicus*, *A. i.* Osapska and *A. pallipes* – France (Figure 1).

Analysis of genetic distance (d) mostly indicates corelation with the geographical remoteness of the separated haplogroups, and it is the biggest between haplogroups that belong to different species (Table 4).

By analysis of nucleotide diversity (π) within the separated haplogroups by COI gene, the highest value of nucleotide diversity was shown by haplogroup *A. torrentium – Juzni Balkan* 4.4% (0.044±0.007), while the lowest was shown by

Table 3
Haplogroups and haplotypes of the processed sequences of the COI gene (*Astacus astacus*, *Austropotamobius torrentium* and *A. pallipes*)

Gen. marc.	Haplogroup		Haplotype
COI	*Astacus astacus*	Europa	Liverovici1, Liverovici2, Liverovici 3, JarugaMrez, Liverovici 4, Krapina, Norway Poland, Trontelj, Krapina
		Lake Buser	Buser1, Buser2
	Austropotamobius torrentium	Southern Balkan	Batania, MilliRamna, KoursovitStruma, Grosnicka, Zlatibor, R. Crnojevica, Crnojevica, Kefaleri, Maras,Toplodolska
		Plitvice	Plitvice
		Kupa	Kupa, Osilnica, GrivackiP
		Danube	DunIvanKralj, DunPoz1, DunDolje, DunLogGailRak, DunGrec, Glinscica, GLazi, Cerkno, Zaplana, Bohinj, Dovje, Grepca, Zala, BreisgauRouder, Wienerwald, Velika
	Austropotamobius pallipes	*A. italicus meridionalis*	Caserta1, Potenza, Perrugia, Caserta2, Mirna, Buzet, Bracana, Sopotnica, Soca, Vipara, GIT3, Codroipo, AlpsCres, Tarcento, Rasa, Moosbachl, DalmacijaZeta, Zeta1, Stanjel
		A.i.casicus	Dragonja, RjecinaKozinaIstra, BEL2, Bergamo
		A.i.italicus + *A.i.carinthiacus*	CUG2, ITCH, Verbania, Iberic
		Osapska	Osapska
		A. pallipes	France

haplogroup „Buser“ of the species *A. astacus* and *A. i. – Carsicus- italicus* 0.3 % (0.003±0.002) (Table 5).

Analysis of the basic elements of population structure is presented in population *A. astacus* and *A. pallipes*, which are

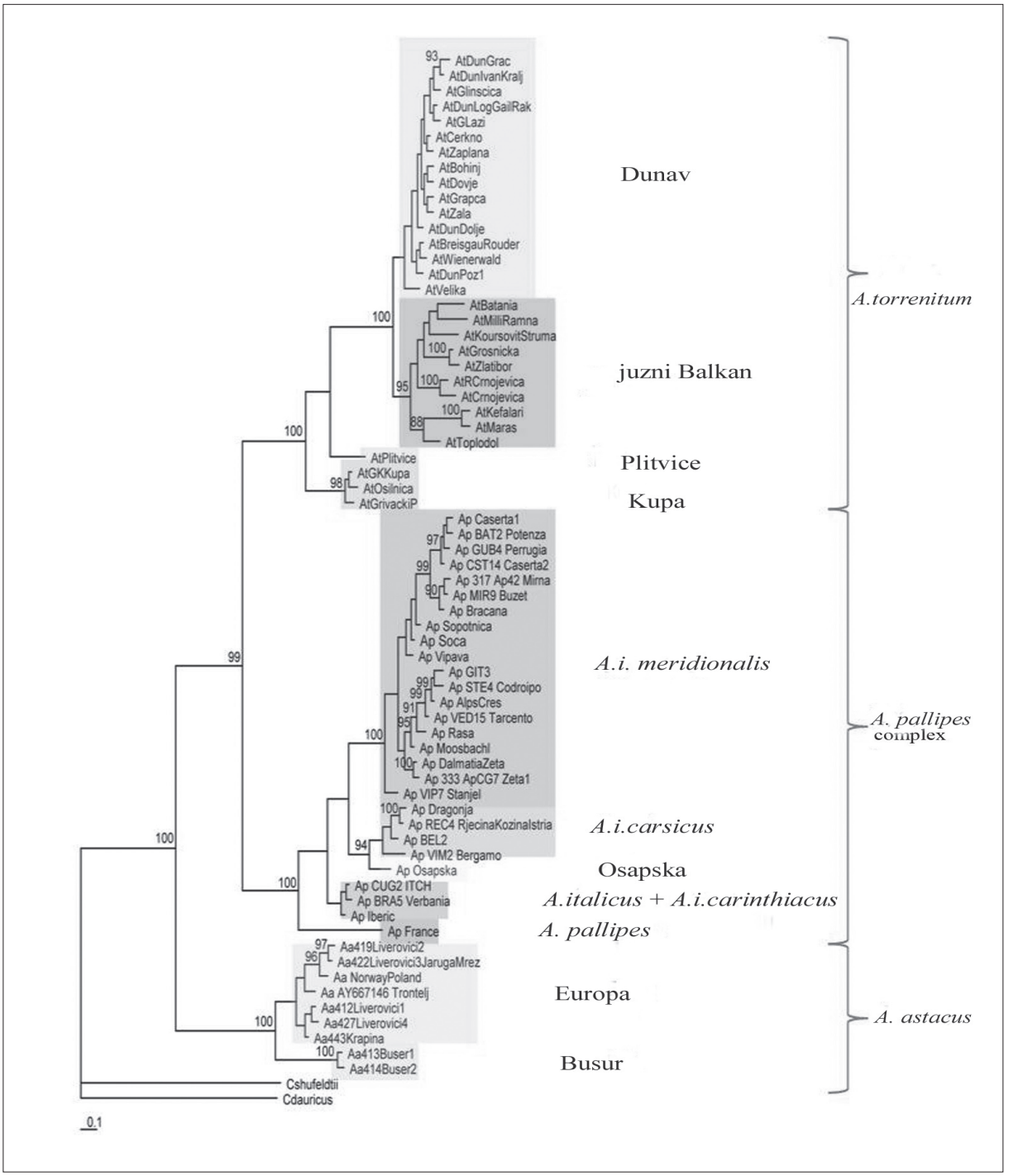

Fig. 1. Haplogroups and the belonging haplotypes of phylograms of COI gene bulit by *Bayesian* method

legally and / or illegally exploited in Serbia and Montenegro in Table 6.

Discussion

Management crayfish in Serbia and Montenegro, except through the analysis of basic indicators of population structures that are shown in Table 6 considered from the aspect of the genetic structure of populations. Based on the relationships of exploited biomass and total biomass crayfish populations of *A. astacus* and *A. pallipes,* which are legally and / or illegally exploited in this area shows a tendency to overexploitation *A.astacus* in Montenegro. The excessive exploitation of *A. astacus* in Montenegro can be observed under exploited biomaase, which is approximately 64% of the total biomass. In populations *A. astacus* from Serbia percentage of exploitation, if we observe the biomass is approximately 47% versus the total biomass (Table 6).

The degree of belonging exploitation in the lower streams of river Zeta cannot be determined with certainty from our research, but it is evident that locals are sometimes using the crayfish as a food (Table 6).

However, the genetic structure of populations exploited obtained new data, which are important for both management and conservation of crayfish in this area. The first significant result of genetic analysis is the clarification of taxonomy of crayfish especially in comes to *A. pallipes.*

In the past, several authors have tried to solve the taxonomy of crayfish from the family Astaciade based on the differences in the morphological characteristics. Within the species *A. pallipes* (Bott, 1950) differentiates three subspecies based on morphological criteria. Subspecies *A. pallipes pallipes* (Lereboullet, 1858) is, according to him, most represented in the area that borders the Pyrenees on one, the Alps on the the other and England and Ireland on the third side. The subspecies *A. pallipes lusitanicus* is limited to Iberian Peninsula, while the subspecies *A. pallipes italicus* inhabits areas in Italy, Slovenia, Austria and Switzerland.

Opposed to this, Karaman (1961) differentiates two species, *A. pallipes* and *A. italicus.* Furthermore, he divides the

Table 4
Genetic distance (d) between isolated haplogroup (subspecies) crayfish

Haplogroup	*A.torrentium* Danube	*A.torrentium* Kupa	*A.torrentium* Southern Balkan	*A.torrentium* Plitvice	*A.astacus* Buser	*A.astacus* Europa	*A.italicus meridionalis*	*A.italicus carsicus*	*A.italicus carital*	*A.italicus* Osapska	*A.pallipes*
A.torrentium Danube		0.018	0.007	0.016	0.028	0.027	0.024	0.022	0.023	0.021	0.022
A.torrentium Kupa	0.092		0.017	0.016	0.029	0.028	0.027	0.023	0.022	0.024	0.024
A.torrentium Southern Balkan	0.042	0.103		0.018	0.025	0.025	0.024	0.02	0.022	0.018	0.022
A.torrentium Plitvice	0.076	0.072	0.099		0.03	0.027	0.025	0.023	0.022	0.022	0.021
A.astacus Buser	0.171	0.183	0.165	0.181		0.012	0.03	0.024	0.028	0.025	0.029
A.astacus Europa	0.172	0.173	0.167	0.166	0.056		0.03	0.023	0.027	0.024	0.027
A.italicus meridionalis	0.151	0.158	0.161	0.146	0.198	0.191		0.013	0.013	0.012	0.018
A. italicus carsicus	0.137	0.137	0.139	0.135	0.153	0.145	0.064		0.011	0.008	0.018
A.italicus carital	0.137	0.131	0.141	0.122	0.183	0.166	0.059	0.045		0.012	0.017
A.italicus Osapska	0.124	0.14	0.117	0.123	0.152	0.143	0.057	0.026	0.044		0.016
A. pallipes	0.134	0.142	0.139	0.12	0.182	0.171	0.099	0.093	0.081	0.078	

Table 5
Nucleotide diversity (π) COI gene isolated haplogroups (subspecies) crayfish

Haplogroup	*A.torrentium* Danube	*A.torrentium* Kupa	*A. torrentium* Southern Balkan	*A.torrentium* Plitvice	*A.astacus* Buser	*A.astacus* Europa	*A.italicus meridionalis*	*A.italicus carsicus*	*A.italicus carital*	*A. italicus* Osapska	*A.pallipes*
Nucleotide diversity (π)	0.008	0.007	0.044	n/c	0.003	0.017	0.022	0.016	0.003	n/c	n/c
Standard error (SE)	0.002	0.03	0.007	n/c	0.002	0.005	0.005	0.005	0.002	n/c	n/c

species *A. italicus* into three subspecies: *A. i. italicus*, *A. i. lusitanicus* and *A. i. carsicus* whose range is in the karst area of the former Yugoslavia. Lately, following the progress of molecular phylogenetic methods (Grandjean et al., 2000), based on genetic distance (d) of the gene for rRNA of 4.6% (0.046 ± 0.009) between two big haplogroups, suggest a division of the complex of the species *A. pallipes* into two separate species: *A. pallipes* and *A. italicus.* Furthermost, within the same work, the authors define three subspecies, based on molecular phylogenetic evidence - *A. i. italicus*, *A. i. carinthiacus* and *A. i. carsicus*. Taking into account genetic distance of the same gene of 4.18% (0.042 ± 0.006) (Fratini et al., 2005), also differentiate two species: *A. pallipes* and *A. italicus* and, similar to (Grandjean et al., 2000) further divide the species *A. italicus* into the same three subspecies (*A. i. italicus*, *A. i. carinthiacus*, *A. i. carsicus*), but also add another subspecies *A. i. meridionalis*, whose range is in the complete southern part of the Apenine peninsula, where it is joined by a haplotype from Slovenia (Zaccara et al., 2005), based on the genetic distance between haplogroups of 6.51% ± 0.42% confirm the results of other research, but by using COI marker. Their result indicate the existence of two subspecies within the species *A. italicus* on the relatively unexplored area around the river Po in Italy. Trontelj et al. (2005) uses COI gene as a genetic marker and distinguishes four haplotypes within *A. torrentium* - (Southern Balkans, SE Alps + Slovenia, Uper Rhine basin, Uper Kolpa Basin) and six haplotypes within *A. pallipes*: (NW Italy, W Europe, Istra I, Istra II, Apennine, SE Alps+W Balkans).

Marker analysis of COI gene from the population of Astacidae in Serbia and Montenegro has shown distinctly diferentiated haplogroups (Table 3), of which four haplogroups create taxon *A. italicus*, while separate ones are taxons *A. pallipes, A. torrentium* and *A. astacus.*

Results obtained in this work unequivocally speak in favour of the hypothesis that there are two separate species within the complex of the species *A. pallipes.* This is acertained through comparinf the resluts of genetic distance of the marker of COI gene between *A. pallipes* haplogroup and *A. italicus* haplogroup with the afore mentioned research, which amounts to 9.9% (0.099±0.017).

In this work, based on COI gene, it has been discovered and proven that a population of *A. italicus*, belonging to the subspecies *meridionalis* lives i the lower flow of the river Zeta in Montenegro. This is a new finding of this species and it represents, so far, the southern border of it's range of distribuiton.

Throught the analysis of the research done by (Fratini et al., 2005), it is clear that the range of species called by the authors *A. i. meridionalis* matches the range of several haplotypes within the haplogroup „Balkan". The range of distribution of the haplogroup „Balkan" is limited to the area of the

Table 6
Characteristics of the population and the rate of exploitation in Serbia and Montenegro

Parameters/species	*A. astacus* Serbia	*A. astacus* Montenegro	*A. pallipes* Montenegro
N/km	102.9	11580	69.85
N total	6174	173 700	3465
	sex structure (mm) %		
Male %	56.78	52.95	50.2
Female %	43.22	47.03	48.7
	age structure (mm) %		
0-40 %	1.52	2.8	-
41-60	7.42	7.9	10
61-80	39.67	15.5	32.2
81-100	25.78	28.3	45
101-120	13.65	207	14.1
121-140	9.78	18.2	-
141-160	2.23	6.6	-
L mean	57.34	77.78	61.27
W mean (g)	19.67	37.88	23.65
Biomas/total in kg	121 400. 00	6 543 279.00	81 947.25
The total annual biomass increment in kg	approximately 700	3 192.606	?
Year old exploitation rate in kg and %	approximately 350 47%	approximately 2 066.820 64%	?

Dinarides on one side and the area of central and southern Italy on the other. *A. i. meridionalis* has a mutual haplotyoe with the dalmatian populations and the populations in central and southern Italy. This genetic similarity can be explained by land connection that existed between the Balkan and Apennine peninsula during the receding of the Adriatic sea level during the Mesinna crisis. An interesting finding of a haplotype in Slovenia which (Fratini et al., 2005) classify under the subspecies *A. i. meridionalis* speaks in favour of that theory. It remains unclear if the haplotypes found in the Apennine peninsula were incorrectly classified into the subspecies *A. i. meridionalis* , and if it would still be more natural if they were classified under the subspecies *A. i. carsicus* which was described by (Karaman, 1961) and confirmed by (Grandjean et al., 2000; Fratini et al., 2005), or all the haplotypes of the Balkan part of the group „Balkan" were incorrectly determined in the earlier researches as *A. i. carsicus* and should be called *A. i. meridionalis*, accordind to their genetoc characteristics. It is a fact that the Apennine and Balkan part of the haplogroup „Balkan" have shown a high level of phylogenetic similarity, so it is expected for them to be one subspecies. This fact is further confirmed in the work of (Trontelj et al., 2005).

Haplogroup «Zapad» presents a group of haplotypes which can be classified into a unique species *Austropotamobius pallipes*. Results in this part of the research almost entirely match the results obtained in other similar researches (Grandjean et al., 2000; Fratini et al., 2005; Zaccara et al., 2005; Trontelj et al., 2005). The limit of the range of the species *A. pallipes* and *A. italicus* in this research's case was somewhat more to the west then quoted by (Zaccara et al., 2005), which is most probably the consenquence of the fact that there was no specimen in that research which, in this case, moved the limit minimally westward. The clarification of the taxonomy, phylogeny and phylogeography complex *A. pallipes* contribute to genetic research and new findings of this species in Bosnia and Herzegovina (Trozic-Borovac, 2011).

The species *Austropotamobius torrentium* has four haplogroups. Populations from Montenegro belong to the haplogroup «juzni Balkan». This is in accordance with the research (Trontelj et al., 2005) which classsifies the populations of *A. torrentium* from the southern Adriatic and northern Aegean (territories of Montenegro, Macedonia, Greece, Bulgaria and Turkey) into a haplogroup which they called Southern Balkans and from which *torrentiums* in the entire Danube basin (most of Europe) originated. Populations of *A. torrentium* from Serbia are, based on this research, also classified into the group Southern Balkans, while the populations from Croatia belong to separate haplogroups. Most of them belong to the haplogroup „Dunav", and other populations in the areas of Plitvice and the river Kupa. Trontelj et al., (2005) consider the area of the river Kupa and Gorski kotar the center of biological diversity of this species. These researches are further confirmed by the researches by (Marn, 2009) which, based on the analysis of 16S and COI gene, ascertain the presence of four haplogroups in the small, protected area of Zumberek – Samoborsko gorje.

Analysis of COI marker (Table 3) indicates that the species *Astacus astacus* has two haplogroups. Haplogroup «Evropa» includes the populations from Montenegro and shares haplotypes with the populations of Astacus from the Danube basin (there is no diversity and disstance), whereas *A. astacus* from Serbia («Buser» lake) has a unique haplotype, so the units are probably older than the other ones. This result is a new fact in the research of phylogeny and phylogeography of the species *A. astacus.* From the aspect of crayfish conservation, the results obtained in this work are of utmost importance, primarily the ones related to defining the special haplogoup of *A. astacus* in the area of Serbia. Except this, from the conservation aspect, the fact of genetic isolation of population of *A. torrentium* from river Crnojevica and the population of *A. i. meridionalis* from the lower flow of the river Zeta in Montenegro, are also important.

The population of *A. i. meridionalis* in Montenegro is the marginal and southernmost population of this subspecies in it's area of diffusion and the entire complex of *A. pallipes.*

Conclusion

The global status of the level of endangerment of the researched crayfish, according to IUCN, *A. pallipes* EN (Endangered), *A. astacus* VU (Vulnerable) (Edsman et al., 2010; Füreder et al., 2010) is in contrast with the current trend of exploitation (both legal and illegal) of the crayfish of the species *A. astacus* and *A. italicus* on the territory of Montenegro and Serbia. The obtained results indicate that the management of the crayfish species in question based exclusively on monitoring the basic structural characteristics is not complete and has to be completed with genetic characteristics of the populations, too. This fact is confirmed in these researches, especially when it comes to *A. astacus* as a commercially most important species. Therefore, the populations of *A. astacus* in Serbia, from the aspect of genetic characteristics, such as belonging to a special haplotype and a low genetic diversity, indicate a necessary protection from any form of further exploitation, regardless of the fact it is the most numerous population of this species in the area (Simic et al., 2008). Likewise, the results indicate the prevention of any kind of exploitation of *A. i. meridionalis* on the territory of Montenegro, for it is an isolated and marginal population of this subspecies from the species complex of *A. pallipes.* The management of populations

of *A. astacus* in the territory of Montenegro should be revised in the future, by decreasing the annual quote of exploitation or a complete ban of exploitiation in a certain period of time.

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