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Genetic survey of alveolar and cystic echinococcoses in Romania: first molecular evidence of *Echinococcus multilocularis* in humans in the country

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Article info	Summary
	Cystic echinococcosis (CE) and alveolar echinococcosis (AE) are considered as one of the most important zoonotic diseases in Romania, where they are subject to mandatory reporting. To obtain more knowledge about the genetic diversity of *Echinococcus* causative agents of these diseases, 11 isolates from humans and ungulate intermediate hosts from the two regions of Romania were genotyped using mitochondrial markers. In clinical samples of five patients from north-eastern Romania (Iasi, Botosani, Vaslui counties), *Echinococcus multilocularis* was identified as causal agent by *cox1* sequence analysis. To the best of our knowledge this finding presents the first molecular evidence of *E. multilocularis* in humans from Romania. Only two cases of AE in patients were previously documented in the country by serological methods. In our four patients the most widespread European variant E5 of *E. multilocularis* was recorded, whereas in isolate from Vaslui county three nucleotide substitutions were detected as compared to the most related E5 haplotype. One of these mutations (411T/G) matched N1 and N2 haplotypes described previously from North America. In six CE samples retrieved from western Romania (Caras-Severin and Timis counties), two human isolates were diagnosed as *Echinococcus canadensis* G7, one as *E. granulosus* s.s. G1 and one as *E. granulosus* s.s. G3 using *atp6* and *rrnS* sequencing. In ungulates, the cattle isolate was allocated to *E. granulosus* s.s. G1 and pig isolate to *E. canadensis* G7. The two G7 findings in humans reinforced the recent view that G7 was underestimated as compared to the *E. granulosus* s.s. regarding human CE threat that can be further employed for identifying sources of infections and establishing suitable preventive measures. **Keywords:** *Echinococcus*; Romania; genotype; human; cattle; pig

Introduction

Cystic echinococcosis (CE) and alveolar echinococcosis (AE) caused by *E. granulosus* sensu lato and *Echinococcus multilocularis*, respectively, present substantial disease burden and public health economic problem in many parts of the world. In developed countries, human CE is more common than AE; 77.5 % of

601 confirmed cases of echinococcosis with species information available for Europe in 2015 were caused by *E. granulosus* s.l. as compared to *E. multilocularis* (EFSA, 2016). In some countries of Eastern Europe and the former Soviet Union (Bulgaria, Romania, Kazakhstan), zoonotic CE infections are emergent since the early 1990´s, with burden approximately four fold times higher than during administration of previous political system (Todorov &

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Boeva, 1999; Torgerson & MacPherson, 2011). The increased CE occurrence in these regions is mainly due to neglect or collapse of veterinary control services and changes in typology of animal husbandry drifted from intensive to familiar management (Battelli, 2009). Romania has been ranked in the forefront of the European countries in the number of human and animal cases of echinococcosis in the mid 1990´s (Neghina *et al.*, 2010). Since 2001, the prevalences of CE infection in livestock animals of Romania varied between 12.7 % and 65.6 % in sheep, 19 % and 40.1 % in cattle, 3.8 % and 6.5 % in pig, and 9.5 % and 12.1 % in horses (Iacobiciu *et al.*, 2005; Mitrea *et al.*, 2010, 2012, 2014).

Genetic variability in the *E. granulosus* s.l. species complex is well perceived as influencing infectivity in humans, intermediate host affiliations, development rate and other biological characteristics of the parasites (Alvarez Rojas *et al.*, 2014). The complex is considered to include genotypes G1-G8, G10 encompassing at least four species currently recognized (*E. granulosus* sensu stricto, *E. equinus*, *E. ortleppi*, *E. canadensis*), and *E. felidis* (Romig *et al.*, 2015). Recently, genotype G2 of *E. granulosus* s.s. was recommended to be deleted from the genotype list based on the complex mitogenome and nuclear evidence (Kinkar *et al.*, 2017).

According to the latest records, the known range of *E. multilocularis* has markedly extended across Europe and it is assumed to be currently distributed over the most of its territory. For countries of central-eastern Europe, evidence supports northward and southeastern expansions from a core endemic area in south-central Europe as documented by findings from Poland, Baltic countries, Slovakia, Hungary, Romania (summarized in Davidson *et al.*, 2012 and Oksanen *et al.*, 2016). Prevalence trends of AE in humans appear to follow the surge in parasite abundance in wildlife coinciding with dramatic increases of fox population that began since 1990´s (Mackenstedt *et al.*, 2015). In several European countries, the steady rise of human AE cases was documented over the last two decades at least in some regions (e.g., Schweiger *et al.*, 2007; Said-Ali *et al.*, 2013; Marcinkutė *et al.*, 2015). For Romania, recent data suggest spreading and emergence of *E. multilocularis* where a mean prevalence of 4.8 % was found in red foxes from Transylvanian counties in central and north-western parts of the country (Sikó *et al.*, 2011). Transmission of the parasite to humans was confirmed by two cases of AE in patients in past (Panaitescu & Pop, 1999; Savlovschi, 2000). Nevertheless, information about genetic variants of *E. multilocularis* circulating in Romania is lacking. The present study was conducted to identify *Echinococcus* spp. from western and north-eastern regions of Romania derived from human, cattle and pig intermediate hosts and to examine haplotype microvariants that circulate in respective areas.

Materials and Methods

A total of 11 *Echinococcus* isolates (nine human and two ungulate isolates) derived from five counties in north-eastern Romania (Iasi, Botosani, Vaslui) and western Romania (Caras-Severin, Timis) were included in this study (characteristics of the isolates are in Table 1). Sampling sites from which isolates were collected are shown in Fig. 1. Fertile hydatid isolates with viable protoscoleces from cattle and pig livers were recovered from abattoir and household in Caras-Severin county of western Romania. Human cysts were excised or drained during conventional surgical intervention of patients in north-eastern Romania and western Romania. Nine human isolates were obtained from livers and one from lungs in surgical departments of hospitals in Iasi (north-eastern Romania) and Timisoara (western Romania). Six patients were females and three were males. The age of the patients has ranged from 17 to 67 years. Cyst contents were examined under light microscopy for the presence of protoscoleces, rinsed in physiological saline and fixed in 70 % ethanol prior to molecular analyses.

Total genomic DNA was retrieved from protoscoleces or germinal layer using the DNeasy tissue kit (QIAGEN, Germany) according to the manufacturer's instructions. Three mitochondrial genes were targeted for PCR amplifications employed in two different

Table 1. *Echinococcus* isolates used in this study.

Isolate	Host	Geographical origin	Age in years, sex	Organ	Additional characteristics
R1	human	Iasi, Iasi county	36, F	Liver	urban, unemployed
R2	human	Iasi, Iasi county	17, F	Liver	urban, student (often visited the grandparent´s house in the countryside)
R3	human	Baltati, Iasi county	51, F	Liver	rural, farmer (dog, no sheep)
R4	human	Botosani, Botosani county	45, M	Liver	rural, farmer (sheep, goats)
R5	human	Tutova, Vaslui county	67, M	Liver	rural, farmer
R6	human	Timis county	32, F	Liver	urban, employed in meat industry
R7	human	Caras-Severin county	20, M	Lung	rural, shepherd
R8	human	Caras-Severin county	41, F	Liver	rural, unemployed
R9	human	Caras-Severin county	55, F	Liver	rural, office worker
R10	cattle	Caras-Severin county		Liver	rural, abattoir
R11	pig	Caras-Severin county		Liver	rural, household system

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Fig. 1. Geographic locations of *Echinococcus* samples from Romania used in the study.

time periods after the sample recoveries. For samples obtained from north-eastern Romania resulting as *E. multilocularis*, partial cytochrome *c* oxidase 1 gene (*cox1*, 789 bp) was analyzed using primers described by Xiao *et al.* (2003). For samples obtained from western Romania resulting as *E. granulosus* s.l., partial ATP synthase subunit 6 (*atp6*, 513 bp) and 12S rRNA (*rrnS*, 295 bp) genes were examined using primers described by Xiao *et al.* (2005) and Dinkel *et al.* (1998). The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; followed by a final extension at 72 °C for 5 min. PCR products were visualized after electrophoresis on 1.5 % (w/v) agarose gels and purified using a Nucleospin Extract II kit (Macherey Nagel, Düren, Germany). Amplicons were sequenced in both directions using a dye terminator cycle sequencing kit (DYEnamic ET terminator; Amersham Biosciences, UK) and analysed with an ABI PRISM 377 automated sequencer (Applied Biosystems, USA). Generated sequences were manually edited, aligned and compared to reference GenBank sequences by nucleotide BLASTN program (https://blast.ncbi.nlm.nih.gov). The branching pattern was generated by MEGA7 software (Kumar *et al.,* 2016) using the neighbor-joining (N-J) method, selecting the most reliable evolutionary method (TN93) according to ModelTest performed under the Bayesian Information Criterion (BIS). Bootstrap analyses were conducted by using 1000 replicates. To infer reticulate evolutionary relationships among specimens of *E. multilocularis* here analysed, median joining network (Bandelt *et al.,* 1999) and parsimony network (Clement *et al.*, 2002) were constructed using PopART software (http://popart.otago.ac.nz). EM-

BOSS Transeq tool (http://www.ebi.ac.uk/emboss/transeq) was used for translation nucleotide sequence into a protein sequence to distinguish synonymous and non-synonymous mutations. The sequences obtained in this study were deposited in GenBank under the accession numbers MF162277-MF162293.

Results

In this study DNA extracted from 11 *Echinococcus* isolates originating from 9 human hosts and 2 animal hosts in Romania was PCR amplified. Species and genotype analyses using the BLAST algorithm identified 5 isolates from humans as *E. multilocularis*, 2 isolates from humans as *E. granulosus* s.s. (G1 and G3 genotypes), 2 isolates from humans as *E. canadensis* (G7 genotype), 1 isolate from cattle as *E. granulosus* s.s. (G1 genotype) and 1 isolate from pig as *E. canadensis* (G7 genotype).

Five *Echinococcus* isolates from humans in north-eastern Romania were analyzed by *cox1* mitochondrial sequences (789 bp). Given that species analysis of nucleotide composition identified *E. multilocularis* in all examined isolates, we have compared obtained sequences with those referenced by Nakao *et al.* (2009) in a study on global species geographic pattern and available in GenBank for *cox1* from Europe (E1, E3, E5), Asia (A1, A5) and North America (N1, N2). Polymorphic nucleotide sites are shown in Table 2. Resulting *cox1* haplotypes for four Romanian isolates (R1-R4) were identical to the E5 isolate (referenced from Slovakia), which represents the most common European variant of *E. multilocularis*. For the R5 isolate derived from Vaslui county, three nucleotide

Table 2. Nucleotide differences in cytochrome c oxidase subunit 1 among *Echinococcus multilocularis* isolates.

Position in *cox1*	12	22	40	44	261	351	364	399	411	436	476	498	531	602
Isolate (origin, accession numbers)														
R1-R4 (Romania)	A	T	G	C	G	G	T	G	T	A	C	A	T	C
R5 (Romania)	.	.	.	.	.	.	.	T	G	.	.	.	C	.
E1 (Austria, GBr AB461412)	.	C	T	.	.	.	.	.	.	.	.	.	.	.
E3 (France, GBr AB461413)	.	.	.	T	.	.	.	.	.	.	.	.	.	.
E5 (Slovakia, GBr AB461414)	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A1 (Kazakhstan, GBr AB461415)	.	.	.	.	.	T	C	.	.	.	.	.	.	G
A5 (China, GBr AB461417)	.	.	.	.	.	T	C	.	.	.	.	G	.	.
N1 (St. Lawrence Island, USA, GBr AB461418)	G	.	.	.	.	T	.	.	G	.	T	G	.	.
N2 (Indiana, USA, GBr AB461419)	.	.	.	.	A	T	.	.	G	G	T	G	.	.

Only positions with differences between isolates are shown. Identical nucleotides are represented by dots.

substitutions (accounting for 0.38 % divergence) were recorded as compared to the most related E5 haplotype. These mutations, namely 399G/T, 411T/G and 531T/C, were synonymous. One of these mutations (at position 411) matched N1 and N2 haplotypes from North America, whereas remaining mutations were unique. Four and five nucleotide differences, respectively, were found in the R5 compared with the additional referenced sequences from Europe, E3 (France) and E1 (Austria), respectively. However, all European samples clustered together in the resulting dendrogram, being separated by sufficiently supported clade (bootstrap value of 68 %) from Asian and North American samples (Fig. 2). Pairwise divergences of the R5 isolate in relation to Asian and North American haplogroups were identical (6 – 7 differences to reference couples accounting for 0.76 – 0.89 % divergence). The structure of phylogenetic haplotype network for *E. multilocularis* isolates in *cox1* is illustrated in Fig. 3.
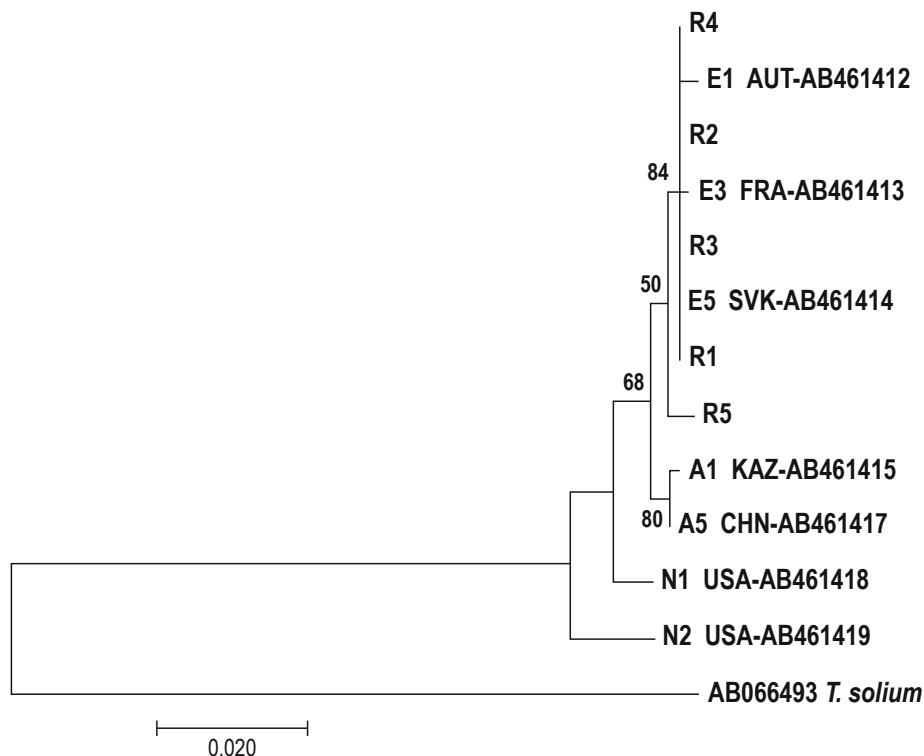

Fig. 2. Neighbor-joining tree inferred from *cox1* (789 bp) sequences showing the relationships among the *Echinococcus multilocularis* isolates from Romania (R1-R5) in comparison to GenBank retrieved reference sequences. *Taenia solium* was used as outgroup. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The tree is drawn to scale, with branch lengths proportional to the evolutionary distances.
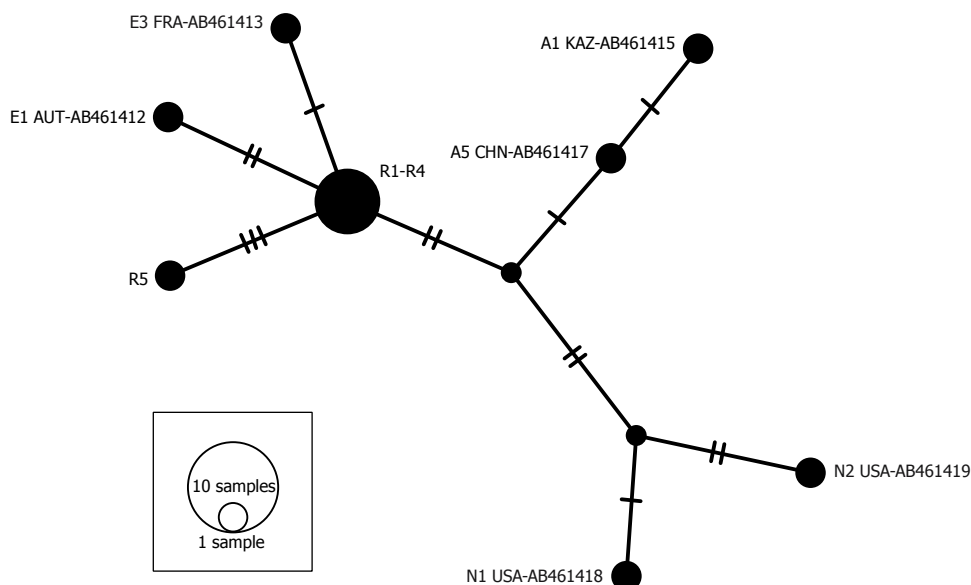
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Fig. 3. Haplotype network of *Echinococcus multilocularis* isolates based on *cox1* sequences. Diameters of circles are proportional to haplotype frequencies, transversal bars at branches represent point mutations, black dots without sample code indicate extant haplotypes.

In the study conducted on 6 isolates (4 from humans and 2 from domestic ungulates) collected from the two counties in western Romania (Caras-Severin, Timis) in mitochondrial *atp6* and *rrnS* gene fragments, within human isolates two were identified as *E. canadensis* G7, one isolate as *E. granulosus* s.s. G1, and one isolate as *E. granulosus* s.s. G3. For ungulates, the pig isolate was defined as possessing the *E. canadensis* G7 structure and the cattle isolate exhibited the *E. granulosus* G1 structure. Resulting *E. granulosus* s.l. genotypes from samples in western Romania are specified in Table 3.

In the *atp6* gene portion (513 bp), three samples showed the pattern characteristic for *E. granulosus* s.s. Amongst them, nucleotide composition of R6 and R9 human isolates was identical to the *E. granulosus* G1-G3 reference bases (GBr AF297617). In addition, the R10 isolate from cattle manifested two base differences, specifically the 342T/C synonymous substitution and the 379G/A non-synonymous substitution (alanine/threonine). These mutations were previously found according to the data on the NCBI

Table 3. Genotypic classification of *E. granulosus* isolates from western Romania.

Isolate	Host	Geographical origin	*atp6*	*rrnS*	Final genotype
R6	human	Timis county	G1-G3	G1	G1
R7	human	Caras-Severin county	G7A	G7	G7
R8	human	Caras-Severin county	G7	G7	G7
R9	human	Caras-Severin county	G1-G3	G3	G3
R10	cattle	Caras-Severin county	G1-G3	G1	G1
R11	pig	Caras-Severin county	G7	G7	G7

database in the Indian sheep isolate (GBr EF394904), and in the Turkish sheep isolate (Šnábel *et al.*, 2009). The *atp6* pattern of the three additional isolates was allocated to *E. canadensis* G7. Of these, sequences of the R8 (human) and the R11 (pig) isolates entirely matched the G7 reference (GBr AY056614). The R7 human isolate exhibited one nucleotide difference 238G/T, corresponding to non-synonymous substitution of alanine/serine (this haplotype is herein referred to as G7A for *atp6*).

To differentiate between genotypes of *E. granulosus* s.s. (*atp6* gene allows only for diagnosis at species level of *E. granulosus* s.l.), *rrnS* gene (295 bp) with resolving power to discriminate G1/G3 genotypes was screened. Fixed nucleotide exchanges 166T/G and 205A/G had delineated the R6 human-derived isolate and the R10 cattle-derived isolate as bearing G1 genotype and the R9 human-derived isolate as bearing G3 genotype. Monomorphic patterns of the remaining R7, R8 and R11 isolates from humans and pig were identical to the G7 reference for *rrnS* (GBr AY462128), thus definitely confirming their genotypic status. A list of diagnosed genotypes in western Romania is provided in Table 3.

Discussion

The present study provides the first report on the genetic characterization of *E. multilocularis* in clinical samples of five patients from north-eastern Romania. In addition, the present data add further evidence for the transmission of *E. granulosus* s.s. and *E. canadensis* G7 in humans and domestic ungulates in the western part of the country.

In Romania, *E. multilocularis* was firstly detected in rodents during surveys conducted in 1991 – 1995 in the subalpine region of the

East Carpathian Mountains (counties Harghita and Covasna in central Romania). Prevalence rates ranged from 0.44 % to 1.67 % in four species of 2,416 examined rodents (Sikó, 1992, 1993; Sikó *et al.*, 1995). Nevertheless, until 2002 *E. multilocularis* was not detected neither in 535 red foxes from one of the study areas (Covasna) nor in 50 foxes from three other counties (Brasov, Cluj, Mures) (Sikó *et al.*, 1995; Gherman *et al.*, 2002). However, in the first complex investigation of the fox population from the large part of Romania the parasite was found in 4.8 % (27/561) of red foxes in 8 of 15 counties (Sikó *et al.*, 2011). The highest *E. multilocularis* prevalences of 10.5 – 14.6 % were found in the counties bordering Hungary and Ukraine, with a tendency of decreasing prevalences towards the central parts. Three adjacent counties in north-eastern Romania allocated to geographical historical region of Western Moldavia (Iasi, Vaslui, Botosani), from which our patients originated, were not included in this fox survey. The question arises from where the parasite may have been introduced into these areas. In Ukraine neighboring northward to Western Moldavia, Kharchenko *et al.* (2008) recorded *E. multilocularis* in 28.8 % (4/14) of foxes. Two foxes collected in both foci in Volyn and Lviv oblasts of western Ukraine were found to be infected. Of these, the latter focus in Staryi Sambir forestry is located closer to Romanian areas with positive patients, but yet in a relatively long distance of approx. 400 km. Moreover, failure to detect metacestodes of the parasite in small mammal populations and lack of reported human AE cases indicate the recent introduction of the parasite in the country, likely connected to migrating fox populations from endemic areas of Hungary, Poland, or Slovakia (Szilágyiová *et al.*, 2015). In Harghita, Bistrita and Covasna counties in central-northern Romania (located 150 – 250 km apart from sites of our AE patients), infection rates of 1.69 – 5.66 % were measured in the above study of Sikó *et al.* (2011). It is thus plausible that *E. multilocularis* is being transmitted in some portion via red foxes also in north-eastern Romania. The Moldova Republic neighboring eastward is classified as a country with uncertain endemicity status (Oksanen *et al.*, 2016; EFSA, 2016), having a single record of *E. multilocularis* from house mouse in 1961 (Abuladze, 1964; Bessonov, 2002). Nevertheless, the country is believed to be endemic for the parasite over at least part of its territory, with 1 estimated annual number of human cases according to Torgerson *et al.* (2010).

For Romania, the compiled number of AE and CE human cases recorded in the EU summary reports has decreased in 2011 – 2015 from 0.27 to 0.09 per 100.000 population, with 18 cases registered in 2015 (EFSA, 2016). Although the exact proportion of human AE cases is unknown from EFSA reports, some AE cases in Romanian patients could have been misclassified as CE, because the notion that cystic echinococcosis is endemic in Romania is more common. Only two cases of AE in patients, originated from north-western Romania (Bihor county) (Panaitescu & Pop, 1999) and from central Romania (Sibiu county) (Savlovschi, 2000), were previously documented in the country. In the first infection, the liver was parasitized and the disease was serologically confirmed at Cantacuzino Institute in Bucharest. Later, the serum was tested repeatedly abroad and was confirmed to be highly positive. The second case was recorded in the spleen of a 49-year-old patient during a surgical operation (Iacobiciu *et al.*, 2005). To the best of our knowledge the present study provides the first molecular evidence of *E. multilocularis* in Romanian humans.

Age at the diagnosis of five herein AE-affected persons in north-eastern Romania ranged from 17 to 67 years (mean age 43.2). Three of 5 AE affected patients were farmers and one case was associated with a 17-year-old student who has regularly spent some time at the grandparent´s countryside house. This is in agreement with the summarized data for AE patients from Europe of Kern *et al.* (2003) who reported 61.4 % of 210 patients as involved in vocational or part-time farming, gardening, forestry, or hunting. Three patients infected with AE originated from the two sites of the Iasi county, and one patient was diagnosed from both Botoșani and Vaslui counties. Whereas the sequence pattern of four isolates was completely homologous to the most widespread variant of *E. multilocularis* identified for Europe in the *cox1* gene, one isolate (R5) at the southernmost location (Tutova, Vaslui county) amongst the examined samples exhibited three nucleotide substitutions. Interestingly, one of these mutations (411T/G) corresponded to N1 and N2 haplotypes from North America described by Nakao *et al.* (2009) and to an older human isolate from Austria obtained upon hepatic surgery in 1981 (Gottstein, personal communication; genotyping of the isolate specified in Šnábel *et al.*, 2010). The peculiar genetic composition of *E. multilocularis* seen in some restricted foci of Europe as Trentino Alto Adige region (Casulli *et al.*, 2005, 2009) and in the present isolate from Vaslui county, coupled with the discontinuous distribution of the parasite in recent Europe supports a hypothesis that the European clade has been derived from isolated populations in glacial refugia such as the Italian, Balkan and Iberian peninsulas (Taberlet *et al.*, 1998). Given that the same nucleotide substitution was detected in the North American clade and the two European isolates from distinct sites (Austria, Romania), retention of the shared ancestral polymorphism due to incomplete lineage sorting seems to be plausible explanation for this pattern. Molecular evidence for natural selection imposed on *cox1* has not been indicated because the nucleotide differences present in the R5 isolate did not lead to amino acid substitutions. Generally, lower genetic diversity, including founder effects, was recorded in peripheral areas of the endemic zone of Europe in several recent studies (Šnábel *et al.*, 2006; Bagrade *et al.*, 2008; Casulli *et al.*, 2009; Knapp *et al.*, 2010).

The molecular discrimination of the species and strains of *Echinococcus granulosus* s.l. are major prerequisites for effective control programs. Five of six hydatid samples genetically typed here originated from Caras-Severin county in western Romania. During 2004 – 2010, an incidence rate of 4.4 cases/100,000 inhabitants was recorded in Caras-Severin that was higher as compared to the period of 1987 – 1991 during which 3 cases/100,000 inha-

bitants were reported (Sikó *et al.*, 2002; Moldovan *et al.*, 2012). The authors explained this rise by the massive increases in the number of stray dogs (with a concomitant poorer control of them) and dog owners during the post-communist period after 1990. Consistently with this issue, Piccoli *et al.* (2013) indicated as main risk for acquiring CE infection close contact with stray dogs, which potentially experienced 96.7 % of 60 patients in south-eastern Romania. As reviewed by Neghina *et al.* (2010), the average prevalence of CE infection in dogs was quite high during the surveyed period 1956 – 1992 (21.6 %, the range of 0 – 83 % in the Romanian regions). More recent data obtained in the observation period 2005 – 2008 measured prevalence rates in dogs from rural areas in the range of 12.5 % – 19.2 % (Seres *et al.*, 2006; Seres *et al.*, 2010). The ongoing active transmission of *E. granulosus* in Romania is seen particularly in areas where pastoral activities are concentrated. Unsupervised home slaughtering of livestock still present especially for sheep and pigs, frequent absence of appropriate anthelmintic treatment of dogs and the above mentioned parasite dispersal by stray dogs are likely to be the most important factors maintaining transmission cycle of the tapeworm in the country (Mitrea *et al.*, 2012).

Among the three human isolates derived from Caras-Severin, two were identified as *E. canadensis* G7 and one belonged to *E. granulosus* s.s. G3. The human isolate recovered from the adjacent Timis county was classified as *E. granulosus* s.s. G1. Amongst isolates retrieved from domestic ungulates in Caras-Severin county, one cattle was allocated to *E. granulosus* G1 and one pig isolate to *E. canadensis* G7. The presence of *E. granulosus* G1-G3 complex (*E. granulosus* s.s.) coincides with areas of high CE prevalence, whereas for *E. canadensis* G6/G7 a low infectivity for humans was suggested because case numbers are usually low in regions where these genotypes predominate (Romig *et al.*, 2015). In a recent phylogeographical study of Kinkar *et al.* (2016) on Mediterraenan and South European *E. granulosus* s.s. G1, Romanian cattle isolate was genetically most closely related to Turkish samples derived from one of central haplotypes than to geographically more close samples. This indicated a substantial role of livestock trade, which has facilitated the parasite dispersal over vast areas, in shaping phylogeographical G1 pattern.

In connection with a single human sample identified here as *E. granulosus* G3, a relatively high proportion of the G3 genotype (28.2 %, 46 of 163 isolates) in the samples typed as *E. granulosus* s.s. was before detected in Romania when markers with the G1-G3 resolution power were used (Bart *et al.*, 2006; Badaraco *et al.*, 2008; Maillard *et al.*, 2009; Casulli *et al.*, 2012; Mitrea *et al.*, 2014; Šnábel *et al.*, 2016). This genetic form was found in sheep, cattle and pig in the country, whereas the present finding provides the first report of G3 in humans from Romania. High rates of G3 infections within *E. granulosus* s.s. were recently recorded also in neighboring Serbia (40 %, 18 of 40 isolates) and Bulgaria (46.7 %, 14 of 30 isolates) (Debeljak *et al.*, 2016; Marinova *et al.*, 2017). Interestingly, in two of the four human samples *E. canadensis* G7

was diagnosed as CE causative agent. Before, only a single G7 case was detected by Piccoli *et al.* (2013) amongst 68 typed human isolates in Romania. Patients and pigs infected with G7 have smaller cysts than those infected with G1, therefore the human infections are being more often identified incidentally (Turčeková *et al.*, 2009; Schneider *et al.*, 2010). Pig constitutes a significant reservoir for maintaining transmission pattern in Romania and the number of G7-registered human cases is likely to be underestimated. Although genotypes of *E. canadensis* are of minor relevance for human health compared to *E. granulosus* s.s., the estimated contribution of 11.07 % (184 of 1661 human cases) to global CE disease according to the worldwide synopsis is not negligible (Alvarez Rojas *et al.*, 2014).

As expected, the overall nucleotide diversity estimate (π) was markedly higher for six *E. granulosus* s.l. examined Romanian isolates (0.0702) than for five *E. multilocularis* isolates (0.0196). Lower genetic variability in *E. multilocularis* is putatively due to the facts that *E. multilocularis* has conservative host spectrum compared to *E. granulosus* adapted to a number of different host species and that *E. multilocularis* is phylogenetically young species (Haag *et al.*, 1997; Bart *et al.*, 2003).

More effective public health policies to reduce *E. granulosus* s.l. transmission should be implemented in Romania, also with regard to prevent precursors to regional and global expansions associated with trade. For example, in 2007 the country accounted for 90 % of all *Echinococcus* positive cattle imported to the Netherlands, the number of which has increased **s**ince the accession of the country to the European Union (Berends *et al.*, 2009). Further investigations of genetic and epidemiological issues on greater number of samples in various areas are needed to better clarify the transmission ecology and geographic pattern of CE and AE in Romania.

Acknowledgements

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