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DIFFERENCES IN THE FEEDING HABITAT USE BY PASSAGE AND BREEDING BIRDS AT THE BRĂDENI FISHPONDS

COSMIN IOAN MOGA[1]*, TIBOR HARTEL[2] and KINGA ÖLLERER[3]

SUMMARY. The aim of this study is to compare the feeding habitat use by passage and breeding waterbirds in the fishponds from Brădeni. The average number of species in autumn was found to be smaller than in spring. However, although in autumn we couldn't detect significant differences regarding the average number of species for the four ponds, in spring these were used differently by the avifauna. The average number of birds that use the ponds for feeding was larger in autumn than in spring, but the difference had no statistical significance. The four ponds were used differently by the individual bird species, both in autumn and in spring. Each pond was used differently for feeding in the two seasons (autumn and spring), both regarding the average species richness and the total number of individuals. The birds showed a preference for the larger ponds, with paludal, submerse and floating vegetation. Management measures should be planned in order to account for the differences in the habitat use shown by the bird species in these fishponds.

Keywords: avifauna, Brădeni fishponds, pond use.

Introduction

Knowing the aquatic habitat use by birds is an important step for their conservation. This becomes even more important in the light of climate change, which is likely to influence the quality of wetlands as far as both breeding avifauna and the passage one is concerned. Thus, it is possible that spatial shifts in migration would occur due to climate change and wetlands that were not previously used, will be more important for some birds (UNEP/CMS 2002). Therefore creating and updating databases, created from field observations will help researchers to better understand and separate the various (climatic, stochastic, populational) reasons of variability in habitat use by birds. The identification of new wetlands that are useful for the passage birds, together with data collection regarding the ecology of the aquatic birds, are necessary steps in understanding the modifications occurring in their distribution because of the climate changes (Boere and Taylor, 2004). The identification

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of new wetland areas that are important for the aquatic bird fauna, and which should be protected in the future, also represents a compensatory measure for the depreciation of the quality of the currently-protected ones (Delany, 1999; Blanco and Carbonell, 2001; Jackson *et al.*, 2004; Rehfisch and Crick, 2003). This process has to be followed by the establishment of an ecological network for these birds in order to secure their conservation (Boere and Taylor, 2004). Catry *et al.* (2004) have demonstrated that the aquatic birds (waterfowl and waders) are faithful to their stopover site. Due to this reason, the identification and the preservation of the quality of the wet areas used for their passage are vital elements for the conservation of this group, also determining reproductive success (Newton, 2004).

Previous Romanian references on the passage aquatic avifauna were mainly based on faunistical studies (e.g. Weber, 1993; Mitruly, 1997; Mesteacănu, Gava and Conete, 2004). Very few contain an analysis of the group (e.g. Fântână and Szabó, 2004). In Europe, in general, papers on the passage of the aquatic birds are scarce, most of the references focusing only on one or two species (e.g. Vogrin 1998a,b, 1999a).

In this study we aim to present the feeding habitat use by bird species in the Brădeni fishponds outside the reproductive season (in spring only a small period of the reproductive season being covered). We included in the analysis both the breeding and the passage avifauna. The knowledge about the use for feeding of the four fishponds by waterbirds is an important prerequisite for a future management plan, considering that the ponds are part of the Podişul Hârtibaciului Natura 2000 (SPA) site and are in process of becoming Nature Reserve according to the Romanian legislation.

In detail we are referring to the following aspects:

Analyzing species richness.

(1) Is there any difference regarding the average number of species between autumn and spring for the four ponds taken together?

(2) Are the four ponds used differently for feeding in autumn and in spring, considering the average number of species / individual ponds?

Analyzing the number of individuals.

(1) Is there any difference regarding total number of individuals and average number of individuals between autumn and spring in the case of the four ponds taken together?

(2) Are the four ponds used differently for feeding by the birds, considering the average number of individuals / each pond, both for autumn and spring?

The use of individual ponds in autumn and spring.

(1) Is there any difference regarding the use of individual ponds in autumn and spring, considering average number of species and average number of individuals?

Material and Methods

Study area. The fishponds from Brădeni (N. 46.07017, E. 24.81736 and 470 m a.s.l.) have a total surface of 171 ha, and were created on a former wetland area along the Hârtibaciului Valley, a tributary of the Olt River. The four ponds considered in the present study (Figure 1) are separated by dams of about 5 m height and 10 m width. Water depth is around 1.50 m.

Pond 1. Has a total area of 51.97 ha, out of which 31.97 ha is open water area, the rest (20 ha) being covered by paludal vegetation. Submerse and floating vegetation is present in about 20 % of the open water area.

Pond 2. Has a total area of 3.76 ha, out of which the open water area covers 2.76 ha and the area covered by paludal vegetation is 1 ha. Submerse and floating vegetation is present in about 70 % of the open water area.

Pond 3. Has a total area of 37.56 ha, out of which the open water area covers 26 ha, 11.56 ha being covered by paludal vegetation. Submerse and floating vegetation is present in about 90 % of the open water area.

Pond 4. Has a total area of 25.52 ha, out of which 23 ha represents the open water area, while 2.52 ha are covered by paludal vegetation. Submerse and floating vegetation is present in about 40 % of the open water area.

For all ponds vegetation cover was estimated visually. The paludal vegetation is mainly represented by *Typha latifolia* and *Phragmites australis*. Submersed and floating macrophyte vegetation is represented by *Ceratophylum* sp., *Myriophyllum* sp. and *Potamogeton* sp.

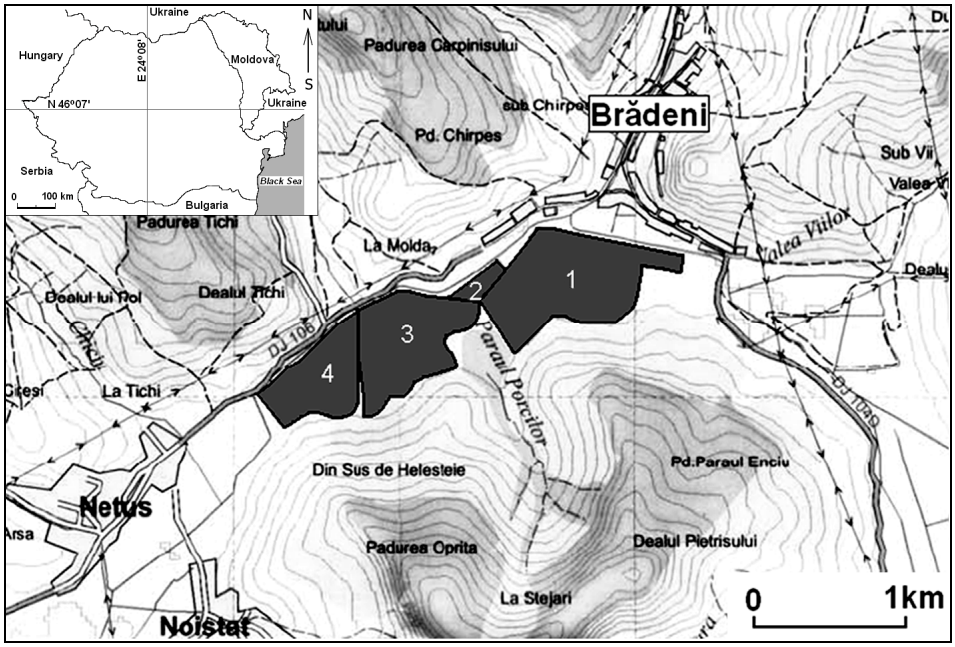

Fig. 1. Study area: Brădeni fishponds

Field study. The studies were carried out during autumn, 2003 (from September 21st until December 1st, when the ponds have frozen) and spring, 2004 (from March 18th until June 26th). We made 11 observations both in autumn and spring.

In the field we used the linear transect method along the ponds, and the point counts method (Biby 2002). Observations were done in the morning, after sunrise till 11 a.m., and also in the evenings, between 6 p.m. and sunset. Field observations were done using a 9-27 x 50 zoom binocular and 20-60 field glass.

Data analysis. For data comparison between the four ponds we used Analysis of Variance and Kruskal-Wallis ANOVA. For the comparison of data collected in autumn and spring we used parametric *t* test and nonparametric Mann-Whitney U test. Normality of data was verified with the Levene Test. All analysis were done with Statistica 6 software package, differences were considered significant at $P < 0.05$.

Results

Analyzing the species richness

Average number of species in autumn was smaller than in spring (Average = 1.61, Median = 1.00, Min = 0.00, Max = 9.00, SD = 2.01, n = 44 in autumn; Average = 3.20, Median = 3.00, Min = 0.00, Max = 9, SD = 2.36, n = 44 in spring) the difference being statistically significant (t = -3.39, df = 86, p = 0.001).

In autumn we found no significant difference regarding the average number of species for the four ponds (n = 44, ANOVA, $F_{(3, 40)} = 1.25$, P = 0.30) most of the species being recorded for Pond 3 (Fig. 2).

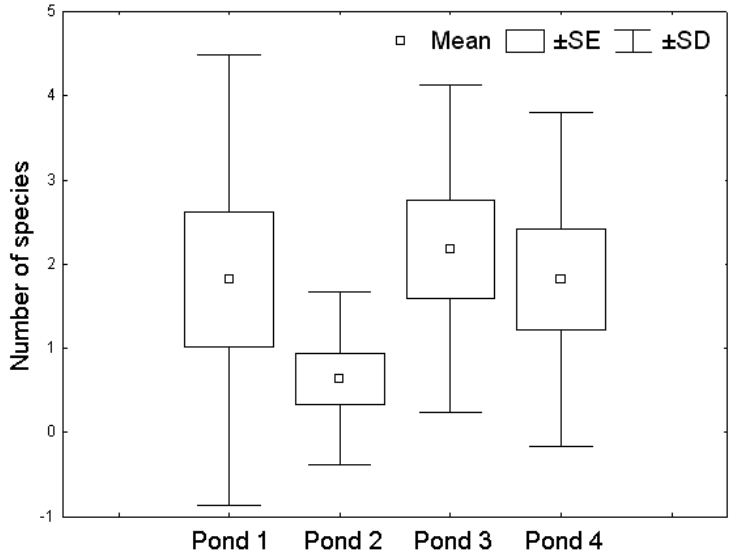

Fig. 2. The number of bird species in the four ponds during autumn

During spring the ponds are used differently by the avifauna (Fig 3), an observation supported by statistical significance (n = 44, ANOVA, $F_{(3, 40)} = 10.27$, P = 0.000038). The largest number of species was recorded for Pond 1 (Fig. 3). Post hoc analysis shows that this pond is more often used for feeding, in comparison with ponds 2 and 4; Pond 2 has more feeding birds than ponds 1 and 3; while Pond 3 gave significant differences only when compared with Pond 2; and Pond 4 only against Pond 1 (P < 0.05 for all cases) (Fig. 3).

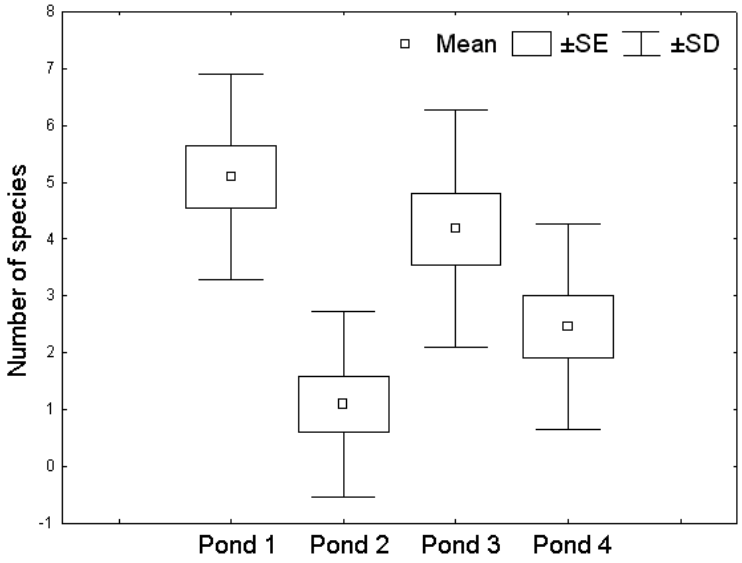

Fig. 3. The number of bird species in the four ponds during spring

Analyzing the number of individuals

During autumn we recorded 3914 birds for the four ponds all together, while in spring only 3142. The average number of birds using the four ponds for feeding is larger in autumn than in spring (Average = 88.95, Median = 23.00, Min = 0.00, Max = 433.00, SD = 131.71, n = 44 for autumn; Average = 71.40, Median = 59, Min = 0.00, Max = 283.00, SD = 70.90, n = 44 for spring), differences having no statistical significance (Mann-Whitney U test, Z = -1.16, P = 0.24).

The four lakes are used differently by the avifauna, both in autumn and in spring (Kruskal-Wallis ANOVA by Ranks, H (3, N = 44) = 11.22, P = 0.01 for autumn; H(3, N = 44) = 18.21, P = 0.0004 for spring) (Fig. 4 and 5). In autumn, the largest average number of bird individuals was observed on Pond 3, while in spring on Pond 1 (Fig. 4 and 5).

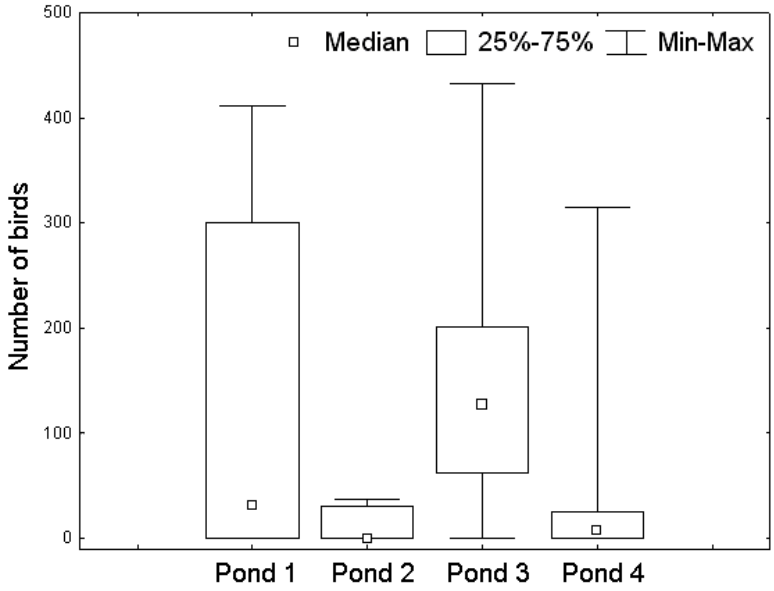

Fig. 4. The number of birds that use the four ponds for feeding in autumn

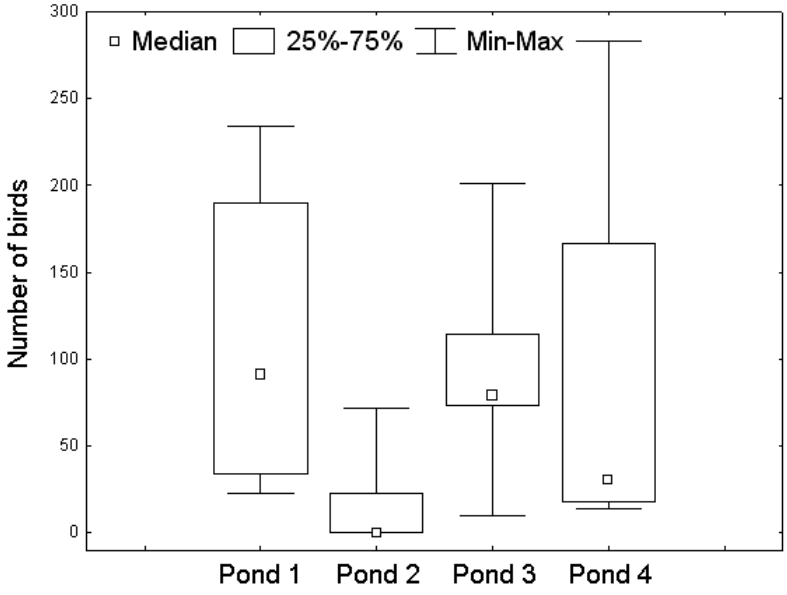

Fig. 5. The number of birds that use the four ponds for feeding in spring

The use of individual ponds in autumn and spring

Pond 1 is used differently for feeding by bird species in autumn and spring, when the largest average of feeding species was observed, a difference with statistical significance (*t* test, t = -3.35, df = 20, p = 0.003) (Fig. 6). The largest average of feeding individuals on this pond was observed in autumn (Fig. 7), (Mann-Whitney U test, Z = -0.29, P = 0.76).

The largest average number of species on Pond 2 was recorded in spring (Fig. 6), but the difference between the two seasons is without statistical significance (t test, t = -0.77, df = 20, p = 0.44). The largest average of feeding individuals on Pond 2 was observed in spring, but again without statistical significance (t test, t = -0.39, df = 20, p = 0.70).

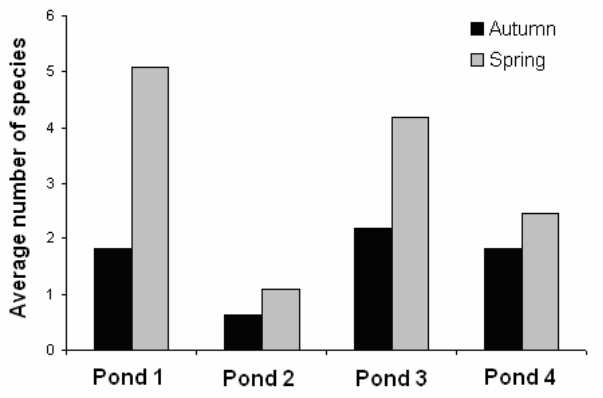

Fig. 6. Distribution of species in relation to the four ponds and study seasons

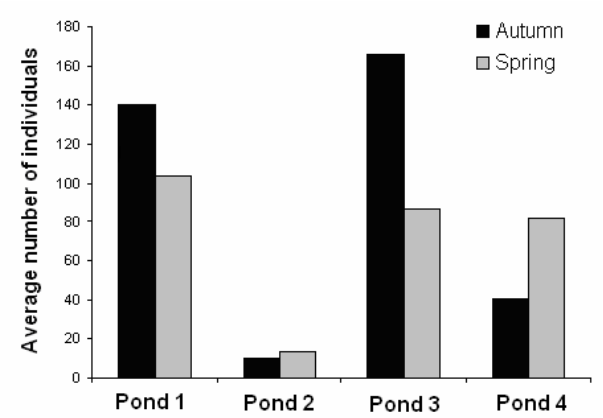

Fig. 7. Distribution of individual birds observed on the four ponds according to the study seasons

On Pond 3, the largest average number of species was recorded in spring (Fig. 6), with statistical significance (t test, t = -2.32, df = 20, p = 0.03). The maximum number of birds on this pond was recorded in autumn (Fig. 7), but the difference between the two seasons had no statistical significance (Mann-Whitney U test, Z = 1.44, P = 0.14).

The largest average number of species on Pond 4 was recorded in spring (Fig. 6), without significant difference between seasons (t test, t = -0.78, df = 20, p = 0.44). The largest average of individuals was recorded also in spring, again without statistical significance (t test, t = -1.05, df = 20, p = 0.30).

Discussion

Species richness analysis

Similar results regarding the passage avifauna for the Brădeni fishponds during autumn and spring passage were already recorded by Moga *et al.* in 2007. Considering *Anseriformes*, the authors showed that the number of *Anatinae* species was significantly higher in spring than in autumn, but there was no significant difference between the two seasons for the *Aythynae* species. Santoul *et al.* (2004), in a study from south-west France, observed the highest species richness in early spring, before the breeding season, a finding similar to that of the present study.

The largest species richness was observed on pond 3 (in autumn) and pond 1 (in spring). Pond 3 is the second in size and has the largest submerse and floating vegetation cover, reaching 90%. Pond 1 is the largest and also has the largest paludal vegetation cover. Differences in pond use for feeding during spring can be explained by the higher species richness in this season, with more birds specialized on certain feeding type. Paracuellos (2006), highlighting the importance of large ponds and lakes with large open water areas for the segregation of waterbird feeding areas, showed that generalist species feed mostly at the edge of the water, while specialized birds more in the open water and the central areas. The same author showed that the occupation of the ponds is negatively correlated with their minimum size, and that endangered species are dependent on large ponds. Paszkowski and Ton (2006), when studying boreal lakes, observed a positive correlation between the number of birds with different feeding specializations and pond sizes. Severo *et al.* (2002), in the wetlands from the Central Mexican Plateau, recorded the largest diversity of migrating ducks on the lakes with high macrophyte cover, affected by eutrophication. Santoul *et al.* (2004) showed that submerse macrophytes are the most important in the distribution of bird species. They also noted that human disturbance reduces both species number and species richness. This might explain the differences in the use of large lakes by waterbirds

Analyzing the number of individuals and the use of individual ponds by birds in autumn and spring

As in the case of species richness and number of individuals, the ponds used for feeding more often are Pond 1 and 3. Regarding the comparative analysis of each pond between the two seasons, we notice that generally the largest species richness /

pond was observed in spring. The largest number of individuals / pond did not show a clear pattern, the values being different for each pond, both in autumn and spring (on ponds 1 and 3 the largest number of individuals was recorded in autumn, while on ponds 2 and 4 in spring).

Moga *et al.* (2007), in the same area, recorded a higher number of *Anas platyrhynchos* individuals in autumn passage than during spring. The same situation was noted by Kranj *et al.* (1998) from north-western Croatia. Similarly to our study, Santoul *et al.* (2004) recorded the highest number of individuals on wetlands from southern France during summer and autumn. Another study with similar results like ours is that of Suter (1994), who recorded the highest bird density on meso-eutrophic and hipertrophic lakes in Sweden. Froneman (2001), while studying a system of farming ponds from Western Cape, South Africa, showed that the size of the pond and the structural diversity of the vegetation are important for the presence and abundance of waterbirds.

Conclusions

The four ponds from Brădeni are used differently by the avifauna, both when comparing the two seasons (autumn and spring) and in each season. These differences were observed when considering average number of species and average number of individuals. The dimension of the pond and the presence of paludal, submerse and floating vegetation are important factors that determine the use of these ponds by the avifauna. The maintenance of different vegetation covers on these ponds creates spatial heterogeneity for the feeding of waterbirds, contributing to their future conservation. Visitor management should aim to decrease disturbance as much as possible on the ponds used intensively for feeding.

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SNOUT-BEETLES (COLEOPTERA, CURCULIONOIDEA) FROM THE COLIBIȚA AREA (BÂRGĂU MOUNTAINS, ROMANIA)

LUCIAN ALEXANDRU TEODOR[1] and MIHAELA CRIȘAN[2]

SUMMARY. We recorded 83 species and subspecies from 49 genera, 12 subfamilies and 4 families of Curculionoidea in the Colibița area (table 1). 9 species or subspecies are rare: *Rutidosoma (Scleropteridius) monticola* (Otto), *Otiorhynchus (Magnanotius) equestris* (Rich.), *O. (Magnanotius) schaumi* Stierl., *O. (Prilisvanus) rugosus krattereri* Boh., *Onyxacalles pyrenaeus* Boh., *Tychius sharpi* Tourn., *Stomodes gyrasicollis* Boh., *Adexius scrobipennis* Gyll., *Plinthus (s. str.) illigeri* Germ.; 6 species or subspecies are endemical in Carpathian area: *Bryodaemon hanakii hanakii* (Friv.), *Otiorhynchus (Magnanotius) deubeli* Ganglb., *O. (Magnanotius) schaumi* Stierl., *O. (Prilisvanus) obsidianus* Boh., *O. (Prilisvanus) opulentus* Germ., *Phyllobius (s. str.) transsylvanicus* Stierl. The best represented are the Palearctic spread species (20%), followed by the European species (14%) and the Eurosiberian species (13%). The Carpathian species (7%) and the Alpine-Carpathian species (6%) are also well represented. High abundance has especially the montainous species, characteristic to the area (tab. 2). Into the lawns, among the mountainous species are abundant the common species. For all habitats the biodiversity and equitability index have high values.

Keywords: Bârgău Mountains, biodiversity, Colibița area, ecology, faunistical studies, Romania, snout-beetles.

Introduction

The Bârgău Mountains represent a relatively low high mountainous unity, situated on the central–northern region of Romania, on the western side of East Carpathians, between two high massifs on the northern and southern sides: the Rodna Mountains on North and the Calimani Mountains on South. The Bârgau Mountains are flanked by two depression areas: Transylvania Depression and Dornelor Depression, therefore, this area possess a gentle climate, being protected from the northern winds by the Rodna Massif but, in the same time is under the influence of western moist air masses. This gentle climate is favorable for to many invertebrate species that have a rich diversity into the studied habitats. The snout-beetles are exclusively phitophagous species and are favourised in this area by the rich flora.

The snout-beetles fauna of the Colibița (Bârgău Mountains) area has never been studied before.

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Material and methods

In this area, Colibița (Bârgău Mountains), we collected snout-beetles from March to August 2008 in several characteristic habitats.

The habitats and location of sampling sites (Fig. 1):

1 – **Beech forest**, **Şoimu Pass** (Fig. 2) at a 700-800 m altitude, (***Pulmonario rubrae-Fagetum***) –forest composed mostly by beech trees mixed with fir trees and spruce fir, often being observed a high dominance of the fir tree. Into the herbaceous layer we have identified *Pulmonaria rubra, Athyrium filix-femina, Dryopteris filix-mas, Brachypodium sylvaticum, Poa nemoralis, Anemone nemorosa, Oxalis acetosella.*

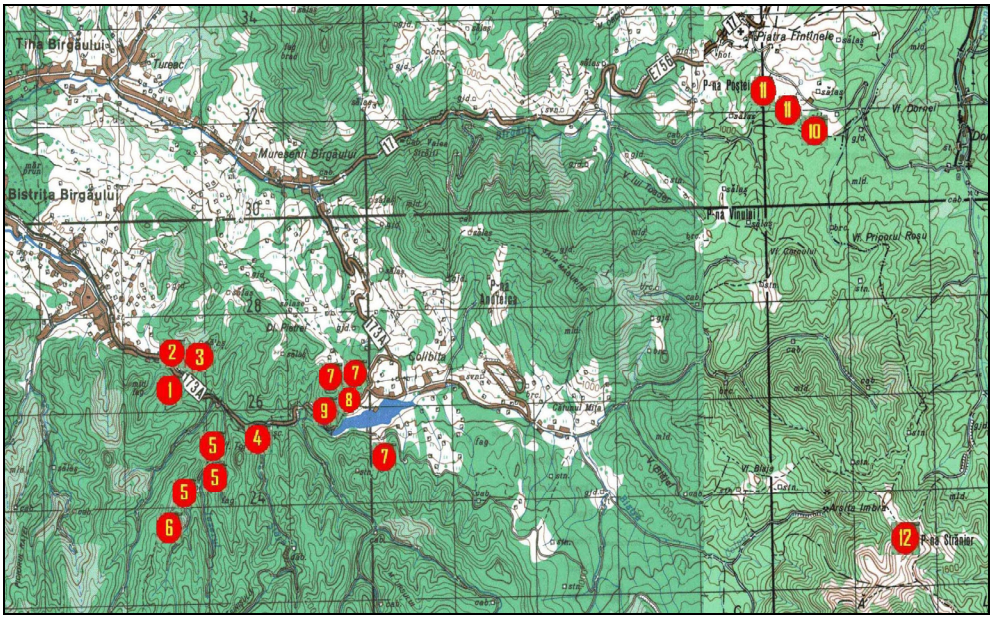

Fig. 1. Location of sampling sites: **Şoimu Pass**: **1** – Beech forest, **2** – Coppice (alder coppice), **3** – Pasture; **Stegea Valley**: **4** – Mixed forest of deciduous and coniferous trees; **Şoimu de Sus Valley**: **5** – Mixed forest of deciduous and coniferous trees, **6** – Hazel trees association; **Colibița**: **7** – Mixed forest of coniferous and deciduous trees, **8** – Mountain meadow (hayfield), **9** –Moist pasture; **Piatra Fântânele**: **10** – Spruce fir forest, **11** – Hayfield; **Tăul Zânelor – Poiana Strănior**: **12** – Pasture.

2 – **Coppice** (alder coppice) – mixture of shrub and tree species, **Şoimu Pass** on the Bistrița Bârgăului Valley (Fig. 3), at a 700 m altitude (***Telekio speciosae- Alnetum incanae***) formed by many mesophylous and meso-hygrophylous species such as: *Alnus incana, Salix sp., Populus sp., Stellaria nemorum, Geranium phaeum, Festuca gigantea, Carex remota, Cirsium oleraceum, Urtica dioica, Trifolium pratense, Vicia sepium, Rubus sp.*

Fig. 2. Beech forest, Şoimu Pass.

Fig. 3. Coppice (alder coppice), Şoimu Pass.

3 – **Pasture** – by the coppice, **Şoimu Pass**, at a 700 m altitude (***Festuco rubrae - Agrostetum capillaris***). We identified species such as: *Dactylis glomerata, Lotus corniculatus, Leontodon hispidus, Carum carvi, Leucanthemum vulgare, Knautia arvensis, Taraxacum officinale, Veronica chamaedrys, Daucus carota, Achillea millefolium, Vicia sepium, Tragopogon orientalis*, *Trifolium* and *Cirsium* species on this location.

4 –Mixed forest of deciduous and coniferous trees: beech, hornbeam, spruce fir and some glades (***Piceo-Fagetum***), **Stegea Valley**, at a 700 m altitude. Into the glades we meet species of *Trifolium, Petasites,* ferns, *Urtica dioica*, *Geum rivale* and several other species.

5 – **Mixed forest of deciduous and coniferous trees**: beech, hornbeam, spruce fir (***Piceo-Fagetum***), **Şoimu de Sus Valley** – a very moist valley, at a 700-800 m altitude. We studied on this location, especially the snout beetles that live on the herbaceous vegetation along the valley ***Telekio speciosae - Petasitetum hybridi association*** (Fig. 4) dominated by *Petasites* species: *Petasites hybridus* and *Petasites albus.* We also identified species such as: *Urtica dioica*, *Rumex acetosa*, *Ranunculus sp.*, and other herbs.

6 – H**azel trees association** (***Coryletum avellanae association*****),** situated by the outskirts of the mixed forest, on a South-Western slope, **Şoimu de Sus Valley**. Among the hazel bushes there is rich tall herbaceous vegetation formed by *Athyrium filix-femina* and *Dryopteris filix-mas, Urtica dioica, Astragalus glyciphillus, Petasites albus* etc.

7 – **Mixed forest of coniferous and deciduous trees** with shrubs: spruce fir trees, beech and hazel trees (Fig. 5), **Colibița** (up the lake and nearby the hut) (***Pulmonario rubrae -Fagetum***). Among the shrubs we identified *Rubus sp*., *Cirsium sp*., *Urtica dioica* and ferns as: *Athyrium filix-femina* and *Dryopteris filix-mas*.

8 – **Mountain meadow** (hayfield) (Fig. 5), **Colibița** (by the hut) – is formed by hygrophilous herbaceous plant associations represented by the following species: *Succisa pratensis, Colchicum autumnale, Gymnadenia conopsea, Linum catharticum, Juncus conglomeratus, Cirsium palustre, C. oleraceum, Equisetum palustre, Symphytum officinale, Angelica sylvestris, Trifolifolium pratense, T. medium, Achillea milefolium, Lotus sp., Medicago sp., Vicia sp., Violla sp., Urtica dioica.*

9 –**Moist pasture**, **Colibița** (***Junco-Caricetum fuscae asociation***) (Fig. 6) – with *Carex fusca* ssp. *nigra, Juncus conglomeratus, Juncus effusus,* species of *Trifolium, Carduus, Vicia.*

Fig. 4. *Telekio speciosae – Petasitetun hybridi,* Şoimu de Sus Valley.

Fig. 5. Mixed forest, and mountain meadow (hayfield), Colibița.

Fig. 6 Moist pasture, Colibița.

Fig. 7 Spruce fir forest, Piatra Fântânele

10 – Spruce fir forest, **Piatra Fântânele** (***Vaccinio-Piceetum***) (Fig. 7) with: *Picea abies, Pinus sylvestris, Vaccinium myrtillus, Lycopodium annotinum, Sorbus aucuparia, Oxalis acetosella*.

11 – Hayfield, **Piatra Fântânele** (***Scorzonero roseae – Festucetum nigricantis assocation***) with: *Hypericum maculatum, Arnica montana, Campanula abietina, Campanula serrata, Trifolim pratense, T. medium, Centaurea sp., Vicia sp., Lotus sp.*

12 – Pasture, **Tăul Zânelor – Poiana Strănior** (***Rumici obtusifoliae-Urticetum dioicae assocation***) – *Rumex* and *Urtica* species and also *Cirsium, Rubus, Centaurea* and *Carduus* species.

We collected the insects by mowing down the vegetation with the entomological net or directly from the host plant, by shaking down the tree and bush branches, using the umbrella net or by using „Barber" catches.

The identification of the snout-beetle species was made into the laboratory, using the stereomicroscope and the special literature (Endrödi, 1961; Angelov, 1976; Freude, Harde and Lohse, 1981, 1983; Dieckmann, 1988; Lohse and Lucht, 1994; Behne, 1998; Colonnelli, 1994; Podlussány, 1998; Alonso-Zarazaga and Lyal 1999, 2002; Stüben and Bahr, 2005; Stüben, 2008; Skuhrovec, 2008, 2009). The identification was made based on the morphological specific characters as well as based on the study of the male genitalia.

We took pictures of the studied habitats as well as of the found snout beetles. For the ecological studies on the snout beetles of the researched area, we calculated and used the following ecological indices: the relative abundance, the ecological diversity index and the equitability index.

Results and discussions

We collected 520 individuals, belonging to 83 species and subspecies, 49 genera, 31 tribes, 12 subfamilies and 4 families of the Curculionoidea group (Table 1 and Fig. 8, 9).

The best represented were the species of the Curculionidae and Apionidae families (Fig. 8), this repport being similar to the representation of these snout-beetles families in Romania (Teodor and Vlad, 2007).

As number of species, the best represented snout beetle subfamily was the Entiminae subfamily (30 species), represented in the Colibița area by numerous species from the Otiorhynchini tribe (Fig. 9, Table 1). Well represented were also the species from Curculioninae (14 species) and Apioninae (12 species) subfamilies, some of them being characteristic for the beech and mixted forests, but favorised by the presence of the pastures and the coppices in the area. Next on our range are: Ceutorhynchinae (7 species), Hyperinae (5 species) and Molytinae (4 species) subfamilies. The poor represeentad subfamilies were: Lixinae and Cryptorhynchinae each with 3 species, Scolytinae (2 species), Anthribinae, Orobitidinae and Rhynchitinae each with one species (Fig. 9).

Table 1.

Snout-beetle species identified in the Colibița area during 2008

No.	Classification/species	N	Date	Hab	rs	Spreading in România	General Spreading
	Superfamily Curculionoidea						
	Family Anthribidae						
	Subfamily Anthribinae						
1	*Anthribus nebulosus* Forster 1770	1	26.VI	10		Tr, Mm	euro-sw-as
	Family Apionidae						
	Subfamily Apioninae						
	Tribe Ceratapiini						
2	*Ceratapion (Acanephodus) onopordi* (W. Kirby, 1808)	1	22.V	2		Tr, Bn, M, Mt,Db	euro-w-c-as
		2	30.VII	8			
3	*Ceratapion (s. str.) gibbirostre* (Gyllenhal, 1813)	2	30.IV	9		Tr, Bn, Mm, M, Mt, Db	pal
	Tribe Kalcapiini						
4	*Taeniapion urticarium* (Herbst, 1784)	3	30.VII	2		Tr, Bn, M, Mt, Db	euro-w-c-as
	Tribe Oxystomatini						
	Subtribe Oxystomatina						
5	*Oxystoma cerdo* (Gerstaecker, 1854)	1	30.IV	9		Tr, M, Mt	euras
	Subtribe Synapiina						
6	*Ischnopterapion (Chlorapion) virens* (Herbst, 1797)	1	30.IV	9		Tr, Bn, Mm, M, Db	pal
		1	30.VII	4			
7	*Ischnopterapion (s. str.) loti* (W. Kirby, 1808)	1	26.VI	11		Tr, Bn, Ot, Db	pal
		2	30.VII	8			
	Tribe Piezotrachelini						
8	*Protapion assimile* (W. Kirby, 1808)	1	30.VII	8		Tr, Bn, Mm, M, Mt	pal
9	*Protapion apricans* (Herbst, 1797)	1	23.V	8		Tr, Bn, Mm, M, Ot, Mt, Db	pal
		2	26.VI	11			
		1	29.VII	8			
		3	30.VII	2, 3			
10	*Protapion gracilipes* (Dietrich, 1857)	2	26.VI	11		Tr, M	euro
		4	30.VII	8			
11	*Protapion fulvipes* (Fourcroy, 1785)	3	26.VI	8, 11		Tr, Bn, Mm, M, Ot, Mt, Db	pal
		4	30.VII	2, 4			
12	*Protapion trifolii* (Linnaeus, 1768)	1	30.VII	3		Tr, Bn, Mm, M, Mt, Db	pal
13	*Pseudoprotapion astragali astragali* (Paykul, 1800)	1	24.V	6		Tr, M	pal
	Family Curculionidae						
	Subfamily Ceutorhynchinae						
	Tribe Ceutorhynchini						
14	*Ceutorhynchus erysimi* (Fabricius, 1787)	1	26.VI	11		Tr, Bn, Mm, Cr, M, Ot, Mt, Db	hol
15	*Datonychus urticae* (Boheman, 1845)	1	1.V	2		Tr, Bn, Mm, Mt	euro-w-as
16	*Nedyus quadrimaculatus* (Linnaeus, 1758)	4	1.V	5		Tr, Bn, Mm, Cr, M, Mt, Db	eurosib
		13	24.V	2, 3, 6			
		1	25.VI	1			
17	*Trichosirocalus barnevillei* (Grenier, 1866)	4	24.V	3		Tr, M	euro-w-c-as
		1	26.VI	8			
	Tribe Phytobiini						
18	*Rhinoncus pericarpius* (Linnaeus, 1758)	2	29.VII	2		Tr, Bn, Mm, Cr, M, Ot, Mt	pal
	Tribe Scleropterini						
19	*Rutidosoma (Scleropteridius) monticola* (Otto, 1897)	1	22.V	1	+	Tr, Bn, Mm, Cr, M, Mt	carp-balk
		1	25.VI	1			

No.	Classification/species	N	Date	Hab	rs	Spreading in România	General Spreading
20	*Scleropterus serratus serratus* (Germar, 1824)	1	22.V	6		Tr, Bn, Cr, Mm, M, Mt	boreo-alp (n-c-euro)
		10	24.V	2			
		1	26.VI	11			
		1	30.VII	4			
	Subfamily Cryptorhynchinae						
	Tribe Cryptorhynchini						
	Subtribe Tylodina						
21	*Acalles camelus* (Fabricius, 1792)	1	30.VII	7		Tr, Bn, Mm, M, Mt	euro
22	*Onyxacalles pyrenaeus* Boheman, 1844	1	23.V	2	+	Tr, Bn, Mm, M, Mt	Pyrenes, Alps, Carpathians
23	*Ruteria hypocrita* (Boheman, 1837)	1	2.V	2		Tr, Bn, Mm, Cr, M, Mt	s-e, c-euro
		1	25.VI	1			
	Subfamily Curculioninae						
	Tribe Anthonomini						
24	*Anthonomus (s. str.) rubi* (Herbst, 1795)	1	24.V	2		Tr, Bn, Mm, Cr, M, Ot, Mt	pal
	Tribe Curculionini						
	Subtribe Curculionina						
25	*Archarius (s. str.) crux* Fabricius, 1776	1	24.V	2		Tr, Bn, Mm, M, Mt	pal
	Tribe Ellescini						
	Subtribe Elescina						
26	*Ellescus bipunctatus* (Linnaeus, 1758)	1	30.IV	9		Tr, Bn, Mm, M, Mt	hol
	Subtribe Dorytomia						
27	*Dorytomus (s. str.) taeniatus* (Fabricius, 1781)	1	23.V	7		Tr, Bn, Mm, M, Mt, Db	pal
	Tribe Mecinini						
28	*Cleopomiarus distinctus distinctus* (Boheman, 1845)	1	26.VI	8		Tr	eurosib
29	*Miarus monticola* Petri, 1912	3	24.V	3		Tr	eurosib
		7	26.VI	10			
	Tribe Rhamphini						
	Subtribe Rhamphina						
30	*Isochnus foliorum* (O. F. Müller, 1776)	1	30.VII	2		Tr, Bn, Mm, M, Ot, Mt	eurosib
31	*Orchestes (Salius) fagi* (Linnaeus, 1758)	1	22.V	2		Tr, Bn, Mm, Cr, M, Ot, Mt	euro
		5	24.V	1			
		3	25.VI	7			
32	*Tachyerges decoratus* (Germar, 1821)	1	30.VII	2		Tr, Bn, M, Mt	euro
33	*Tachyerges stigma* (Germar, 1821)	1	24.V	6		Tr, Bn, Mm, Cr, M, Ot, Mt	eurosib
	Tribe Tychiini						
	Subtribe Tychiina						
34	*Tychius (s. str.) picirostris* (Fabricius, 1787)	1	26.VI	8		Tr, Bn, Mm, M, Mt, Db	hol
		1	30.VII	3			
35	*Tychius (s. str.) rufipennis* Ch. Brisout de Barneville, 1862	1	26.VI	8		Tr, Bn	med
36	*Tychius (s. str.) sharpi* Tournier, 1873	1	30.VII	8	+	Tr, M	euro
37	*Tychius (s. str.) stephensi* Schönherr, 1836	1	24.V	3		Tr, Bn, Mm, M, Mt, Db	hol
	Subfamily Entiminae						
	Tribe Alophini						
38	*Graptus triguttatus* (Fabricius, 1775)	2	22.V	2		Tr, Bn, Mm, Cr, M, Mt,	euro
		1	24.V	2		Db	
	Tribe Omiini						
39	*Bryodaemon hanakii hanakii* (I. Frivaldzky, 1865)	9	25.VI	7		Tr, Mm	carp (Romania, Ukraine)
	Tribe Otiorhynchini						

No.	Classification/species	N	Date	Hab	rs	Spreading in România	General Spreading
40	*Otiorhynchus (Cryphiphorus) ligustici* (Linnaeus, 1758)	1	24.V	6		Tr, Bn, Cr, Ot	hol
41	*Otiorhynchus (Namertanus) pauxillus* Rosenhauer, 1847	1	24.VI	5		Tr, Bn, Mm, Cr, M, Mt	euro
		1	29.VI	1			
		1	29.VII	5			
42	*Otiorhynchus (Nihus) scaber* (Linnaeus, 1758)	1	22.V	6		Tr, Bn, Mm, Cr, M, Mt	euro
		4	23.V	7			
		3	24.VI	5			
		15	25.VI	1, 7			
		2	26.VI	10			
		1	29.VII	5			
		4	30.VII	4, 7			
43	*Otiorhynchus (Magnanotius) equestris* (Richter, 1821)	1	24.V	5	+	Tr, Mm, M	s-e, c-euro
44	*Otiorhynchus (Magnanotius) deubeli* Ganglbauer, 1896	9	1.V	2, 5		Tr, Mm	carp (Romania, Slovakia, Ukraine, Hungary)
		8	22.V	5			
		33	24.V	2, 5			
		1	29.VII	5			
		3	30.VII	4			
45	*Otiorhynchus (Magnanotius) schaumi* Stierlin, 1861	1	1.V	7	+	Tr, Mm. M, Mt	carp (Romania, Ukraine, Hungary)
46	*Otiorhynchus (s. str.) coecus coecus* Germar, 1824	1	30.VII	4		Tr, Bn, Mm, Cr, M, Ot, Mt	n-med
47	*Otiorhynchus (Prilisvanus) obsidianus* Boheman, 1843	8	1.V	5		Tr, Mm, Cr, M, Mt	carp (Romania, Hungary, Poland, Slovakia, Ukraine)
		5	22.V	5			
		7	24.V	2, 5			
		8	24.VI	3, 12			
		19	25.VI	7			
		2	30.VII	4			
48	*Otiorhynchus (Prilisvanus) rugosus krattereri* Boheman, 1843	2	22.V	5	+	Tr, Mm, M, Ot, Mt	carp-balk (Romania, Hungary, Slovakia, Ukraine, Bulgaria)
		1	25.VI	7			
49	*Otiorhynchus (Prilisvanus) opulentus* Germar, 1834	2	1.V	5		Tr, Mm, Cr, M, Ot, Mt, Db	carp (Romania, Hungary , Ukraine, Poland)
		4	22.V	1, 2, 5			
		1	23.V	3			
		8	24.V	2, 5			
		2	24.VI	3, 5			
		17	25.VI	1, 2, 7			
		5	29.VII	1, 2, 5			
		5	30.VII	2, 3			
	Tribe Peritelini						
50	*Stomodes gyrosicollis* Boheman, 1843	1	25.VI	7	+	Tr, Bn, M, Mt	n-med
	Tribe Phyllobiini						
51	*Phyllobius (Metaphyllobius) glaucus* (Scopoli, 1763)	1	22.V	5		Tr, Bn, Mm, Cr, M, Ot, Mt	eurosib
		12	24.V	2, 5			
52	*Phyllobius (Nemoicus) oblongus* (Linnaeus, 1758)	3	24.V	2		Tr, Bn, Mm, Cr, M, Ot, Mt	eurosib
		1	25.VI	2			
53	*Phyllobius (s. str.) betulinus betulinus* (Bechstein & Scharfenberg, 1805)	14	25.VI	7		Tr, Bn, Cr, M, Ot	n-med
		1	26.VI	8			
		1	29.VII	1			
54	*Phyllobius (s. str.) transsylvanicus* Stierlin, 1894	1	25.VI	7		Tr, Bn, M, Mt	carp (Romania, Moldavia Rep., Ukraine, Slovakia)
		1	26.VI	10			

No.	Classification/species	N	Date	Hab	rs	Spreading in România	General Spreading
	Tribe Polydrusini						
55	*Polydrusus (Chlorodrosus) amoenus* (Germar, 1824)	5	24.VI	12		Tr, Mm, Cr, M, Ot	eurosib
		2	26.VI	11			
56	*Polydrusus (Eustolus) pterygomalis* Boheman, 1840	1	25.VI	7		Tr, Bn, Mm, Cr, M, Ot, Mt	eurosib
57	*Polydrusus (Metallites) impar impar* Gozis, 1882	14	26.VI	10		Tr, M	w, c, s-e, s-euro
58	*Polydrusus (s. str.) fulvicornis* (Febricius, 1792)	3	24.V	2		Tr, M, Mt	eurosib
		2	25.VI	2			
		3	29.VII	2			
	Tribe Sciaphilini						
59	*Sciaphilus asperatus* (Bonsdorff, 1785)	1	23.V	7		Tr, Bn, Mm, M, Mt	euro-n-am
		1	24.V	2			
	Tribe Sitonini						
60	*Sitona (s. str.) hispidulus* (Fabricius, 1776)	1	24.V	3		Tr, Bn, Mm, M, Ot, Mt	pal
61	*Sitona (s. str.) humeralis* Stephens, 1831	1	24.V	6		Tr, Bn, Mm, Cr, M, Mt	pal
62	*Sitona (s. str.) inops* Gyllenhal, 1832	1	30.VII	8		Tr, Mm, M, Ot, Mt	euro-v-c-as
63	*Sitona (s. str.) lepidus* Gyllenhal. 1834	1	25.VI	2		Tr, Bn, Mm, Cr, M, Mt, Db	hol
		1	30.VII	3			
64	*Sitona (s. str.) suturalis* Stephens, 1831	2	30.IV	9		Tr, Bn, Mm, M, Ot, Mt	pal
		1	24.V	3			
		3	26.VI	11			
65	*Sitona (s. str.) waterhousei waterhousei* Walton, 1846	1	30.VII	8		Tr, Bn, Mm, M, Mt	w-pal
	Tribe Tanymecini						
	Subtribe Tanymecina						
66	*Chlorophanus viridis viridis* (Linnaeus, 1758)	1	24.VI	12		Tr, Bn, Mm, M, Mt	euro
	Subfamily Hyperinae						
	Tribe Hyperini						
67	*Hypera (s. str.) miles* (Paykull, 1792)	1	25.VI	8		Tr, Bn, Mm, Cr, M, Ot,	eurosib
		2	26.VI	11		Mt	
68	*Donus (s. str.) intermedius intermedius* (Boheman 1842)	1	23.V	8		Tr, Bn, Mm, Cr, M, Mt	alp-carp
		1	29.VII	8			
69	*Donus (s. str.) ovalis* (Boheman, 1842)	1	24.V	6		Tr, Bn, M, Mt	s-e, c-euro
		1	24.VI	3			
70	*Donus (s. str.) oxalidis* (Herbst, 1795)	1	24.VI	12		Tr, Bn, Mm, Cr, M, Ot, Mt	alp-carp
71	*Donus (s. str.) velutinus* (Boheman, 1842)	1	24.VI	12		Tr, Bn, Mm, M	alp-carp
	Subfamily Lixinae						
	Tribe Lixini						
72	*Larinus (Larinomesius) obtusus* Gyllenhal, 1836	8	24.VI	3		Tr, Bn, Mm, M, Mt, Db	euro-w-c-as
		1	26.VI	8			
		12	30.VII	3			
73	*Larinus (Phyllonomeus) jaceae* (Fabricius, 1775)	2	24.VI	12		Tr, Bn, Mm, Cr, M, Db	pal
		25	26.VI	11			
	Tribe Cleonini						
74	*Cleonis pigra* (Scopoli, 1763)	1	1.V	8		Tr, Bn, M, Ot, Mt, Db	pal
	Subfamily Molytinae						
	Tribe Hylobiini						
	Subtribe Hylobiina						
75	*Lepyrus capucinus* (Schaller, 1783)	1	30.IV	9		Tr, Bn, Cr, M, Ot, Mt	euro
		1	23.V	9			
	Tribe Molytini						
	Subtribe Molytina						
76	*Liparus (s. str.) glabrirostris* (Küster, 1849)	9	1.V	5		Tr, Bn, Mm, Cr, M, Ot,	alp-carp
		1	22.V	5			
		26	24.V	5		Mt	

No.	Classification/species	N	Date	Hab	rs	Spreading in România	General Spreading
	Subtribe Plinthina						
77	*Adexius scrobipennis* Gyllenhal, 1834	7	22.V	1, 5, 6	+	Tr, Bn, Mm, M	Caucasus, Alps, Carpathians
		3	25.VI	1			
78	*Plinthus (s. str.) illigeri illigeri* Germar, 1824	1	25.VI	6	+	Tr, Bn	alp-carp
79	*Trachodes hispidus* (Linnaeus, 1758)	1	22.V	6		Tr, M, Mt, Db	c-euro
		1	29.VII	2			
		1	30.VII	4			
	Subfamily Orobitidinae						
80	*Orobitis cyanea* (Linnaeus, 1758)	1	25.VI	8		Tr, Bn, Cr, M	euro-w-c-as
		1	29.VII	8			
	Subfamily Scolytinae						
	Tribe Hylastini						
81	*Hylastes ater* (Paykull, 1800)	2	22.V	2		Tr, Mm, M,	euro
		5	25.VI	2			
	Tribe Scolytini						
82	*Scolytus rugulosus* (Müller, 1818)	1	29.VII	2		Tr, Mt, Db	euro
	Family Rhynchitidae						
	Subfamily Rhynchitinae						
	Tribe Rhynchitini						
	Subtribe Rhynchitina						
83	*Lasiorhynchites (s. str.) olivaceus* (Gyllenhal, 1833)	1	30.VII	2		Tr, M	med

Abbreviations:

Hab = researched habitats: 1. Beech forest – *Pulmonario rubrae-Fagetum* (Şoimu Pass), **2. Coppice (alder coppice)** – *Telekio speciosae - Alnetum incanae* (Şoimu Pass), **3. Pasture** – *Festuco rubrae - Agrostetum capillaris* (Şoimu Pass), **4 – Mixed forest of deciduous and coniferous trees -** *Piceo-Fagetum* (Stegea Valley), **5.** – **Mixed forest of deciduous and coniferous trees -** *Piceo-Fagetum* and along the valley *Telekio speciosae - Petasitetun hybridi* association (Şoimu de Sus Valley), **6.** H**azel trees association** –*Coryletum avellanae* association (Şoimu de Sus Valley), **7. Mixed forest of deciduous and coniferous trees** – *Pulmonario rubrae-Fagetum* (Colibiţa), **8. Mountain meadow** (hayfield) – (Colibiţa**), 9. Moist pasture –** *Junco-Caricetum fuscae* (Colibiţa), **10. Spruce fir forest** – *Vaccinio-Piceetum* (Piatra Fântânele), **11. Hayfield** – *Scorzonero roseae – Festucetum nigricantis* (Piatra Fântânele), **12. Pasture –** *Rumici obtusifoliae -Urticetum dioicae* (Tăul Zânelor – Poiana Strănior);

N = number of colected individuals, **rs** = rare species;

Spreading in Romania: Tr = Tansylvania, **Bn** = Banat, **Mm** = Maramureş, **Cr** = Crişana, **M** = Moldavia, **Ot** = Oltenia, **Mt** = Muntenia, **Db** = Dobrudja;

General spreading: alp-carp = Alpine-Carpathian species; **boreo-alp (n-c-euro)** = boreo-alpine species (northern and central European); **carp** = Carpathian species; **carp-balk** = species spreading in Carpathian and Balkan area; **c-euro** = central European species; **euro** = European species; **euras** = Eurasian species; **eurosib** = Eurosiberian species; **euro-n-am** = species spread in Europe and in North America; **euro-sw-as** = European and south-western Asian species; **euro-w-as** = European and western Asian species ; **euro-w-c-as** = European, western and central asian species; **hol** = species with Holarctic spreading; **med** = Mediterranean species; **n-med** = northern Mediterranean species; **pal** = species with Palearctic spreading; **s-e, c-euro** = south-eastern and central European species; **w,c,s-e, s-euro** = western, central, south-eastern and south European species; **w-pal** = species with west Palearctic spreading.

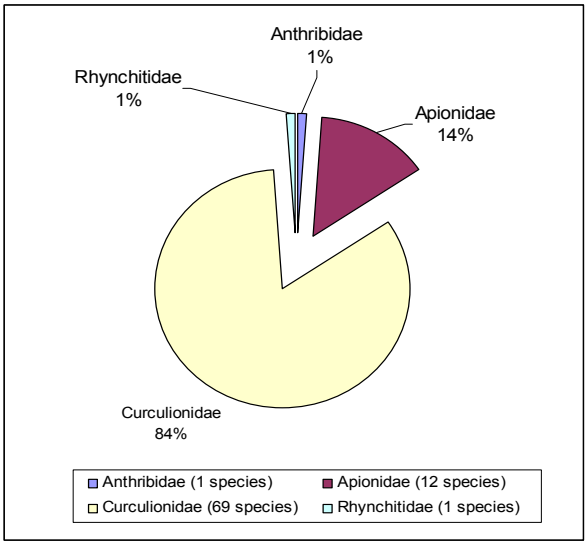

Fig. 8. Composition of the collected material according to Curculionoidea families.

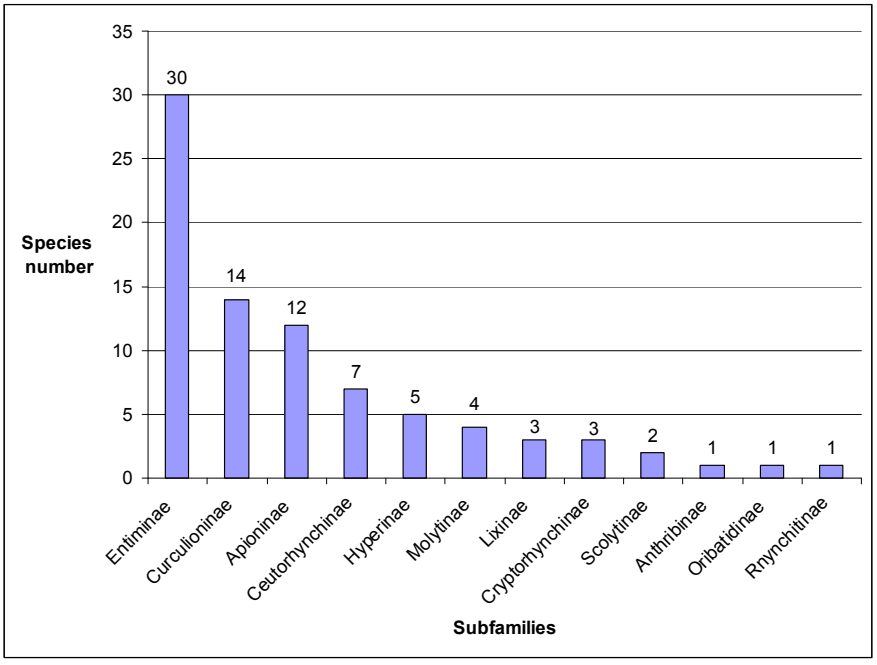

Fig. 9. Composition of the collected material according to Curculionoidea subfamilies.

The important snout-beetle species found in Colibita area

From that 83 snout-beetle species and subspecies identified in Colibița area, 13 species have a smaller area: 6 are Carpathian endemic species, two are Carpathian-Balkan species and 5 are Alpine-Carpathian species. Here are also 9 rare species (tab. 1).

Carpathian endemic species

Bryodaemon hanakii hanakii (I. Frivaldzky, 1865)

Studied material: 9 individuals: 4 ♂♂, 5 ♀♀ - 25.VI.1008, mixed forest of coniferous and deciduous trees – *Pulmonario rubrae-Fagetum* (Colibița, up the lake).

General spreading: Romania: Maramureş Mountains and Rodna Mountains; Ukraine: Černa Hora (Podlussány, 1998). We identified this subspecies for the first time in Bârgău Mountains.

Biology and eecology**:** montainous species, that lives into mixed forests of deciduous and coniferous trees, on mosses: *Pleurozium schreberi* (Willd.) and in coniferous and deciduous leaf layers by the mosses; it's biology is not known yet (Podlussány, 1998).

Otiorhynchus (Magnanotius) deubeli Ganglbauer, 1896 (fig. 10)

Studied material: 55 individuals: 1♀ - 1.V, 1♀ - 24.V, 2008, coppice (alder trees) – *Telekio speciosae- Alnetum incanae* **(** Şoimu Pass on the Bistrița Bârgăulu Valley); 2 ♂♂, 1♀ - 30.VII. 2008, mixed forest of deciduous and coniferous trees – *Piceo-Fagetum* (Stegea Valley); 6♂♂, 2♀♀ - 1.V, 4♂♂, 4♀♀ - 22.V, 21♂♂, 11♀♀ - 24.V, 1♂, 1♀ - 29.VII, 2008, mixed forest (*Piceo-Fagetum*), along the valley – *Telekio speciosae- Petasitetun hybridi* (Şoimu de Sus Valley).

General spreading: Carpathian Mountains (Romania, Slovakia, Ukraine, Hungary).

Biology and ecology: mountainous species that lives on *Petasites species*; it's biology is not known yet.

Otiorhynchus (Prilisvanus) obsidianus Boheman, 1843

Studied material: 41 individuals: 1♀ - 24.V.2008, coppice (alder trees) – *Telekio speciosae- Alnetum incanae* (Şoimu Pass on the Bistrița Bârgăului Valley); 2♀♀ - 24.VI.2008, pasture – *Festuco rubrae - Agrostetum capillaris* (Şoimu Pass); 2♀♀ - 30.VII.2008, mixed forest–*Piceo-Fagetum* (Stegea Valley); 3♂♂, 2♀♀ - 22.V., 4♂♂, 2♀♀ - 24.V, 2008, mixed forest (*Piceo-Fagetum*), along the valley– *Telekio speciosae - Petasitetun hybridi* (Şoimu de Sus Valley); 11♂♂, 8♀♀ - 22.V.2008, mixed forest – *Pulmonario rubrae-Fagetum* (Colibița, up the lake); 2♂♂, 4♀♀ - 24.V. 2008, pasture – *Rumici obtusifoliae-Urticetum dioicae* (Tăul Zânelor – Poiana Strănior).

General spreading: Carpathian Mountains (Romania, Hungary, Poland, Ukraine).

Biology and ecology**:** mountainous, poliphagous species, frequently present on *Urtica dioica*; biology not known yet.

Otiorhynchus (Prilisvanus) opulentus Germar, 1834 (fig. 11)

Studied material: 45 individuals: 1♂ - 22.V, 1♀ - 25.VI, 1♀ - 29.VII, 2008, beech forest – *Pulmonario rubrae-Fagetum* (Şoimu Pass); 1♂ - 22.V, 1♀ - 23.V, 3♂♂, 4♀♀ - 24.V, 1♂, 4♀♀ - 25.VI, 1♂, 2♀♀ - 29.VII, 4♀♀- 30.VII, 2008, coppice (alder trees) – *Telekio speciosae- Alnetum incanae* **(**Şoimu Pass on Bistriţa Bârgăului Valley); 1♀ - 23.V, 1♀ - 24.VI, 1♀ - 30.VII, 2008, pasture– *Festuco rubrae - Agrostetum capillaris* (Şoimu Pass); 1♂, 1♀ - 1.V, 1♂, 1♀ - 22.V, 1♂ - 24.V, 1♂ - 24.VI, 1♂ - 29.VII, 2008, mixed forest (*Piceo-Fagetum*), along the valley – *Telekio speciosae- Petasitetun hybridi* (Şoimu de Sus Valley); 6♂♂, 5♀♀ - 25.VI.2008, mixed forest– *Pulmonario rubrae-Fagetum* (Colibiţa, up the lake and by the hut);

General spreading: Carpathian Mountains (Romania, Hungary, Ukraine, Poland).

Biology and ecology: poliphagous species, present on Picea abies, *Urtica dioica* and diffrent *Salix* and *Rubus* species; biology not known.

Otiorhynchus (Magnanotius) schaumi Stierlin, 1861

Studied material: 1♀ - 1.V.2008, mixed forest– *Pulmonario rubrae-Fagetum* (Colibiţa, surroundings).

General spreading: Carpathian Mountains (Romania, Hungary, Ukraine)

Biology and ecology: mountainous species, biology and ecology unknown.

Phyllobius (s. str.) transsylvanicus Stierlin, 1894 (fig. 12)

Studied material: two individuals: 1♀ - 25.VI.208, mixed forest– *Pulmonario rubrae-Fagetum* (Colibiţa, up the lake); 1♂ - 26.VI.2008, spruce tree forest – *Vaccinio-Piceetum* (Piatra Fântânele).

General spreading: Carpathian Mountains (Romania, Moldovia Republic, Ukraine, Slovakia).

Biology şi ecology: mountainous, poliphagous species, lives on different deciduous species; biology unknown.

Carpathian - Balkan species

Rutidosoma (Scleropteridius) monticola (Otto, 1897)

Studied material: two individuals: 1♀ - 22.V, 1♂ - 25.VI, 2008, beech forest – *Pulmonario rubrae-Fagetum* (Şoimu Pass).

General spreading: Carpathian and Balkan Mountains (Romania, Hungary, Bulgaria, Bosnia & Herzegovina, Croatia).

Biology and ecology: mountainous, monophagous species on *Oxalis acetosella,* that has just one generation each year.

Fig. 10. *Otiorhynchus (Magnanotius) deubeli* Ganglb., 8,5 mm length - (original).

Fig. 11. *Otiorhynchus (Prilisvanus) opulentus* Germ., 8 mm length – (original).

Fig. 12. *Phyllobius (s. str.)* 8 mm length – (original).

Fig. 13. *Liparus (s. str.) glabrirostris* (Küst.), *transsylvanicus* Stierl., 19 mm length – (original).

Otiorhynchus (Prilisvanus) rugosus krattereri Boheman, 1843

Studied material: 3 individuals 2♀♀ - 22.V.2008, mixed forest (*Piceo-Fagetum*), along the valley – *Telekio speciosae - Petasitetun hybridi* (Şoimu de Sus Valley); 1♀ - 25.VI.2008, mixed forest – *Pulmonario rubrae-Fagetum* (Colibița, up the lake).

General spreading: Carpathian and Balcan Mountains (Romania, Hungary, Slovakia, Ukraine, Bulgaria).

Biology and ecology: mountainous species, ecology and biology unknown.

Alpine-Carpathian species

Donus (s. str.) intermedius intermedius (Boheman 1842)

Studied material: two individuals: 1♀ - 23.V, 1♀ - 29.VII, 2008, mountainious medow– hay field (Colibița by the hut).

General spreading: Alps and Carpathian Mountains

Biology and ecology: muntainous, poliphagous species, on *Centaurea jacea, Salvia verticilata, Mentha officinalis*. It has just one generation /year.

Donus (s. str.) oxalidis (Herbst, 1795)

Studied material: 1♀ - 24.VI.2008, pasture– *Rumici obtusifoliae-Urticetum dioicae* (Tăul Zânelor – Poiana Strănior).

General spreading: Alpes and Carpathian Mountains

Biology and ecology: montainous, poliphagous species on *Petasites, Adenostyles, Senecio* and *Chaerophyllum* species. It has just one generation /year.

Donus (s. str.) velutinus (Boheman, 1842)

Studied material: 1♀ - 24.VI.2008, pasture– *Rumici obtusifoliae-Urticetum dioicae* (Tăul Zânelor – Poiana Strănior).

General spreading: Alpes and Carpatian Mountains

Biology and ecology: montainous, poliphagous species on *Aconitum napellum, Doronicum austriacum, Rumex alpinus, Saxifraga rotundifolia* (Skuhrovec, 2009). It has just one generation /year.

Liparus (s. str.) glabrirostris (Küster, 1849) (fig. 13)

Studied material: 36 individuals: 3♂♂, 6♀♀ - 1.V, 1♂ - 22.V, 12♂♂, 14♀♀ - 24.V, 2008, mixed forest *Piceo-Fagetum*), along the valley – *Telekio speciosae - Petasitetun hybridi* (Şoimu de Sus Valley).

General spreading: Alpes and Carpathian Mountains

Biology and ecology: muntainous, poliphagous species on *Petasites* şi *Heracleum* species. It has one generation/ year.

Plinthus (s. str.) illigeri Germar, 1824

Studied material: 1♂ - 25.VI.2008, hazel bushes – *Coryletum avellanae*, on the outskirts of the mixed, forest, on a S – W exposed slope (Şoimu de Sus Valley).

General spreading: Alps şi Carpathians Mountains

Biology and ecology: mountainous species, biology and ecology unknown.

Rare species

Rutidosoma (Scleropteridius) monticola (Otto, 1897) (see Carpathian – Balkan species)

Onyxacalles pyrenaeus Boheman, 1844

Studied material: 1♂, coppice (alder trees) – *Telekio speciosae- Alnetum incanae* (Şoimu Pass on Bistriţa Bârgăului Valley).

General spreading: Pyrenes, Alpes and Carpathian Mountains.

Biology and ecology: mountainous specis, found on the leaf layers of the decidous forests (*Fraxinus excelsior, Fagus sylvatica, Sorbus aucuparia, Sorbus chamaemespilus, Salix caprea, Rosa canina* as well as on coniferous leaf layer (*Picea abies*), (Knutelski, 2001, 2005; Stüben and Bahr, 2005; Stüben 2008).

Tychius sharpi Tournier, 1873

Studied material: 1♀, mountainious medow – hay field (Colibiţa, by the hut).

General spreading: Europe.

Biology şi ecology: mountainous species, monophagous on *Trifolium montanum*. It has one generation/year.

Otiorhynchus (Magnanotius) equestris (Richter, 1821)

Studied material: 1♀, mixed forest (*Piceo-Fagetum*), along the valley – *Telekio speciosae- Petasitetun hybridi* (Şoimu de Sus Valley)

General spreading: Central and South-Eastern Europe.

Biology and ecology: mountainous species, found on *Asplenium* species; biology unknown.

Otiorhynchus (Prilisvanus) rugosus krattereri Boheman, 1843 (see Carpathian – Balkan species)

Otiorhynchus (Magnanotius) schaumi Stierlin, 1861 (see Carpathian species).

Stomodes gyrasicollis Boheman, 1843

Studied material: 1♂ - 25.VI.2008, mixed forest – *Pulmonario rubrae-Fagetum* (Colibiţa, up the lake).

General spreading: north – Mediterranean species.

Biology and ecology: oligophagous species, found on *Medicago* and *Trifolium* species, biology unknown.

Adexius scrobipennis Gyllenhal, 1834

Studied material:: 1♂, 2♀♀ - 22.V, 2♂♂, 1♀ - 25.VI, 2008, beech forest – *Pulmonario rubrae-Fagetum* (Şoimu Pass); 1♀ - 22.V.2008, mixed forest (*Piceo - Fagetum*), along the valley – *Telekio speciosae- Petasitetun hybridi* (Şoimu de Sus Valley); 1♂, 2♀♀ - 22.V.2008, hazel bushes – *Coryletum avellanae*, on the outskitrs of the mixed forest, on a S – W slope (Şoimu de Sus Valley).

General spreading: Caucasus, Alpes and Carpathian Mountains.
Biologie şi ecologie: moutainous species, ecology and biology unknown.

Plinthus (s. str.) illigeri Germar, 1824 (see Alpine – Carpathian species).

Zoogeographic analysis

Analyzing the general spreading of the identified snout beetle–species in this area (tab. 1, fig. 10) we observe that from this point of view, the snout beetles are very divers. Tough, the best represented were the Palearctic spread species (20%), followed by the European species (14 %) and the Eurosiberian species (13%). Well represented in the area were also the Holarctic species, those spread in Europe and in the Western and Central Asia and the Carpathian species each having a representation of 7 % and Alpine-Carpathian species (6 %). The other species categories had a lower representation into the area (fig. 14). It must be noticed the presence of the Carpathian species (7%) and Alpine-Carpathian species (6 %).

Ecological contributions

The data concerning the relative abundance of the snout-beetle species into the area reveals the fact that a high abundance into the studied habitats had especially the mountainous species, characteristic to the area (tab. 2). Among the mountainous species, into the meadow areas, the common species were quite abundant.

Into the **beech forest**, Şoimu Pass the abundant species was *Adexius scrobipennis* (26,09%), followed by *Orchestes (Salius) fagi* (21,74%).

Into the **coppice** (alder trees), Şoimu Pass, the abundant species is *Otiorhynchus (Prilisvanus) opulentus* (21.51%), followed by *Phyllobius (Metaphyllobius) glaucus* (11,83%) and *Scleropterus serratus* (10,75%).

On the **pasture**–by the coppice, Şoimu Pass are well represented are *Larinus (Larinomesius) obtusus* (47,72%) and *Trichosirocalus barnevillei* (9,52%).

Into the **mixed forest, Şoimu de Sus Valley,** abundant species are *Protapion fulvipes* and *Otiorhynchus (Magnanotius) deubeli* (23,077%).

Into the **mixed forest of decidous and coniferous trees** habitat**, Şoimu de Sus Valley**, abundant are *Otiorhynchus (Magnanotius) deubeli* (39,2%) and *Liparus (s. str.) glabrirostris* (28,8%).

In to **the hazel tree association,** the greatest abundance have species *Nedyus quadrimaculatus* (36.8%) and *Adexius scrobipennis* (15.8%).

Into **the mixed forest of deciduous and coniferous trees, Colibiţa,** abundant are: *Otiorhynchus (Nihus) scaber* (24,70%), followed by *Otiorhynchus (Prilisvanus) obsidianus* (22,35%) and *Phyllobius (s. str.) betulinus* (16,47%).

Into the **mountainous meadow Colibiţa** the most abundant is *Protapion gracilipes* (14,28%) followed by *Ceratapion (Acanephodus) onopordi*, *Ischnopterapion (s. str.) loti*, *Protapion apricans*, *Protapion fulvipes*, *Neoglanis (s. str.) intermedius* and *Orobitis cyaneus*, (7,14% each).

Into **the moist pasture, Colibița**, the abundant species are: *Ceratapion (s. str.) gibbirostre*, *Sitona (s. str.) suturalis* and *Lepyrus capucinus* (22,22% each).

Into **the spruce fir tree forest, Piatra Fântânele**, the abundant species are: *Polydrusus (Metallites) impar* (56%) and *Miarus monticola* (29%).

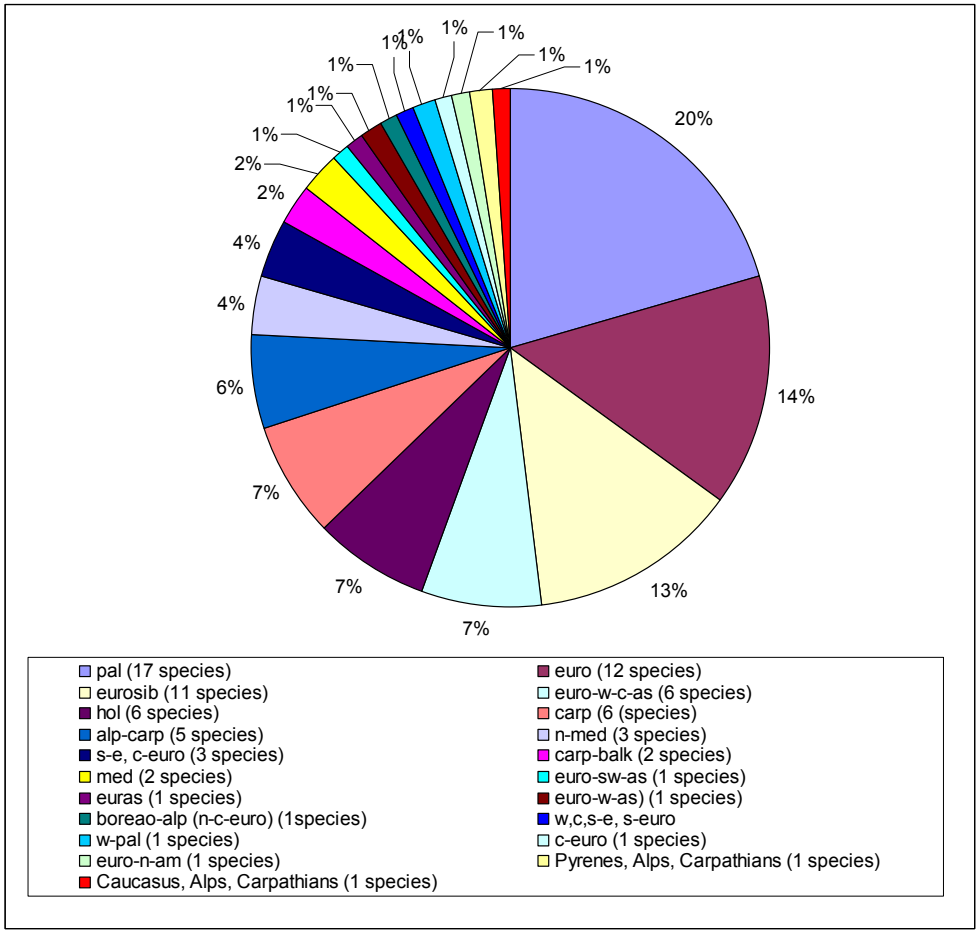

Fig. 14. The Colibita area snout- beetles zoogeographic spectrum

Abbreviations: alp-carp = alpine-Carpathian species; **boreo-alp (n-c-euro)** = boreo-alpine species (northern and central European); **carp** = Carpathian species; **carp-balk** = Carpathian-Balkan species; **c-euro** = central European species; **euro** = European species; **euras** = Eurasian species; **eurosib** = Eurosiberian species; **euro-n-am** = species spread in Europe and in North America; **euro-sw-as** = European and south - western Asian species; **euro-w-as** = European and western Asian species; **euro-w-c-as** = European, western and central Asian species; **hol** = Holarctic spread species; **med** = Mediterranean species; **n-med** = northern Mediterranean species; **pal** = Palearctic spread species; **s-e, c-euro** = south eastern and central European species ; **w,c,s-e, s-euro** = western, central, south-eastern and southern European species; **w-pal** = western palearctic spread species.

Table 2

Relative abundance (A%) of snout-beetle species from the Colibița area in 2008

Species	Habitats											
	1	2	3	4	5	6	7	8	9	10	11	12
	A%	A%	A%	A%	A%	A%	A%	A%	A%	A%	A%	A%
Anthribus nebulosus										4,00		
Ceratapion onopordi		1,07						7,14				
Ceratapion gibbirostre									22,22			
Taeniapion urticarium		3,23										
Oxystoma cerdo									11,11			
Ischnopterapion virens				7,69					11,11		2,50	
Ischnopterapion loti								7,14				5,88
Protapion assimile								3,57				
Protapion apricans		1,07	4,76					7,14			5,00	
Protapion gracilipes		1,07						14,28			5,00	
Protapion fulvipes		1,07		23,08				7,14			2,50	
Protapion trifolii			2,38									
Pseudoprotapion astragali						5,26						
Ceutorhynchus erysimi											2,50	
Datonychus urticae		1,07										
Nedyus quadrimaculatus	4,35	5,38	2,38		3,20	36,80						
Trichosirocalus barnevillei			9,52					3,57				
Rhinoncus pericarpius		2,15										
Rutidosoma monticola	8,69											
Scleropterus serratus		10,7		7,69		5,26					2.50	
Acalles camelus							1,17					
Onyxacalles pyrenaeus		1,07										
Ruteria hypocrita	4,35	1,07										
Anthonomus rubi		1,07										
Curculio crux		1,07										
Ellescus bipunctatus									11,11			
Dorytomus taeniatus							1,17					
Cleopomiarus distinctus								3,57				
Miarus monticola			7,14							28,00		
Isochnus foliorum		1,07										
Orchestes fagi	21,74	1,07					3,53					
Tachyerges decoratus		1,07										
Tachyerges stigma						5,26						
Tychius picirostris			2,38					3,57				
Tychius rufipennis								3,57				
Tychius sharpi								3,57				
Tychius stephensi			2,38									
Graptus triguttatus		3,23										
Bryodaemon hanakii hanakii							10,59					
Otiorhynchus ligustici						5,26						
Otiorhynchus pauxillus	4,35				1,60							
Otiorhynchus scaber	13,04			7,69	1,60	5,26	24,70			8,00		
Otiorhynchus equestris					0,80							
Otiorhynchus deubeli		2,15		23,08	39,20							
Otiorhynchus schaumi							1,17					
Otiorhynchus coecus coecus				7,69								
Otiorhynchus obsidianus		1,07	4,76	15,38	15,20		22,35					35,29

Species	Habitats											
	1	2	3	4	5	6	7	8	9	10	11	12
	A%	A%	A%	A%	A%	A%	A%	A%	A%	A%	A%	A%
Otiorhynchus rugosus krattereri					1,60		1,17					
Otiorhynchus opulentus	13.04	21,51	7,14		5,60		12,94					
Stomodes gyrasicollis							1,17					
Phyllobius glaucus		11,83			1,60							
Phyllobius oblongus		4,30										
Phyllobius betulinus	4,35						16,47	3,57				
Phyllobius transsylvanicus							1,17			4,00		
Polydrusus amoenus											5,00	29,41
Polydrusus pterygomalis							1,17					
Polydrusus impar										56,00		
Polydrusus fulvicornis		8,60										
Sciaphilus asperatus		1,07					1,17					
Sitona hispidulus			2,38									
Sitona humeralis						5,26						
Sitona inops								3,57				
Sitona lepidus		1,07	2,38									
Sitona suturalis			2,38						22,22		7,50	
Sitona waterhousei								3,57				
Chlorophanus viridis viridis												5,88
Hypera suspiciosa								3,57			5,00	
Neoglanis intermedius								7,14				
Neoglanis ovalis			2,38			5,26						
Neoglanis oxalidis												5,88
Neoglanis velutinus												5,88
Larinus obtusus			47,62					3,57				
Larinus jaceae											62,50	11,77
Cleonis pigra								3,57				
Lepyrus capucinus									22,22			
Liparus glabrirostris					28,80							
Adexius scrobipennis	26,08				0,80	15,80						
Plinthus illigeri						5,26						
Trachodes hispidus		1,07		7,69		5,26						
Orobitis cyaneus								7,14				
Hylastes ater		7,53										
Scolytus rugulosus		1,07										
Lasiorhynchites olivaceus		1,07										

Abbreviations:

Habitats: 1. Beech forest – *Pulmonario rubrae-Fagetum* (Şoimu Pass), **2. Coppice (alder coppice)** – *Telekio speciosae - Alnetum incanae* (Şoimu Pass), **3. Pasture** – *Festuco rubrae - Agrostetum capillaris* (Şoimu Pass), **4 – Mixed forest of deciduous and coniferous trees -** *Piceo-Fagetum* (Stegea Valley), **5. – Mixed forest of deciduous and coniferous trees -** *Piceo-Fagetum* and along the valley *Telekio speciosae - Petasitetun hybridi* association (Şoimu de Sus Valley), **6. Hazel trees association** –*Coryletum avellanae* association (Şoimu de Sus Valley), **7. Mixed forest of deciduous and coniferous trees** – *Pulmonario rubrae-Fagetum* (Colibiţa), **8. Mountain meadow** (hayfield) – (Colibiţa), **9. Moist pasture –** *Junco-Caricetum fuscae* (Colibiţa), **10. Spruce fir forest** – *Vaccinio-Piceetum* (Piatra Fântânele), **11. Hayfield** – *Scorzonero roseae – Festucetum nigricantis* (Piatra Fântânele), **12. Pasture –** *Rumici obtusifoliae - Urticetum dioicae* (Tăul Zânelor – Poiana Strănior);

Into the **hayfield Piatra Fântânele**, the abundant species is *Larinus (Phyllonomeus) jaceae* (62.5%).

Into the **pasture** of **Tăul Zânelor - Poiana Strănior** abundant are *Otiorhynchus (Prilisvanus) obsidianus* (35.29%) and *Polydrusus (Eustolus) pterygomalis* (29.41%).

The greatest biodiversity of the snout–beetles was signaled on the mountainous meadow of Colibița (2,837), followed by the coppice of Şoimu Pass (2,756). It is important to see that the biodiversity index is high for every habitat of the studied area (tab. 3, fig. 15). The lowest value of the biodiversity index was recorded into the spruce tree forest of Piatra Fântânele (1,141), yet this value also indicates a great biodiversity. Also, the equitability index values (tab. 3, fig. 15) indicate the fact that the snout–beetle populations of the studied habitats are stable, so, these habitats are well preserved and not strongly influenced by men. Even into the pastures and hayfields where they are some human interference, the biodiversity and equitability index have quite high values (tab. 3, fig.15).

Table 3

The values of Shannon-Wiener (H') ecological diversity index and equitability index (e) and the numeric distribution of Curculionoidea species end individuals in Colibița area

Habitats	1	2	3	4	5	6	7	8	9	10	11	12
Species number	9	29	14	8	11	11	14	19	6	5	10	7
Indiviuals number	23	93	43	13	125	19	85	28	9	25	40	17
H'	1.971	2.756	1.956	1.951	1.625	2.054	2.016	2.837	1.735	1.141	1.456	1.646
e	0.8972	0.827	0.7412	0.9384	0.6779	0.8566	0.7639	0.9635	0.9684	0.7088	0.6324	0.8458

Abbreviations: Habitats 1-12 – idem Table 2.

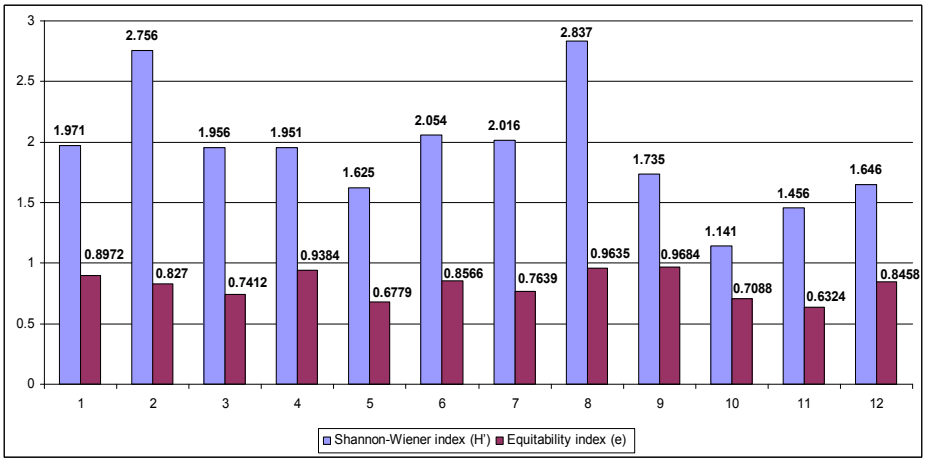

Fig. 15. Values of Shannon-Wiener ecological diversity index **(H')** and equitability index **(e)** for Curculionoidea in Colibița area.

Abbreviations: Habitats 1-12 – idem Table 2.

Conclusions

- In the studied area we identified 83 species and subspecies, from 49 genera, 31 tribes, 12 subfamilies and 4 families from the Curculionoidea suprafamily. All the identified species and subspecies are signaled for the first time in this area.
- The best represented were the species of the Curculionidae family (84%) and Apionidae family (14%).
- As number of species, the best represented snout-beetle subfamily was Entiminae (30 species), followed by Curculioninae subfamily (14 species) and Apioninae subfamily (12 species). Poor represented were the following subfamilies: Anthribinae, Orobitidinae and Rhynchitinae, with one species each.
- We identified 9 rare snout-beetle species: *Rutidosoma (Scleropteridius) monticola* (Otto), *Otiorhynchus (Magnanotius) equestris* (Rich.), *O. (Magnanotius) schaumi* Stierl., *O. (Prilisvanus) rugosus krattereri* Boh., *Onyxacalles pyrenaeus* Boh., *Tychius sharpi* Tourn., *Stomodes gyrasicollis* Boh., *Adexius scrobipennis* Gyll. and *Plinthus (s. str.) illigeri* Germ.
- We signaled into the area 6 Carpathian endemic species and subspecies: *Bryodaemon hanakii hanakii* (Friv.), *Otiorhynchus (Magnanotius) deubeli* Ganglb., *O. (Magnanotius) schaumi* Stierl., *O. (Prilisvanus) obsidianus* Boh., *O. (Prilisvanus) opulentus* Germ. and *Phyllobius (s. str.) transsylvanicus* Stierl.
- For the endemic subspecies *Bryodaemon hanakii hanakii* (Friv.), known so far as being present just in Černa Hora (Ukraine) and in Maramureş Mountains and Rodna Mountains (Romania), we signaled the presence of a population in the Bârgău Mountains (Colibița area).
- From the zoogeographic point of view, into this area, the best represented species are the Palearctic spread species (20%), followed by the European spread species (14%) and the Eurosiberian species (13%). The Carpathian species (7%) and the Alpine-Carpathian species (6%) are also well represented.
- High abundance into the studied habitats, have especially the mountainous species, characteristic to the area. Into the lawns, among the mountainous species are abundant the common species.
- The highest biodiversity of the snout-beetles was signaled into the mountainous pasture of Colibița (2,837), followed by the coppice of Şoimu Pass (2,756).
- The values of the equitability index, reveals the fact that the snout-beetle populations from the studied habitats are stable.
- The human influences do not change the biodiversity and the ecological balance of the habitats into the area.

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SYNTAXONOMIC REVISION OF *QUERCUS VIRGILIANA* TEN. AND *QUERCUS PEDUNCULIFLORA* K. KOCH FORESTS FROM ROMANIA

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SUMMARY. The taxonomical status of the species *Quercus virgiliana* Ten. and *Quercus pubescens* Willd. was interpreted differently in time. Given the clear morphological characters that differentiate the two taxa, namely achene morphology, scales at the base of the cup and leaf morphology, as well as the specific ecological conditions and distribution area, we consider their treating as valid species to be justified. The syntaxonomical revision of these forests was done using both literature data and field research. In Transylvania, the first species, *Quercus virgiliana*, was reported from only a few locations. Based on our research in the Natura 2000 Site from Petiş, we have described a new association with this species: *Carici montanae-Quercetum virgilianae*. Since the phytocoenoses with *Quercus virgiliana* from Southern Romania, namely from Dobruja and the Southern Mehedinţi plateau, have a floristic structure that is richer in thermophilous species and distinctive ecology, we have assigned them to the association *Carpino orientalis-Quercetum virgilianae*. The tree layer is dominated by *Quercus virgiliana* and *Quercus pubescens*. By analyzing the Romanian phytocoenoses which include the second species, *Quercus pedunculiflora*, we have concluded that they can be classified in two associations. The first one, *Tilio tomentosae – Quercetum pedunculiflorae* comprises the stands from plateaus and moderate slopes in Dobruja and Moldavia. The tree layer is dominated by the species *Quercus pedunculiflora* and *Tilia tomentosa*. The second association, *Fraxino pallisae-Quercetum pedunculiflorae* is specific to the meadows of Bârlad, Siret, Prut and Buzău rivers. The tree layer is dominated by *Quercus pedunculiflora*, *Fraxinus pallisae* and *Fraxinus angustifolia.*

Keywords*: Quercus virgiliana*, *Quercus pedunculiflora,* Romania, syntaxonomy, thermophilous forests.

Introduction

The taxonomical status of the species *Quercus virgiliana* Ten. and *Quercus pubescens* Willd. was interpreted differently with time. In his paper, Prodan (1939) considers *Quercus virgiliana* to be a subspecies of *Quercus pubescens* Wild., and *Quercus pedunculiflora* C. Koch to be the subspecies *osteotricha* Borb. et Csató of *Quercus robur* L.

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Al. Borza, in his "Conspectus florae Romaniae" (1947) treats the two taxa as valid species. The same taxonomical interpretation is found later in the papers published by Al. Beldie (1952, 1977), Ciocârlan (2000, 2009), Sanda et al. (2003), regarding the Romanian flora.

In Flora Europaea (1993), O. Schwarz presents only *Quercus pedunculiflora* C. Koch as valid species, while the species *Quercus virgiliana* Ten. is considered to be a synonym of *Quercus pubescens* Wild.

In this paper, we undertake a critical syntaxonomic revision of forests with *Quercus pedunculiflora* and *Quercus virgiliana* from Romania, using literature data as well as field data from our research.

Materials and methods

The work methodology included the study of specific literature and the field research of the plant communities through relevés. In our research we have used the method elaborated by Braun-Blanquet (1964), widely used in Central Europe.

Species cover in the phytocoenosis (relevé) was estimated using Braun-Blanquet's 6 interval scale (+: covers < 1%, 1: covers between 1-5%, 2: covers between 5-25%, 3: covers between 26-50%, 4: covers between 51-75%, 5: covers > 75% of the surface of the analyzed phytocoenosis (relevé).

The phytocoenoses were assigned to plant associations according to the presence of characteristic and local differential species. For forest plant communities, the characteristic and differential species are usually found in the herb layer.

We have carried out the relevés at large distances from each other in order to be able to inventory most of the habitat's biodiversity. The plant associations were included, according to characteristic species, in alliances, orders and classes.

In Tab. 1 we present the structure of forest associations with *Quercus virgiliana* and *Q. pedunculiflora* from Romania.

Results and discussion

Phytocoenotically, the species *Quercus virgiliana* had previously been scantly studied. The first description of a *Quercus virgiliana* stand in Romania was done by Paşcovschi (1942) from Buzău County (Runceni Forest). In this location, the species is accompanied by *Quercus pubescens*, *Q. petraea*, *Carpinus betulus*, *Ulmus campestris*, *Acer campestre*, *A. tataricum* and few other common shrub species. Unfortunately the composition of the herb layer is not specified.

Subsequently, Dihoru, Ţucra and Bavaru (1965) followed by Cristureanu and Ţuculescu (1970) have described from Dobrogea (Nature Reserves Fântâniţa and Hagieni) phytocoenoses with *Quercus virgiliana* and *Carpinus orientalis* that they describe in detail from a floristic point of view, emphasizing also the herbaceous species from these stands.

A more detailed floristic description of *Quercus virgiliana* stands is available in the paper published by Roman (1974), covering the vegetation from the Southern Mehedinți Plateau. He considered such coenoses as a subassociation within the association *Cotino-Quercetum pubescentis* Zolym. et al. 1958. The xerophyllous character of these stands is proven by the presence of Southmediteranean-Balkan elements such as *Fraxinus ornus*, *Cotinus cogygrya*, *Carpinus orientalis* and *Syringa vulgaris*.

Although the presence of *Quercus virgiliana* was reported from more counties of Moldavia (Bacău, Galați, Vaslui), no independent plant associations have been described with it. In Transylvania, the species *Quercus virgiliana* was reported from only a few localities (Bejan, Miniş, Lempeş Hill and Petiş), but without the description of the phytocoenoses that included the species (Csürös-Kaptalan 1970).

On the basis of our phytocoenological research in the Natura 2000 Site at Petiş, we have identified a new forest association, moderately thermophilous and moderately acidophilous, that we have named *Carici montanae-Quercetum virgilianae* (Tab. 1 col. 1). It vegetates on sandy soils (luvisols), with a small humus content and slightly acid reaction.

The tree layer is dominated by *Quercus virgiliana* and has a crown cover of 65-70%. In this layer there are also present isolated individuals of *Quercus pubescens* and *Tilia cordata*.

The shrub layer is dominated by *Ligustrum vulgare*, *Crataegus monogyna*, *Prunus spinosa*, *Pyrus pyraster*, *Rosa canina.* Only sporadically there are present young individuals of the species *Quercus pubescens*, *Q. virgiliana* and *Q. cerris* accompanied by other species that are characteristic to the alliance *Carpinion* such as: *Carpinus betulus*, *Tilia cordata*, *Ulmus minor* and *Acer campestre*.

The herb layer of the phytocoenosis is dominated by meso-xerophilous species such as *Carex montana*, *Chamaecytisus hirsutus* ssp. *leucotrichus*, *Brachypodium silvaticum*, *Brachypodium pinnatum*, *Clinopodium vulgare*, *Vincetoxicum hirundinaria*, *Asparagus officinalis*, *Teucrium chamaedrys*, *Veronica orchidea*, *Hypericum perforatum*, *Sedum maximum*, *Phleum phleoides*, which have, together, a cover of 40%.

Along them there can also be found some meso-xerophilous meadow species such as *Euphorbia cyparissias*, *Anthericum ramosum*, *Scabiosa ochroleuca*, *Linaria genistifolia*, *Allium fuscum*, *Centaurea bibersteinii*, *Coronilla varia*, *Achillea collina* and *Inula ensifolia*, proving even more the meso-xerophyllous character of this association.

The area at the base of the slope where the forest was cleared is covered by coenoses dominated by *Botriochloa ischaemum* and includes the species *Chrysopogon gryllus*.

The northern side of Petiş hill is covered by phytocoenoses of *Carpinus betulus* and *Quercus petraea*.

Considering the narrow distribution of *Quercus virgiliana* in Transylvania and its particular floristic structure - different from similar stands in Southeastern Romania, its conservation within the Natura 2000 site is thoroughly justified.

The phytocoenoses of *Quercus virgiliana* from the Southern part of Romania - Dobruja (Dihoru et al. 1965, Cristureanu et Ţuculescu 1970) and respectively the Southern Mehedinţi Plateau (Roman 1970) have a similar structure, rich in thermophilous (submediterranean) species and a particular ecology. They grow on skeletal soils-Rendzic Leptosols or Levigate Chernozems, with a slightly alkaline reaction (pH= 7,6).

We include all these communities in the association *Carpino orientalis-Quercetum virgilianae* Dihoru et al. 1965 (Tab. 1 col. 2).

Their tree layer is dominated by the species *Quercus virgiliana* and *Quercus pubescens,* while the shrub layer is dominated by thermophilous species such as *Carpinus orientalis*, *Cotinus coggygria*, *Syringa vulgaris*, *Cornus mas*, *Crataegus monogyna* and *Euonymus verrucosa*.

Within the herb layer there are present species such *Orchis simia*, *Tamus communis*, *Echinops bannaticus*, *Carex halleriana*, *Lithospermum purpurocoeruleum*, *Dictamus albus*, *Tanacetum corymbosum* and *Asparagus verticillatus,* that confer the meridional character of this association.

We remark that the phytocoenoses of *Quercus virgiliana* from Hungary which are floristically similar to the above-mentioned have been included in the association *Tamo-Quercetum virgilianae* Borhidi & Kevey 1996.

In what regards *Quercus pedunculiflora,* species with a Southeastern European distribution area (Bulgaria, Greece, former Yugoslavia, Romania and Turkey), the first phytocoenoses were described by Al. Borza (1937) in a study concerning the forests of Besserabia. At that time, quasi natural forests of *Quercus pedunculiflora,* with individuals having trunks of 50-60 cm diameter, still existed in Besserabia, in the area of the localities Cotugeni, Dobruşa and Manzâr.

These stands were situated on meadows and the slightly sloped neighboring hills, on Eutric Cambisols or Luvic Chernozems, with profound structure and rich in humus. They were grouped by Borza within the association *Quercetum pedunculiflorae* Borza 1937, that he considered to be specific to dry meadows with continental climate, coming in contact with herbaceous steppe vegetation.

The *Quercus pedunculiflora* forests were remarkable because of the vigorous trees, with conspicuous dark-green glossy and thick leaves, which were hirsute on the underside, having large, starred and bifurcate bristles. In the floristic structure of these stands there were fewer thermophilous species compared to the xerophilous forests which Borza grouped in the associations *Querceto-Lithospermetum cotinetosum* and *Quercetum pubescentis bessarabicum* (Borza 1937).

After the Second World War, the floristic and geobotanical studies have brought more data on the presence of *Quercus pedunculiflora* in the Southern and Eastern part of the country, data which was used by Borza (1960) in the geobotanical division of Romania.

Based on the distribution of *Quercus pedunculiflora* forests he has delimited the Balkan-Moesiac province: from southern Oltenia through Muntenia to the Sothern part of Moldavia – the forest-steppes of Covurlui and Bârlad, extending northwards as a narrow strip to the Iaşi and Suceava regions and towards South-East until Northern Dobruja and the Danube Delta.

Quercus pedunculiflora is also a companion species in the structure of xerophilous phytocoenoses dominated by *Quercus virgiliana* and *Q. pubescens* (Dihoru et al. 1965) and in *Fraxinus pallisae* (Kraush 1965) forests.

Later on, on the basis of presence or dominance of local species, the Romanian geobotanists have described more syntaxa, such as: *Querco pedunculiflorae-Tilietum tomentosae* Doniţă 1970, *Quercetum pedunculiflorae-cerris* Doltu, Popescu, Sanda 1980, *Centaureo stenolepi-Quercetum pedunculiflorae* Doniţă 1970, *Irido pseudocyperi-Quercetum pedunculiflorae* Chifu et al. 2001 and *Aro orientalis-Querceto pedunculiflorae* Chifu et al. 2004.

In Bulgaria, such forests have been described under the names *Quercetum frainetto-pedunculiflorae* Stoianov 1955 and *Quercetum frainetto-cerris*-subas. *Quercetosum pedunculiflorae* Goncev 1965, their floristic structure being similar to the forests described in Romania.

The classification of these associations in a separate alliance, *Quercion pedunculiflorae* Sanda et al. 1980 is only in part justified from a floristic point of view, as most of the species considered to be characteristic for the alliance (Chifu et al. 2006) are also mentioned within the alliance *Aceri tatarici-Quercion* Zólyomi et Jakucs 1957.

We specify that in the monograph elaborated by Horvat et al. (1974) „Vegetation Südösteuropas" the phytocoenoses with *Quercus pedunculiflora* from Romania and Bulgaria are included in the alliance *Aceri tatarici-Quercion* Zólyomi et Jakucs 1957, in the order *Quercetalia pubescentis* Br.-Bl. 1932. We consider this syntaxonomical classification to be valid.

From the floristic and pedoecological analysis of *Quercus pedunculiflora* phytocoenoses from Romania, we have concluded, according to the code of phytosociological nomenclature (Weber et al. 2000) that they belong to two distinct associations.

The first association, *Tilio tomentosae-Quercetum pedunculiflorae* Doniţă 1968 (Syn: *Violo suavis-Quercetum pedunculiflorae* Doniţă 1970, *Centaureo stenolepi-Quercetum pedunculiflorae* Doniţă 1970, *Aro orientali-Quercetum pedunculiflorae* Chifu et al. 1998, *Quercetum pedunculiflorae* Borza 1937), comprises *Quercus pedunculiflora* forests from plateaus or slightly sloped hills, less exposed to the sun, growing on Levigate Chernozems, from Dobruja (Doniţă 1970) and Moldavia (Chifu et al. 2004).

In the tree layer of the association, the regional species *Quercus pedunculiflora* and *Tilia tomentosa* are dominant, with an average cover of 50%. In the shrub layer, there are present: *Acer tataricum*, *Fraxinus ornus*, *Carpinus orientalis*, *Cornus mas*, *Euonymus europaeus*, while in the grass layer there can be found the species: *Paeonia*

peregrina, *Asparagus tenuifolius*, *Carex michelii*, *Bromus benekenii*, *Viola hirsuta*, *Melica uniflora*, *Lithospermum purpurocoeruleum* and *Arum orientale*. They confer a Southeastern European character to the association.

The second association, *Fraxino pallisae-Quercetum pedunculiflorae* Oprea 1997 (Syn: *Fraxino angustifoliae-Quercetum pedunculiflorae* Chifu et al. 1998), has been identified in the meadows of Bârlad, Siret, Prut and Buzău (Sanda 1970, Oprea 1997, 2004, Chifu et al. 1998), on alluvial soils and Levigate Chernozems moist with phreatic water, developed on loess-like deposits (Tab. 1 col. 3).

In the tree layer dominant species are: *Quercus pedunculiflora*, *Fraxinus angustifolia* and *Fraxinus pallisae*, with a cover of about 55%. In the shrub and grass layers there are meso-xerophilous species characteristic for the alliance *Aceri tataricae-Quercion* Zolym. & Jakucs 1957, as well as hygro-mesophilous species characteristic for the alliance *Alno-Padion*, which makes the classification of this association in the coenosystem difficult.

Since in the summer period the soil in these stations is well drained, due to the loess-like substrate (Oprea 2004), we consider that the association suits better within the alliance *Aceri tataricae-Quercion*, then in the alliance *Alno-Padion.*

We have arranged the forest associations with *Quercus virgiliana* and *Quercus pedunculiflora* from Romania in the following coenosystem:

Cls. Quercetea pubescenti-petraeae Jakucs 1961

Ord. Orno-Cotinetalia Jakucs 1961

Al. Syringo-Carpinion orientalis Jakucs et Vida 1959

As. *Carpino orientalis-Quercetum virgilianae* Dihoru, Ţucra et Bavaru 1965 nom. invers.

Ord. Quercetalia petraeae-pubescentis Jakucs 1961

Al. Aceri tatario-Quercion Zolyomi et Jakucs 1957

As. *Carici montanae-Quercetum virgilianae* Coldea et Filipaş 2009 (in press)

As. *Tilio tomentosae-Quercetum pedunculiflorae* Doniţă 1968

As. *Fraxino pallisae-Quercetum pedunculiflorae* Oprea 1997

Table 1.

Forest associations with *Quercus virgiliana* and *Quercus pedunculiflora*

Association nr. **Number of relevés** **Altitude m.s.m.**	**1** **5** **437-476**	**2** **20** **50-230**	**3** **99** **40-325**	**4** **31** **10-43**
1. Tree layer				
Quercus virgiliana	V	V	-	-
Quercus pubescens	I	V	I	-
Quercus pedunculiflora	-	I	V	V
Acer tataricum	-	-	IV	II

Tilia tomentosa	-	I	III	-
Tilia cordata	I	-	I	-
Tilia platyphyllos	-	-	I	-
Quercus cerris	I	-	-	-
Quercus dalechampi	-	II	I	-
Quercus robur	-	-	I	II
Cerasus avium	-	-	I	-
Carpinus betulus	III	-	II	I
Acer campestre	II	I	IV	I
Fraxinus angustifolia	-	-	-	IV
Fraxinus pallisae	-	-	-	IV
Quercus polycarpa	-	II	-	-
Sorbus domestica	-	II	-	-
Fraxinus excelsior	-	-	II	-
Sorbus torminalis	-	-	II	I
Ulmus procera	-	-	II	-
Populus alba	-	-	-	I
Quercus frainetto	-	I	-	-
2. Shrub layer				
Cotinus coggygria	-	V	I	-
Carpinus orientalis	-	V	I	-
Crataegus monogyna	V	II	IV	II
Ligustrum vulgare	IV	I	III	II
Prunus spinosa	IV	I	I	II
Prunus pyraster	III	II	III	II
Rosa canina	III	-	I	I
Ulmus minor	I	-	I	II
Fraxinus ornus	-	III	II	-
Syringa vulgaris	-	I	-	-
Cornus mas	-	III	IV	I
Euonymus verrucosa	-	III	II	-
Viburnum lantana	-	II	II	-
Euonymus europaeus	-	-	III	II
Corylus avellana	-	-	I	I
Cornus sanguinea	-	-	II	II
3. Herb layer				
Carex montana	V	-	-	-
Chamaecytisus h. leucotrichus*	V	-	-	-
Euphorbia cyparissias	V	-	-	-
Veronica orchidea	IV	-	-	-
Orchis simia	-	IV	-	-
Tamus communis	-	III	-	-
Laser triloba	-	IV	-	-
** Paeonia peregrina*	-	-	III	-
Myrrhoides nodosa	-	-	II	-
Veratrum nigrum	-	-	III	-
** Arum orientale*	-	I	III	-
Ornithogalum fimbriatum	-	-	II	-
Brachypodium sylvaticum	V	I	IV	II

Clinopodium vulgare	V	I	II	I
Vincetoxicum hirundinaria	V	III	II	II
Teucrium chamaedrys	V	II	I	-
Agrimonia eupatoria	V	-	II	-
Fragaria viridis	V	III	II	-
Phleum montanum	V	I	-	-
Asparagus officinalis	IV	-	I	I
Hypericum perforatum	IV	I	I	I
Sedum maximum	IV	I	I	-
Anthericum ramosum	IV	-	-	I
Melampyrum bihariense	IV	-	I	-
Torilis japonica	IV	-	II	-
Elymus repens	IV	-	I	II
Dactylis polygama	III	-	IV	I
Asperula cynanchica	III	-	-	-
Stachys recta	III	-	I	-
Scabiosa ochroleuca	III	-	-	-
Linaris genistifolia	III	-	-	-
Clematis vitalba	III	-	I	I
Campanula sibirica	III	-	-	-
Festuca heterophylla	III	-	-	-
Chrysopogon gryllus	II	-	I	-
*Dianthus c. * glabriusculus*	II	-	-	-
Brachypodium pinnatum	II	-	I	-
Allium fuscum	II	-	-	-
Silene otites	II	-	I	-
Poa nemoralis	II	I	I	-
Cruciata glabra	II	-	I	-
Centaurea bibersteinii	II	-	-	-
Trifolium alpestre	II	-	I	-
Hieracium sabaudum	II	-	-	-
Silene viridiflora	II	-	-	-
Geum urbanum	II	I	V	II
Alliaria petiolata	II	I	IV	I
Coronilla varia	II	-	I	I
Achillea collina	II	-	-	-
Allium schoenoprasum	II	-	-	-
Peucedanum carvifolia	I	-	-	-
Delphinium fissum	-	II	-	-
Echinops bannaticus	-	II	-	-
Carduus candicans	-	II	-	-
Carex halleri	-	II	-	-
Acanthus longifolius	-	I	-	-
Lithospermum purpurocoeruleum	-	III	III	II
Lunaria annua	-	II	-	-
Dictamus albus	-	III	-	-
Coronilla elegans	-	II	-	-
Geranium sanguineum	I	II	I	-
Tanacetum corymbosum	-	I	I	-

Potentilla micrantha	-	II	I	-
Polygonatum latifolium	-	IV	II	II
Crocus moesiacus	-	II	-	-
Achillea chrythmifolia	-	II	-	-
Bromus riparius	-	II	-	-
Cleistogene serotina	-	II	-	-
Orlaya grandiflora	-	II	-	-
Nectaroscordum dioscoridis	-	-	I	-
Viola jordanii	-	-	II	-
Asparagus tenuifolius	-	-	III	III
Carex michelii	-	I	II	-
Viola hirta	-	I	III	-
Fallopia dumetorum	-	-	II	-
Melica uniflora	-	-	II	-
Anthriscus cerefolium	-	I	III	II
Galium aparine	-	-	IV	II
Bromus benekenii	-	-	I	-
** Centaurea stenolepis*	-	-	I	-
Asparagus verticillatus	-	II	I	-
Poa angustifolia	-	-	I	II
** Viola suavis*	-	-	I	I
Leonurus cardiaca	-	-	II	I
Mercurialis ovata	-	I	I	-
Pulmonaria obscura	-	-	II	I
Carex precox	-	-	I	-
Verbascum phoeniceum	-	-	II	I
Vinca herbacea	-	I	I	-
Lamium purpureum	-	-	II	I
Doronicum hungaricum	-	-	I	-
Valeriana officinalis	-	-	I	I
Astragalus glycyphyllus	-	I	II	I
Veronica chamaedrys	-	I	I	I
Scrophularia nodosa	-	-	II	I
Bromus inermis	-	-	I	-
Elymus hispidus	-	I	I	-
Veronica hederifolia	-	-	II	-
Peucedanum alsaticum	-	II	I	-
Iris graminea	-	-	I	-
Campanula persicifolia	-	I	I	-
Lathyrus niger	-	-	I	I
Primula veris	-	-	I	-
Lactuca quercina	-	-	I	-
Pulmonaria mollis	-	-	I	-
Polygonatum odoratum	-	I	I	II
Scutellaria altissima	-	-	I	-
Galium schultesii	-	-	I	-
Stellaria holostea	-	-	II	-
Geranium robertianum	-	-	I	-
Lilium martagon	-	-	I	-

Acer platanoides	-	-	I	-
Convallaria majalis	-	-	I	I
Viola reichenbachiana	-	-	I	I
Cherophyllum temulum	-	-	I	-
Carex brevicollis	-	-	I	-
Melica picta	-	-	I	-
Stachys sylvatica	-	-	I	-
Serratula tinctoria	-	-	-	II
Rubus caesius	-	-	-	III
Lysimachia nummularia	-	-	-	III
Rumex sanguineus	-	-	-	II
Viburnum opulus	-	-	-	II
Cruciata laevipes	-	-	-	II
Ranunculus repens	-	-	-	II
Glechoma hederacea	-	-	-	III
Carex remota	-	-	-	III
Cardamine impatiens	-	-	-	III
Frangula alnus	-	-	-	I
Aristolochia clematitis	-	-	-	III
Lychnis coronaria	-	-	-	I
Trifolium medium	-	-	-	II
Ranunculus auricomus	-	-	-	II
Carex spicata	-	-	-	I
Carex sylvatica	-	-	-	I
Sium latifolium	-	-	-	II
Agrostis stolonifera	-	-	-	III
Lycopus europaeus	-	-	-	I
Carex hirta	-	-	-	II
Stachys officinalis	-	-	-	I
Thalictrum lucidum	-	-	-	III
Glechoma hirsuta	-	-	-	II
Asparagus pseudoscaber	-	-	-	II
Vitis sylvestris	-	-	-	II
Periploca greca	-	-	-	I
Galium rubioides	-	-	-	I
Malus sylvestris	-	-	-	I
Symphytum officinale	-	-	-	II
Urtica dioica	-	-	-	I
Physalis alkekengi	-	-	-	I
Stachys palustris	-	-	-	II
Amorpha fruticosa	-	-	-	I
Glycyrrhiza echinata	-	-	-	I

1. *Carici montanae-Quercetum virgilianae*, Coldea et Filipaş, 2009 (sub tipar)
2. *Carpino orientalis-Quercetum vigilianae*, Dihoru et all., 1965
3. *Tilio tomentosae-Quercetum pedunculiflorae*, Doniţă 1968
4. *Fraxino palissae -Quercetum pedunculiflorae*, Oprea, 1997

Conclusions

1. Considering the morphological features that differentiate the two taxa, namely achene morphology, scales at the base of the cup and leaf morphology, as well as the specific ecological conditions and distribution area, we consider their treating as valid species to be justified.
2. The syntaxonomical revision of these forests was done using both literature data and field research. Based on our research in the Natura 2000 Site from Petiş, we have described a new association with this species: *Carici montanae-Quercetum virgilianae*. We have assigned the phytocoenoses with *Quercus virgiliana* from Southern Romania (Dobruja and the Southern Mehedinți plateau), to the association *Carpino orientalis-Quercetum virgilianae*.
3. The phytocoenoses with *Quercus pedunculiflora* can be included in two associations. The first association, *Tilio tomentosae - Quercetum pedunculiflorae* is found in stands from plateaus and moderate slopes of Dobruja and Moldavia. The second association, *Fraxino pallisae - Quercetum pedunculiflorae* is specific to the meadows of Bârlad, Siret, Prut and Buzău rivers.

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THE EFFECTS OF TEMPERATURE ON GROWTH AND LIPID FATTY ACID COMPOSITION IN CYANOBACTERIUM *SYNECHOCYSTIS* SP. STRAIN AICB 51

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SUMMARY. The *Synechocystis* sp. AICB 51 strain is a mesophilic cyanobacterium able to use the inorganic carbon added to the growth medium as $NaHCO_3$. The optimization of the growth factors and the quality of the biomass constituents represent two essential factors for the development of certain applications. The effect of temperature (24-36°C) on the growth process in batch system was studied, as well as the fatty acids composition. The optimal growth temperature is of approx. 30°C, in fluorescent light with 630 $\mu mol \cdot m^{-2} \cdot s^{-1}$ irradiance. A specific growth rate of 0.8 $days^{-1}$ and a doubling time of 1.2 days were observed. The gas chromatography analysis of the methyl esters has displayed a profile with a low number of molecular species, the lack of the polyunsaturated fatty acids and a high amount of myristic acid (14:0). The composition variations caused by the growth of the AICB 51 *Synechocystis* strain in different temperatures (24-36°C) were noticeable, particularly in the supra-optimal interval (33-36°C). An increase of palmitic acid content and a decrease of palmitoleic acid (the dominant monounsaturated acid) were observed in this temperature interval, possibly due to a lowered activity of the temperature-dependent desaturases.

Keywords: cyanobacterium, fatty acids, gas chromatography, growth, temperature effects.

Introduction

The *Synechocystis* sp. strain AICB 51 (Fig.1) is a mesophilic and alkaline-tolerant cyanobacterium which displays high growth rates when large quantities of $NaHCO_3$ are present in the growth medium. Thus, this strain is potentially valuable for biotechnological applications in the field of residual carbon dioxide (CO_2) fixation into the biomass. Additionally, since the AICB 51 strain originated in Africa, it is likely to be adapted to relatively high temperatures.

The aim of the present study was to identify the optimal growth temperature for the AICB 51 strain and to study the fatty acid composition of the biomass, assayed at different temperatures.

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Material and methods

Strain and culture conditions. Synechocystis sp. strain AICB 51 (Fig. 1) was provided by the Algae and Cyanobacteria Culture Collection (AICB) of the Babeş-Bolyai University, Cluj-Napoca. The strain was grown photoautotrophically in a batch bioreactor (Applikon) culture of 900 ml, in Zarrouk (Z) growth medium (Dragoş *et al.,* 1997), under continuous mechanical stirring (400 rpm) and without CO_2 addition. The cultures were continuously illuminated with fluorescent light (irradiance of 630 $\mu mol \cdot m^{-2} \cdot s^{-1}$). The batch culture growth experiments were carried out at temperatures of 24, 27, 30 and 36°C, respectively (±0.4°C).

Measurement of cell culture growth. The growth of cyanobacterial cultures was quantified by daily measurements of optical density at 600 nm using a Cell Density Meter Model 40, Fisher Instruments and is expressed as $\log_2 OD_t/OD_0$. These values were plotted according to optical density and used to determine the exponential growth rate and the doubling time (Sorokin, 1973; Wood *et al.,* 2005).

Quantification of photosynthetic pigments. The cells were pelleted by centrifugation (3000 x g, 10 min.) and subjected to mechanical lysis prior to acetone extraction of chlorophyll *a* and carotenoids. The amounts of chlorophyll *a* and carotenoids, expressed as mg/l, were photometrically quantified (absorption peaks at 665 and 480 nm, respectively) based on the specific absorption coefficients (Arnon, 1949; Lichtenthaler and Wellburn, 1983). The phycobiliproteins were extracted in 0.5 M Sørensen's phosphate buffer (pH 6.2). The phycocyanin content of the extract was also photometrically quantified (absorption peak at 620 nm) using Gantt and Lipschultz (1974) formulas.

Lipid extraction and gas chromatography quantification of fatty acids. The cellular pellet obtained by centrifugation (3000 x g, 10 min) was subjected to a chloroform: methanol (2:1 v/v) extraction using the Folch method (Folch *et al.,* 1957; Kates, 1972). After separation, the liquid phase was completely evaporated at 65°C under nitrogen atmosphere. The methyl esters obtained through transesterification with methanol and acetyl chloride were extracted three times in *n*-hexan. The analysis of methyl esters was performed with a Hewlett-Packard 5890 D gas chromatograph, using nitrogen as gas carrier (2 ml/min) and a flame ionization detector. The analysis was conducted with linear programming of temperature (10°C/min, from 125°C to 250°C) in a separation column HP-INNO Wax (30m x 0.25 mm) with a polar separation phase. The methyl esters of the fatty acids were identified by comparing their retention time (t_r) with the corresponding values of standard samples assayed in the same conditions. The relative content (molar percentage) of the esterified fatty acids was estimated by the analysis of the chromatographic peak areas with a data processor.

Results

The growth of *Synechocystis* sp. strain AICB 51 under continuous mechanical stirring (400 rpm) resulted in higher growth rates, optimizing both the cell exposure to light in the batch culture and the gas transfer at the air-water interface. The batch cultures have rapidly reached the exponential growth phase in approximately one day from inoculation (Fig. 2).

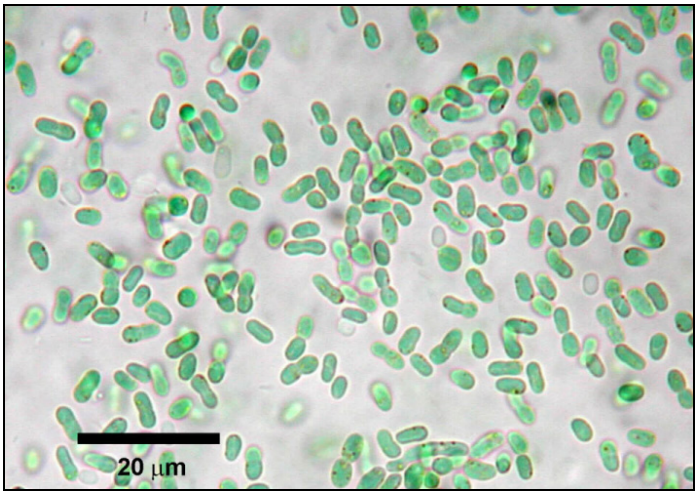

Fig. 1. Light microscopy aspect of *Synechocystis* sp. AICB 51 strain.

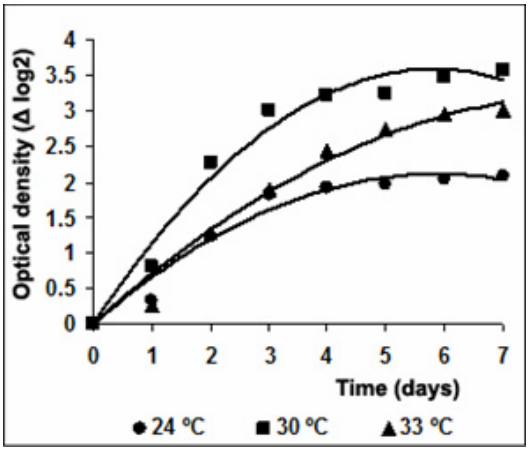

Fig. 2. Growth curves of *Synechocystis* sp. AICB 51 strain in batch culture (Applikon bioreactor). Only three temperatures were selected for the graphical representation; optimal growth at 30°C.

The growth rate was temperature-dependent, its maximum being observed at 30°C (0.83 day^{-1}) (Fig. 3). At this temperature the biomass doubling time was approx. 1.2 days (Fig. 3). With no additional administration of CO_2, the pH increased from 8.8 to 9.8 due to the consumption of the bicarbonate ion in the nutrient solution and its fixation into cell biomass. The supraoptimal temperatures (33-36°C) caused a decrease of the exponential growth rate, an increase of the doubling time (Fig. 3) and a reduction of the pigment content of the cells, especially phycocyanin (Fig. 4).

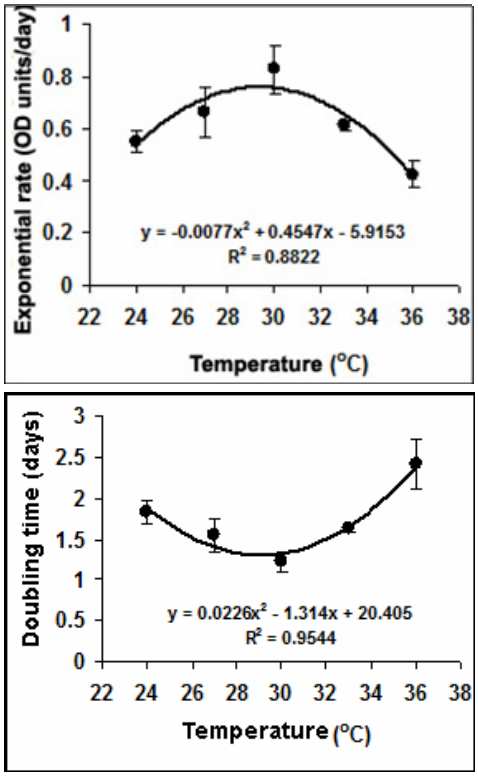

Fig. 3. The temperature-dependent evolution of exponential growth rate (upper panel) and of doubling time (lower panel) in *Synechocystis* sp. AICB 51 cultures. Both parameters were calculated based on optical density measurements and indicate an optimum temperature of approx. 30°C.

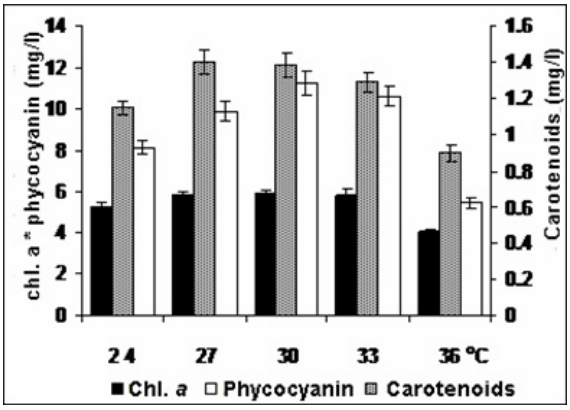

Fig. 4. The pigment concentration levels in the cyanobacterial culture grown at different temperatures.

Gas chromatographic analysis of fatty acid methyl esters extracted from cell mass revealed the presence of five major species of fatty acids (Tab. 1): 3 types of saturated fatty acids (myristic-14:0, palmitic-16:0 and stearic-18:0) and 2 types of monounsaturated fatty acids (palmitoleic-16:1 and oleic-18:1). The stearic acid was detected in small amounts (0.3-0.7%), while the polyunsaturated fatty acids were not detected at all (Tab. 1). The most abundant fatty acids were the myristic acid (11.1-15.6%), the palmitic acid (31-37.5%) and palmitoleic acid (48.9-54.5), regardless of the growth temperature. This pattern places the *Synechocystis* sp. AICB 51 strain within the group I of the four cyanobacterial groups described by Kenyon (1972) and Kenyon *et al.,* (1972) based on the fatty acid composition of the cell.

Temperature-dependent variations of the fatty acid composition of the cell were observed for both saturated and unsaturated fatty acids within the temperature interval of 24-36 ^{o}C. In general, the amount of myrstic acid (14:0) decreased with temperature increase. The cellular content of palmitic acid was constant within the temperature range of 24-30^{o}C, but increased at supraoptimal temperatures (33-36^{o}C) (Tab. 1). Different temperature-induced changes were observed in the composition of monounsaturated fatty acids (16:1 and 18:1). Thus, palmitoleic acid content showed a slow growth tendency in the 24-30^{o}C temperature interval but decreased significantly at supraoptimal temperature (33-36^{o}C), while oleic acid remained at relatively constant levels in the 24 - 33^{o}C temperature interval, with a significant increase at 36^{o}C. On the whole, the degree of fatty acid unsaturation of the cyanobacterial cells decreased at supraoptimal growth temperatures.

Table 1

The fatty acids composition of *Synechocystis* sp. AICB 51 grown at different temperatures in Zarrouk medium.

Fatty acids		Retention time	% of total fatty acid content				
			24^{0} C	**27^{0} C**	**30^{0} C**	**33^{0} C**	**36 ^{0}C**
Myristic	$C_{14:0}$	6.08	15.63	14.90	11.72	15.28	11.10
Palmitic	$C_{16:0}$	8.06	31.05	31.07	31.50	37.57	35.93
Palmitoleic	$C_{16:1}$	8.31	51.56	5245	54.54	45.02	48.91
Stearic	$C_{18:0}$	9.76	0.31	0.30	0.46	0.23	0.70
Oleic	$C_{18:1}$	9.97	1.36	1.26	1.77	1.89	3.34
Total fatty acids			99.91	99.98	99.99	99.99	99.98
Unsaturated fatty acids			52.92	54.04	56.31	46.91	52.25
Saturated fatty acids			46.99	45.97	43.68	53.08	47.73
Unsaturated/Saturated ratio			1.13	1.17	1.29	0.88	1.09

Discussions

Synechocystis sp. AICB 51 strain is a unicellular mesophilic cyanobacterium that shows a high rate of growth at temperatures closed to 30 ^{o}C under high irradiance, when sufficient supply of $NaHCO_3$ is added to the growth medium. The growth performance is comparable to that of other similar unicellular strains (Sakamoto *et al*., 1997).

Our data on the fatty acid composition of *Synechocystis* sp. AICB 51 strain showed very few molecular species, the absence of polyunsaturated fatty acids and an increased level of myristic acid (14:0).

Kenyon (1972) and Kenyon *et al*. (1972) classified cyanobacteria into 4 groups based on their fatty acid composition. Subsequently, Murata *et al.* (1992) confirmed this classification, arguing that it is sustained by the desaturation reactions of the fatty acids in cyanobacteria. They also showed that this classification does not overlap with the traditional morphological one, each of the 4 groups including both unicellular and filamentous species/strains. This system and its chemotaxonomic significance were confirmed in all cyanobacterial groups. (Caudales and Wells, 1992; Caudales *et al.,* 1995, 2000; Cohen and Vonshak, 1991; Li *et al.*, 1998; Li and Watanabe, 2004; Otsuka *et al.,* 1999; Suda *et al.,* 2002). The strain *Synechocystis* sp. AICB 51 can be assigned to group 1 which, according to Murata *et al.*, (1992), comprises cyanobacteria with monounsaturated but not polyunsaturated fatty acids. A similar composition of fatty acids was observed by Kenyon (1972) in several rod-shaped "*Synechococcus*-like" cyanobacteria and subsequently in a limited number of thermophilic (Maslova *et al.*, 2004) and marine strains (Matsunaga *et al*., 1995). The absence of polyunsaturated fatty acids was also observed in certain filamentous cyanobacteria, such as *Oscillatoria limnetica* (Oren *et al*., 1985).

The myristic acid is ubiquitous in various morphologically and taxonomically different cyanobacteria (Caudales and Wells, 1992; Caudales *et al*., 2000; Kenyon, 1972; Li *et al*., 1998, 2001; Li and Watanabe, 2004; Porankiewicz *et al*., 1998; Otsuka *et al*., 1999) but it usually represent only a small fraction of the total fatty acid content (0.2-0.8%). A higher amount of myristic acid (14-23%), comparable to our data for the AICB 51 strain (11-15.6%), was observed in a limited number of cyanobacteria, such as *Synechococcus* or *Synechococcus*-like strains, some of which are thermophilic or marine (Kenyon, 1972; Maslova *et al*., 2004; Matsunaga *et al*., 1995). In certain strains of *Synechococcus,* an increase in the myristic acid was found in the death phase of growth and also under high irradiances (Maslova *et al*., 2004).

Compositional changes caused by incubating strain *Synechocystis* AICB 51 at different temperatures (24-36 ^{o}C) were observed especially in the supraoptimal temperature range (33-36 ^{o}C). In this range, the palmitic acid content increased and the palmitoleic acid (the dominant monounsaturated acid) content decreased, possibly due to the temperature-induced inhibition of the desaturase enzymatic activity. Increased palmitic acid content (44.6% at 24 ^{o}C and 53% at 35 ^{o}C) associated with increasing temperature was previously reported in *Arthrospira platensis* (Pham Quoc and Dubacq, 1997).

It is well-known that fatty acid desaturation in plasma membrane and thylakoid membranes is a critical step in cyanobacterial acclimation to low ambient temperatures, as a compensatory mechanism to increase cell membrane fluidity (Mikami and Murata, 2003; Murata *et al*., 1992; Sakamoto *et al*., 1997; Sarcina *et al*., 2003; Singh *et al*., 2002). Four desaturases have been described in cyanobacteria, three of

which are temperature dependent (Honsthong *et al*., 2003; Los *et al*., 1997). Our data, including the decrease of the unsaturation level of fatty acids in *Synechocystis* sp. AICB 51at supraoptimal growth temperatures might be caused by an inhibition in the desaturase activity. The decrease of the myristic acid fraction in the total fatty acid content, when the *Synechocystis* sp. AICB 51 strain was exposed to increasing temperatures (24-36^{o}C) remains to be elucidated.

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== SHORT COMMUNICATION ==

OXIDATIVE STRESS ENZYMES AS BIOMARKERS OF HEAVY METAL POLLUTION IN INTERSTITIAL INVERTEBRATES

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SUMMARY. Chemical contamination of fresh and marine water has a strong influence on the metabolic status of aquatic organisms. The expression level and catalytic activity of enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) etc., could be sensitive biomarkers for the oxidative stress induced in aquatic organisms by abnormal levels of biotic and abiotic factors. The present work aimed to study the effect of increasing lead concentration under controlled conditions on the levels of SOD activity in living microinvertebrates and to find the most appropriate class of interstitial invertebrates that respond promptly to the heavy metal contamination. The interstitial water samples were collected at Station Scărişoara on Arieş River, during March 2010. The microinvertebrates found have been sorted and identified as belonging to Oligochaeta, Nematoda and Copepoda (Cyclopida) groups. The organisms of each group have been separated and incubated in the presence of lead concentration similar to that determined in the original environment (30 µg Pb/dm^3). Biological samples consisting of living microinvertebrates have been analysed for SOD activity at 0 and 24 hours, and after 7 days of incubation, respectively. Our preliminary results suggest a modulation of activity of SOD, by lead ions present in the surroundings of living microinvertebrates tested.

Keywords: Arieş River, biological indicators, lead, Oligochaeta, superoxide dismutase.

Introduction

The activity of oxidative stress enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) glutathione reductase (GR) and glutathione-S-transferase (GST) could be important markers to estimate the impact of heavy metal pollution on metabolic status of an organism. High intracellular concentration of reactive oxygen species (ROS) indicates an oxidative stress that might be caused by various biotic or abiotic factors. It has been shown that SOD activity might be a sensitive indicator of oxidative stress generated in the bodies of water invertebrates by chemical contamination and/or by abnormal physical parameters of their surroundings (Cantú-Medellín *et al.*, 2009).

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To date, there are few studies on interstitial microinvertebrates as potential bioindicators for freshwater pollution. Most of invertebrate models used as indicators for organic or inorganic pollution in sediments and marine water are macroscopic arthropods, such as crabs (Martin-Diaz *et al.*, 2007), and bivalves, such as mussels (Vlahogianni *et al*. 2007; Vidal-Liñán *et al.*, 2010). For the assessment of soil pollution, earthworms (especially *Eisenia foetida*) are currently used as biological indicators (Sanchez-Hernandez, 2006).

Our previous research has demonstrated that the increase of SOD activity in whole body extract from interstitial invertebrates collected in sampling stations along Arieş River (Central Western Romania) could be correlated with high lead (Pb) concentration detected in the sampled water.

The aim of present research was to study the effect of lead concentration under controlled conditions on the levels of SOD activity in living microinvertebrates and to find the most appropriate class of interstitial invertebrates that respond promptly to the heavy metal contamination.

Materials and methods

Preparation of biological samples for superoxide dismutase (SOD) activity assay

The interstitial water samples were collected at Station Scărişoara on Arieş River (Apuseni Mountains, Central Western Romania), during March 2010. The physical and chemical parameters measured in sampled water were pH 7.95, conductivity of 622 μS/cm and 5.8^{o}C. The microinvertebrates found have been sorted and identified as belonging to Oligochaeta, Nematoda and Copepoda (Cyclopida) groups. The organisms of each group have been separated and incubated in the presence of lead concentration similar to that determined in the original environment (30 μg Pb/l). As control groups, organisms incubated in lead-free water were used. Biological samples (living invertebrates) have been analyzed for SOD activity at 0 and 24 hours, and after 7 days of incubation, respectively.

Biological samples were prepared for SOD assay according to a method described by de Oliveira *et al.* (2005) as follows. Whole organisms for each group were lysed in 0.5 M sucrose, buffered at pH 7.4 with 20 mM Tris–HCl (7 ml/g of tissue), at 4^{0}C. Phenylmethanesulfonyl fluoride (PMSF), as protease inhibitor, was prepared as 100 mM stock solution in isopropanol. PMSF was added to the lysis buffer (10 μl /ml of buffer). Samples were homogenized in lysis buffer using a Potter-Teflon homogenizer. The homogenates were incubated for 30 minutes on ice and centrifuged at 10,000×g, 4^{0}C, for 10 min. The supernatants were collected and the total protein content of the lysates was determined colorimetrically using Coomassie Brilliant Blue G-250 (Sigma) as a dye (Bradford, 1976) and bovine serum albumin as a standard.

Determination of SOD activity

To evaluate the SOD activity in lysates a indirect method was performed using a SOD Assay Kit (Biochemika, Sigma-Aldrich). The SOD activity was measured by the inhibition of the reduction of highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) by superoxide anion (O_2^-) produced by xanthine oxidase activity. Upon reduction of WST-1 with the superoxide anion, a water-soluble formazan dye is developed that could be measured colorimetrically at 450 nm. The rate of the reduction with O_2^- are linearly related to the inhibiton of xanthine oxidase activity by SOD activity. As the absorbance at 450 nm is proportional to the amount of superoxide anion, the SOD activity as an inhibition activity can be quantified by measuring the decrease in the color development at 450 nm with a reference wavelength of 630 nm (Ukeda et al., 2002). The assay was performed in 96-well plates by using a Stat Fax 2100 Microplate Reader (Awareness Technology, Palm City, FL). For each sample, SOD activity was determined for the same amount of protein (10 μg). Blanks and samples were prepared according to the manufacturer's instructions. The activity of SOD was expressed as % inhibition of xanthine oxidase activity.

Statistical analysis of SOD activity results

SOD activity for each experimental group was determined in triplicate. The final results represent mean ± S.D of three measurements. For statistical analysis, a value of $P<0.05$ was considered significant. The differences between the SOD activities in different samples at the same season were analyzed by one-way ANOVA with Bonferroni correction for multiple comparisons using GraphPad Prism v.4.02 for Windows, GraphPad Software (San Diego, CA).

Results and discussions

The results of SOD activity measured in whole body extracts of tested invertebrates were shown below, in Figures 1A (for Cyclopoida), 1B (for Oligochaeta) and 1C (for Nematoda).

Only for Oligochaeta individuals we have found a statistically significant difference of SOD activity in Pb-incubated organisms, at 24 h time point, compared to the control group ($P<0.001$).

The aquatic invertebrates are promising model organisms for studying the effects of inorganic pollutants (heavy metals) and organic (pesticides, oil products) on natural ecosystems. Morevoer, the biochemical parameters tested in invertebrates sampled in mineral, organic or mixed polluted sites, could indicate a certain degree of water (Pempkowiak *et al*., 2006) or sediments pollution (Martín-Díaz *et al*., 2007). Many authors support the integrated approach of biomarkers such as oxidative stress enzymes and antioxidants to unequivocally establish the degree of pollution perceived by organisms, either invertebrates or vertebrates (Morales-Caselles *et al*., 2008; Antunes *et al*., 2010).

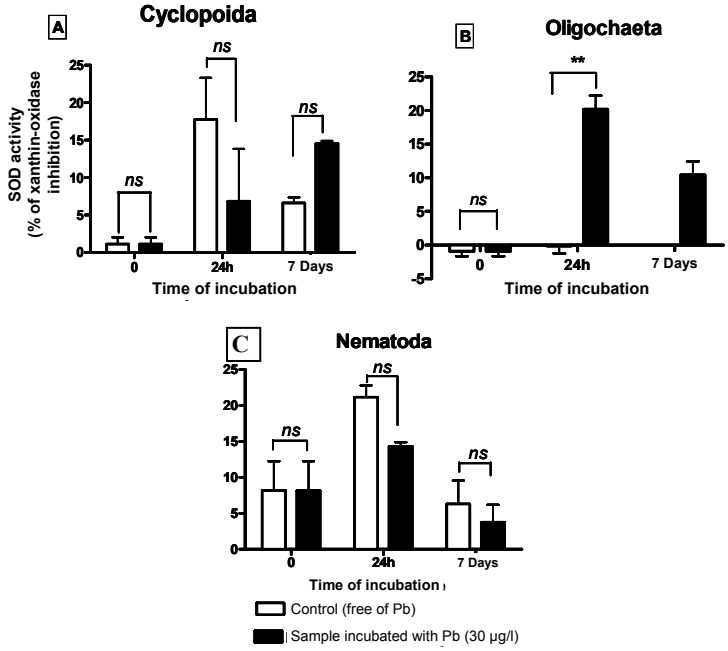

Fig. 1. SOD activity in whole-body lysates obtained from the invertebrates belonging to Cyclopoida (A), Oligochaeta (B) and Nematoda (C) group at different time interval of incubation with Pb. data are presented as mean± S.D of three measurements; *ns*: not statistically significant results; **: P<0.01.

The effect of heavy metals, such as cadmium (Cd) or nickel (Ni), on the chemical and enzymatic parameters (SOD, glutathione peroxidase - GPx, glutathione-S-transferase - GST, acetilcholinesterase – AchE, reduced gluathione - GSH and reduced to oxidized glutathione ratio - GSH/GSSG) in marine copepod *Tigriopus japonicus* has been tested in laboratory (Wang and Wang, 2009; Wang and Wang, 2010). Similar to our observations regarding the Pb effect, the authors noticed that under high levels of Cd and Ni the activities of SOD (and GST) have been increased in the first 24 hours of incubation (with Cd), and first 7 days of incubation (with Ni), respectively. In the same time, the activity of GPx was inhibited. The stimulatory or inhibitory effects of tested heavy metals have been directly correlated with the tested concentrations. Biochemical parameters of oxidative stress in the observed individuals reached the normal levels found in the control organisms until day 12 of exposure.

Among annelids, earthworms (*Eisenia foetida*) and aquatic worm, *Tubifex tubifex*, are known biological indicators for the assessment of soil and water contamination, respectively (Sanchez-Hernandez, 2006; Paris-Palacios *et al*., 2010). However, mostly the organic pollutants are tested in these organisms. In this light, our observation that Oligochaeta individuals tested for SOD activity proved to react promptly and visibly to Pb ions as stress factor, is in accordance with known literature.

Conclusions

The experiments described in this paper have shown an increase of SOD activity in tissue extracts from Oligochaeta organisms tested. We did dot find any significant differences for glutathione reductase (GR) activity in tested invertebrates (data not shown). One explanation might be that SOD is a first-line defence enzyme in oxidative stress induced by unusual values of physical parameters or by high levels of inorganic (or organic) pollutants. It appears that the fast involvement of SOD in neutralizing the ROS would be a quick response strategy to face abiotic stress caused by high concentration of Pb in the environment of tested organisms.

Based on the results presented here, we propose the microscopic Oligochaeta as a promising biological indicator group to assess the freshwater lead pollution.

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== SHORT COMMUNICATION ==

THE EFFECT OF *AGRIMONIA EUPATORIA* AQUEOUS EXTRACT ON CANCER CELLS- A PRELIMINARY STUDY

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SUMMARY. Cancer is the second leading death cause worldwide. Medicinal plants are proving very promisisng in treating various forms of cancers. Here we report the effect of *Agrimonia eupatoria* aqueous extract on MCF-7 breast cancer cells.

Keywords: agrimony, cancer cells, medicinal plants

Introduction

Cancer is the second leading cause of death worldwide. There is a global effort for researching and discovering new drugs for treating cancer and considerable achievements and progress have been made in early detection of the disease, more efficient treatment with prolonged life of patients. In spite of this tremendous progress, the need of better drugs and treatment remains an urge on the entire Globe.

In parallel with the research efforts in the chemical industry there is a growing interest for research and development of new drugs from plants.

There are many plants used for cancer treatment or as adjuvant in cancer patients used in traditional medicine of worldwide cultures and some of them have made their way to clinical trials and drug stores. To date, the most used and efficient drugs in cancer control are obtained from plants (e.g. Taxol, Paclitaxel, etoposide, vincristine, vinblastine).

Recently, a tremendous number of new potential plants with anticancerous activities have been discovered. The real challenge now lies not only in proving the benefits of some plants but also in defining what these benefits are and developing the methods to expose them by scientific means (Tapsell *et al*., 2006).

In Romania, there is a tradition of using the medicinal plants for treating different diseases, including cancer.

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Agrimonia eupatoria is part of the *Rosacee* Family. Is an herbaceous plant and is referred to as common agrimony, church steeples or sticklewort or "turita-mare". His medicinal properties were known by Romans which used the agrimony to treat different wounds. Nowadays it is used by modern herbalist for kidney, liver and bladder disorders, sore throat, dysentery, abdominal pain and as a cure for insomnia. In Romania is used in the treatment of the respiratory inflammations, the hepatic cirrhosis, gastro-intestinal disorders as well as a powerful agent against biliar calculi and as a tonic.

His effects as a hypoglycemiant and anti-inflammatory and antiviral are well documented (Jung *et al.*, 2006; Swanston-Flatt *et al.*, 1990; Correia *et al*., 2007; Min *et al*., 2001).

The anticancer activity of agrimony is stated in a few articles. His effect as an antitumor agent using the purified tannin, agrimonin, or different extracts of the plant organs and his capacity to induce apoptosis on a cancer promyelocytic cell line has been proven. (Murayama *et al*., 1992; Miyamoto *et al*., 1987; 1988; Koshiura *et al*., 1985).

There are no aprofundated studies on the antitumoral mechanism of action of the *Agrimonia eupatoria* plant. Here we report the effect of *Agrimonia eupatoria* aqueous extract on a breast cancer cell line.

Materials and methods

Plant extract. Agrimonia eupatoria was harvested during the late summer 2009, identified and a voucher specimen was deposisted in the "Vasile Fati" Botanical Garden herbarium.The extract was obtained as previously described (Geuenich *et al*., 2008). Breifly, 10g of the plant leaves were cut in very small pieces and then added to 100ml of boiling distilated water for 15 min. The extract was centrifuged at 5000g, 10min. The supernatant was sterile filtered, aliquoted and deposited at -20^{0}C.This supernatant was considered 100% concentration.

Cancer cell line. In this study we used MCF-7 breast cancer cell line. The cell line was a kind gift from Dr. Csaba Vizler, Institute of Biochemistry, Biological Research Center of Hungarian Academy of Sciences, Szeged. MCF-7 cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10%FBS, 2mM L-glutamine, penicillin/streptomycin at 37^{0}C with 5% CO2.

MTT Test. This is a cell proliferation test based on the capacity of mitocondrial reductase enzyme from the living cells to reduce the yellow MTT (dipheniltetrazolium bromide) to purple formazan. Brefly the cells were seeded in 12 well plates at a concentration of 4x10^5 cells/well. Next day the cells were treated with the extracts. After 2h or 24 h in each well were added 100 µl of MTT stock solution /1ml medium. Then the cells were incubated 3 hours at 37^{0}C with 5 % CO2 and then equal volume of lysis solution was added. Cells were lysed for 5min, centrifuged at 13000 rpm for 2 min and then the absorbance was measured at 570 nm with background substraction at 650 nm.

DAPI staining. DAPI (4', 6-Diamino-2-phenylindole dihydrochloride) is a nuclear and chromosomal counterstain for use in fluorescent techniques. The cells were grown on coverslips for 24 h and then treated with 10% *Agrimonia eupatoria* extract for 24 h. The cells were fixed in methanol for 15 min at -20^{0}C and then stained with 300 nM DAPI solution for 5min. Staining was followed by 3 washings with distilled water, 10 min each. Then the coverslips were mounted on slides with mounting medium and analyzed at the Nikon inverted microscope equipped with UV excitation filter and camera for picture acquisition.

Results and discussions

MTT tests were done in three variants: (a) cells were treated with *Agrimonia eupatoria* extract for 24 h and 2 h respectively and then assayed (b) cells were treated with plant extract for 24 h, the medium was changed and cells assayed after 24 h (c) cells were treated for 24 h or 2 h, then medium was changed and cells were assayed after another 48 h.

In the first case we observed a cell proliferation but in case of (b) treatment where the medium was changed after the treatment and cells were assayed after 24h was observed a reduction in the absorbance values that means less cells were alive and the extract had a cytotoxic effect (Fig.1). The most cytotoxic effect was observed in case (c) where after 2 h or 24 h treatment with plant extract, medium was changed and left for 48h before assay of the cells. In this case it was observed a considerably reduction in absorbance values for 5% and 10% aqueous plant treatment (Fig.2).

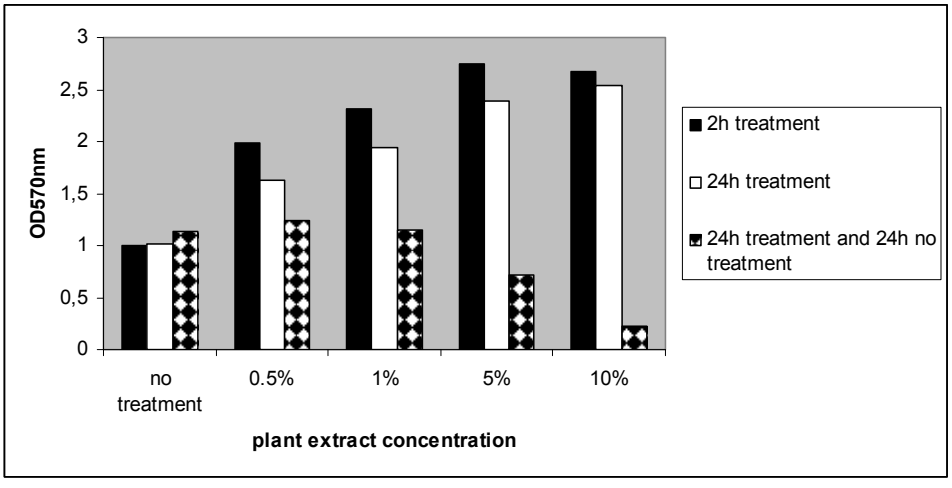


Fig. 1. Cell proliferation on MCF-7 breast cancer cells upon treatment with *Agrimonia eupatoria* aqueous extract. The cells were treated for 24 h or 2 h and the MTT test performed or cells were treated for 24 h with plant extract, medium refreshed and left on the cells for 24 h and then MTT test was performed.

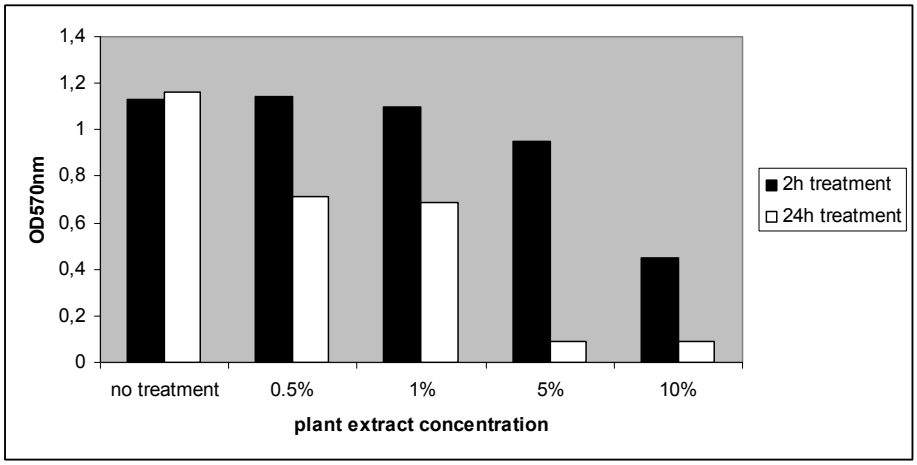
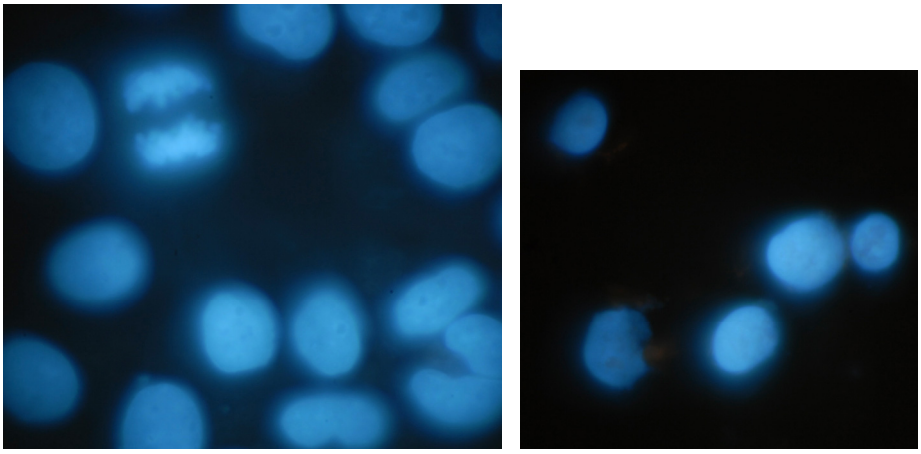


Fig. 2. Cell proliferation on MCF-7 breast cancer cells upon treatment with *Agrimonia eupatoria* aqueous extract. The cells were treated with plant extract for 24 h or 2 h and then medium was refreshed and left on the cells for 48 h and then the MTT test was performed.



No treatment **24h plant extract treatment**

Fig. 3. DAPI staining of MCF-7 breast cancer cells. No treated cells compared with treated cells for 24 h with plant extract

So, a cell proliferation effect can be observed for plant extract treatment for 2 h or 24 h when the MTT test was performed immediately after treatment but when the cells are kept longer in culture after the treatment the cytotoxic effect is considerably.

The nuclei of the no treated and treated cells were analysed by DAPI staining. On the coverslips with cells that had no treatment normal nuclei can be observed and cells underwent mitosis with no alterations. In the case of 10 % plant extract treatment we observed a tremendous change with smaller nuclei and cells that exibit an abnormal nuclei shape and no mitosis could be identified (Fig.3). This is in contrast with MTT results for 24h treatment but this can be explained by the fact that MTT test gives signal from all the cells that are still alive even if they are apoptotic. We think that after 24 h plant extract treatment the cells are entering apoptosis but more tests are under progress to confirm this hypothesis.

In conclusions our prelimiray study on the effect of *Agrimonia eupatoria* aqueous plant extract on MCF-7 cancer cells demonstrates cell proliferation effect or cytotoxic effect depending on the period of time the plant extract is left on the cells. These two opposing effects have to be investigated more closely especialy because of the limitation of information that MTT test can give. The proliferation effect proved to be a signal from cells that are probably in apopatosis according to the DAPI staining images.

It was previously demonstrated the cytotoxic effect of agrimony (*Agrimonia pilosa*) on a promielocitic cell line and here we demonstrate the cytotoxic effect of *Agrimonia eupatoria* extract on a breast cancer cell line and in this way we contribute to the knowledge about cytotoxicity effects of this plant species.

The effect that we observed for *Agrimonia eupatoria* aqueous plant extract on MCF-7 breast cancer cells is a promising one and its investigation can lead to discovery of an valuable cancer fighting plant.

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IDENTIFICATION OF METHICILLIN-RESISTANT, COAGULASE-NEGATIVE *STAPHYLOCOCCUS* STRAINS USING THE API STAPH COMMERCIAL SYSTEM

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SUMMARY. Community-acquired and hospital-acquired systemic staphylococcal infections are two major causes of mortality worldwide. A total of 125 staphylococcal isolates collected in Sibiu between July 2008 and January 2010 were used in this study. Twenty three of them were confirmed using the *mecA/nucA* duplex PCR technique; ten strains contains the *nucA* gene and were considered *Staphylococcus aureus*. The rest of 13 methicillin-resistant strains were tested using the API Staph commercial identification system (bioMérieux, France).

Keywords: API Staph, gene, PCR, *Staphylococcus*.

Introduction

In the last twenty years the inappropriate safety policy (e.g., increased number of invasive procedures, the widespread use of broad-spectrum antimicrobial agents) has led to the emergence of coagulase-negative staphylococci, especially the *Staphylococcus epidermidis* (Kloos and Bannerman, 1994).

One of the earliest schemes for classification of staphylococci and micrococci was that proposed by Baird-Parker (1963, 1965), which remained the method of choice until Kloos and Schleifer identified several new *Staphylococcus* strains (1975). In numerous studies the latter typing scheme has been applied to coagulase-negative staphylococcal clinical isolates.

In all cases, a relatively large number of biochemical and physiological tests were used. In this study a commercial kit of 19 tests was used which can be easily interpreted within 24 hours, based on the scheme proposed by Kloos and Schleifer (1975). The API Staph system can be used for the identification of the *Staphylococcus, Kocuria* and *Micrococcus* genera. This system is based on standardized and miniaturized

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biochemical tests with a specially adapted database (Baldellon and Megraud, 1985). The reliability of this system is guaranteed by using a low bacterial concentration (0.5 McFarland) inoculum, avoiding mixed cultures and subcultures.

The use of the mentioned system requires a preliminary identification of the tested strains as members of the *Micrococcaceae* family with classical techniques: Gram staining, colony morphology, catalase test, etc. (Brun *et al.*, 1978).

The aims of this study were the identification of the isolated staphylococcus strains and the determination of their methicillin resistance comparing the API Staph system to the molecular detection methods.

Materials and methods

Bacterial strains. Bacterial strains were isolated in Sibiu between July 2008 and January 2010 deriving from various pathological samples: pus, otic, conjunctival, nasal, and urethral secretions, sputum, etc. They were identified by using conventional microbiological methods: bacterial culture, biochemical- and antibiotic-sensitivity tests.

According to the direct determination molecular techniques a colony was suspended in 15 µl of pure water and added to the PCR mix. According to the results of the duplex PCR the methicillin-resistant strains were selected. The bacterial DNA was isolated and re-tested by PCR. Lysis buffer contains 0.2 mg/ml lizostaphin, 20 mM Tris/HCl, 2 mM EDTA, 1% Triton X-100 at pH 8 (Macherey-Nagel, 2009). Later proteinase K enzyme was added and the DNA was purified by using Nucleospin® Tissue kit (Macherey-Nagel, Düren, Germany).

Oligonucleotide samples. Oligonucleotide primers used for PCR reaction in the case of the *mecA* gene were: $mecA_1$ - 5'-AAAATCGATGGTAAAGG TTGGC corresponding to nucleotides 1282 to 1303 and $mecA_2$ - 5'-AGTTCTGCAGTACCGGATTTGC being complementary to nucleotides 1793 to 1814 (Murakami *et al.*, 1991). In case of the *nucA* gene the sequences of the two primers were: $nucA_1$ - 5'-GCGATTGATGGTGATACGGTT and $nucA_2$ - 5'-AGCCAAGCCTT GACGAACTAAAGC. Primers bind to the 447 base pair long *nucA* gene which encodes the thermonuclease A protein. The primer 1 binds between 49 and 69 nucleotide positions, and the primer 2 binds between 304 and 327 nucleotide positions generating a 279 bp long fragment (Brakstad *et al.*, 1992). The oligonucleotide primers were synthesized by the Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary.

PCR technique. PCR mix contains: 5x concentrated Green reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP (from each deoxiriobonucleotide-triphosphate), 1 µM $nucA_1$ primer, 1 µM $nucA_2$ primer, 1 µM $mecA_1$ primer, 1 µM $mecA_2$ primer, GoTaq® Flexi DNA polymerase 1.25 U, 10 µl bacterial suspension or 100 ng purified DNA, and ultrapure/UV water (Purelab Ultra Genetic, ELGA LabWater, High Wycombe, UK) up to 50 µl final volume. The DNA polymerase, the reaction buffer and the magnesium chloride were the part of the GoTaq® Flexi DNA polymerase kit

(Promega Corporation, Madison, USA). The dNTP mix was purchased from the same producer. The samples were amplified by using Mastercycler® and Mastercycler® EP gradient S thermocyclers (Eppendorf, Hamburg, Germany). The used thermal profile was previously described (Colcieru *et al.*, 2010).

Agarose gel electrophoresis. For the separation of the amplified fragments were used CONSORT H_1-SET electrophoresis cell (distance between electrodes: 10 cm) and CONSORT H_U-10 electrophoresis cell (distance between electrodes: 15 cm) connected to a Consort E 835 power supply set to 52 V or 80 V, respectively. We used 50 ml or 90 ml 1% agarose gel. Wells were loaded with 10 µl of PCR mix. Generuler™ 100 pb DNA Ladder (Fermentas, Vilnius, Lithuania) or 1 kb DNA Ladder (Axigen Biosciences, Union City, USA) molecular weight markers were used.

Identyfication system. API Staph consists of a strip containing dehydrated test substrates in individual microtubes. The tests are reconstituted by adding to each tube an aliquot of API Staph Medium that has been inoculated with the studied strain. Furthermore the strip is incubated for 18-24 hours at 35-37°C and the results are read and compared with a supplied reference. The identification is facilitated by using the API Staph Analytical Profile Index or the identification software.

The inoculum is prepared by 18-24 h incubation of the target microorganism on a simple blood agar medium followed by the incubation in the supplied 0.5 McFarland turbidity liquid medium. The test stripe inoculation is carried out by using a sterile pipette. In the cases of ADH (arginine dehydrogenase) and URE (urease) wells there must be added sterile mineral oil in order to ensure anaerobic conditions. After incubation some tests require a developing step. In the case of the VP test (acetoin production) VP1 and VP2 reagents must be added to the wells, as well as NIT1 and NIT2 reagents in the case of NIT (nitrate reduction) test and ZYME A and ZYME B reagents in the case of PAL (alkaline phosphatase) test.

When using the Analytical Profile Index the obtained reaction pattern must be coded into a numerical profile**.** On the results-sheet the wells are separated into groups, every three well representing a group. In the group every well has a number of 1, 2 or 4. In each group the numbers of the wells showing positive reactions are added finally creating a 7-digit profile number (Fig. 1).

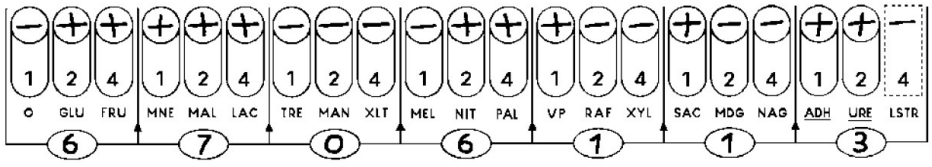

Fig. 1. An example of API Staph numerical profile.

In this study the identification of strains was preformed by using the 4.0 version of the API Web software introducing the result (+/-) of the biochemical tests. Table 1 presents the evaluation criteria used in the identification process (API Staph, 2002).

Table 1.

The API Staph evaluation criteria

Test	Substrate	Reactions/ Enzymes	Result	
			Negative	Positive
0	No substrate	Negative control	Red	-
GLU	D-Glucose	(Positive control) Acidification due to carbohydrate utilisation	Red	Yellow
FRU	D-Fructose			
MNE	D-Mannose			
MAL	Maltose			
LAC	Lactose			
TRE	D-Trehalose			
MAN	D-Mannitol			
XLT	Xylitol			
MAL	D-Melibiose			
NIT	Potassium nitrate	Reduction of nitrate to nitrite	NIT1 + NIT2/ 10 min	
			Colourless	Red
PAL	β-naphtyl-acid phosphate	Alkaline phosphatase	ZYME A + ZYME B /10 min	
			Yellow	Violet
VP	Sodium pyruvate	Acetyl-methyl carbinol production	VP1 + VP2 / 10 min	
			Colourless	Violet- pink
RAF	Raffinose	Acidification due to carbohydrate utilisation	Red	Yellow
XYL	Xylose			
SAC	Sucrose			
MDG	α-methyl-D-glucoside			
NAG	N-acetyl-glucosamine			
ADH	Arginine	Arginine-dihydrolase	Yellow	Orange –red
URE	Urea	Urease	Yellow	Red -violet

Results and discussion

The efficiency of the API Staph system in coagulase-negative staphylococci identification was firstly evaluated by Gemmell and Dawson (1982). After their findings the staphylococcus identification system is so rapid and nearly so accurate as the conventional methods.

A total of 13 methicillin-resistant, coagulase-negative staphylococcus strains were identified by using this commercial system. The resulted biochemical profiles were validated by using the API Web software (ver. 4.0). The most similar profiles were used for identification.

There were identified five different strains: *S. epidermidis* (*n*=4), *S. haemoliticus* (*n*=4), *S. xylosus* (*n*=3), *S. hominis* (*n*=1) and *S. warneri* (*n*=1). The codified biochemical profiles and their similarity values are presented in Table 2.

Table 2.

The identified staphylococcus strains and their similarity to the standard profile.

Isolate	Strain	Origin of the sample	Numerical profile	ID %
1	*S. warneri*	Wound secretion	6734113	49,70
8	*S. xylosus*	Conjunctival secretion	6735112	91,70
9	*S. haemolyticus*	Wound secretion	2635051	97,70
15	*S. haemolyticus*	Conjunctival secretion	6733051	82,70
20	*S. xylosus*	Conjunctival secretion	6372450	94,70
26	*S. xylosus*	Wound secretion	6372400	80,40
33	*S. haemolyticus*	Nasal secretion	6636051	47,10
51	*S. epidermidis*	Nasal secretion	6706011	93,20
54	*S. epidermidis*	Conjunctival secretion	6706010	91,10
55	*S. hominis*	Otic secretion	6712052	45,50
68	*S. epidermidis*	Wound secretion	6706013	93,10
69	*S. epidermidis*	Conjunctival secretion	6706011	93,20
89	*S. haemolyticus*	Wound secreton	6616051	53,30

In this study the API Staph system proved to be a highly adequate and inexpensive method: the inoculation and interpretation of test stripes are rapid (the incubation period is 18-24 h) and effortless processes. The results of the biochemical reactions can be easily explained, it does not require sophisticated apparatus.

According to the literature the API Staph system is characterised by a high identification rate (Radebold and Essers, 1980). Our results show that all isolates identified as coagulase-negative *Staphylococcus* strains by using the *mecA/nucA* duplex PCR were confirmed by the API Staph system.

Using the biochemical testing methods 13 *nucA*-negative (lacking the *nucA* gene, which encodes a thermostable nuclease specific for *Staphylococcus aureus*) strains were confirmed as coagulase-negative staphylococcus. The presence of the *mecA* gene indicates the methicillin resistance of the tested strain. Fig. 2 shows the result of the *nucA/mecA* duplex PCR. In the case of the 51st, 54th, 55th, 68th and 69th isolates only the 533 bp fragment of the *mecA* gene was amplified and they were determined as methicillin-resistant, coagulase-negative staphylococcus strains (MRSS). The 49th, 60th, 70th, 71st and the 74th isolates are methicillin-sensitive *Staphylococcus*

aureus strains (MSSA), because only the 279 bp long fragment, specific for the *nucA* gene, was amplified. The 66th and 73rd isolates were determined as methicillin-resistant *Staphylococcus aureus* strains (MRSA). The remained 14 strains were determined as methicillin-sensitive, coagulase-negative staphylococcus strains (MSSS).

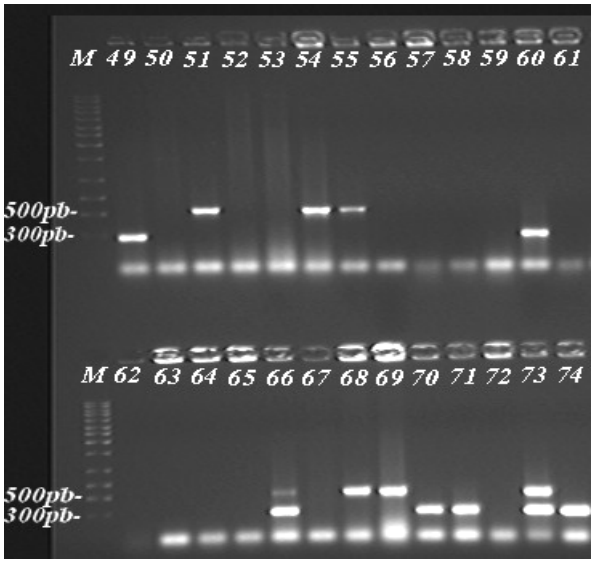

Fig. 2. Agarose gel electrophoresis of the *nucA/mecA* duplex PCR products (the unbound primers form a strong band).

According to the literature there is a correlation between the identified staphylococcus strains and the origin of the pathological samples. Most of the identified isolates were *S. epidermidis* and *S. haemolyticus*.

We can conclude that the frequency rate of the methicillin resistance among the studied coagulase-negative staphylococci corresponds to the available statistical data (Diekema *et al*., 2001).

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