INTRODUCTION
Analysis of subfossil Cladocera (Crustacea: Branchiopoda) is widely used in paleolimnology given its potential to reconstruct past environmental conditions (Korhola and Rautio, 2001; Nevalainen and Rautio, 2014; Zawiska et al., 2015). Cladocerans are used as indicators of several abiotic and biotic environmental variables (Rumes et al., 2011; Chen et al., 2014), as they are very sensitive to changes in total phosphorus concentrations (Amsinck et al., 2005; Chen et al., 2010), water depth (Korhola et al., 2005; Nevalainen et al., 2011; Galka et al., 2014), temperature (Lotter et al., 1997; Korhola, 1999; Mirosław-Grabowska and Zawisza, 2013; Nevalainen et al., 2013; Zawiska et al., 2015), pH (Locke and Sprules, 2000; Zawiska et al., 2013).

The crucial step in subfossil Cladocera analysis is the correct taxonomical identification of the remains at the species level, which is usually based on the use of light microscope (magnification 100-400x) and several determination keys (Alonso, 1996; Szeroczyńska and Sarmaja-Korjonen, 2007; Korosi and Smol, 2012). As the light microscopy allows to observe the remains in two dimensions, the body sculpture appears to be only a pattern on the surface of the carapace. The microstructural characteristics of the chitinous remains can be observed in three-dimensional appearance only by scanning electron microscope (SEM), which enables to create images by scanning the surface with a focused beam of electrons (Goldstein et al., 2003). The magnifications obtained with SEM are much greater than those of light microscopy and reach 100,000x.

SEM is commonly used in taxonomy of living Cladocera to describe morphological features such as the limb setae, the lateral pores or the shell denticles (Sinev et al., 2005; Sinev and Elmoor-Loureiro, 2010). Cladocera for SEM observations are usually collected from water by using a plankton net and dried using either a wide range of alcohol percentages (70%, 90%, 95%, 100%) (Duigan, 1992; Nandini et al., 2009), or the strong reagent hexamethyldisilazane (Laforsch and Tollrian, 2000; Sousa et al., 2015; Juračka et al., 2016). Saha et al. (2011) recently presented a new simplified procedure, where specimens collected from water samples are washed in distilled water, dried in the room temperature for 30 mins, coated with gold palladium and examined with SEM.

SEM images are also frequently used in paleolimnology to study sediment properties, such as the origin of carbonates (terrestrial or autogenic), porosity, composition of lamination and microfossil taxa identification (Kemp et al., 2001; Martín-Serrano et al., 2009; Wetzel, 2013; Kirillova et al., 2016). They are also very useful in observing small morphological details of microorganism and
often help to identify remains such as diatoms (Battarbee, 2001) or testate amoebae (Beyens and Meisterfeld, 2001). Therefore, the methodology for preparing the subfossil remains of these organism for SEM observation is well established (Jiang et al., 2015). On the contrary, Cladocera subfossil remains are still rarely observed under the SEM (Kirillova et al., 2016). In fact, although Cladocera skeleton is composed of fairly hard chitin, the typical thickness variation depending on the species, body part and lake environment conditions, make the Cladocera preparation for SEM more complicated and time-consuming (Andrade-Morraye et al., 2004). The sediment samples should be firstly prepared according to standard procedure (Frey, 1986), then remains have to be picked up from the samples and washed several time with distilled water. After that remains should be put to osmium tetroxide for 2 h, washed in distilled water again and submitted to the ethanol dehyrdation sequence (Andrade-Morraye et al., 2004). When the remains are acquired from unconsolidated sediments they have to be submitted to dehydration in graded alcohols solutions (Kirillova et al., 2016).

In our research we aimed at testing whether the simplified method proposed by Saha et al. (2011) for aquatic samples could be applied also to subfossil Cladocera remains in order to obtain good quality pictures.

METHODS

Subfossil Cladocera remains for SEM observation were obtained from sediment samples using two approaches. In the first one fresh sediment from different lakes located in Central and South America was analysed (i.e. from Lake Comendador, Lake Chicabal, Lake Quezil (Guatemala), Lake Emiliano Zapata (Yucatan Peninsula, Mexico), Lake Los Negritos, Lake Verde (Salvador), Lake Madre Vieja, (Honduras), Lake San Martin, (Argentina). Remains were picked directly from the unconsolidated upper first cm of surface sediments (1 cm²), diluted with distilled water and put into a petri dish. Cladocera remains were pick out using a pipette (in the drop of water) under the dissecting microscope and directly put on the SEM microscope stubs covered with a carbon adhesice tape.

In the second approach the sediment samples for SEM observation were obtained from European Lakes, i.e. Atnsjøen (Norway), Czechowskie (Poland) and Suchar IV (Poland). Samples were taken from sediment cores and chemically prepared for subfossil Cladocera analysis according to standard procedures (Szeroczyńska and Sarmaja-Korjonen, 2007). The amount 1 cm² of fresh sediment was treated with hot 10% KOH for 20 min using a magnetic stirrer in order to deflocculate the material and remove humic substances. Thereafter the carbonates were removed using 10% HCl. The remaining material was sieved through 33 µm mesh and diluted in 10 cm² distilled water. The subfossil remains were removed consecutively with a pipette from the cleaned sediment and directly put on the SEM microscope stubs covered with a carbon adhesive tape.

The remains obtained from both superficial fresh sediments and cleaned core material were left to dry at the room temperature for 48 hs. When dried they were put into the sputter coater SC7620 for 120 s and coated with a gold-palladium. The sample coating with an electric conducting material is necessary in order to avoid the accumulation of electrostatic charge at the sample surface (Sinev et al., 2005; Sinev and Elmoor-Loureiro, 2010). After the specimens were coated they were put into the scanning electron microscope (Jeol JSM-6610LV) chamber and observed in high vacuum, using SEI mode, voltage 20 kV.

RESULTS

Figures 1-8 show 29 good quality images of subfossil Cladocera remains belonging to 12 Cladocera species. The pictures were taken at magnification ranging from 200x to 11,000x. The remains from both fresh sediments and from the sediment cores have different state of preservation, independently from the age of the sample. The remains of Chydorus spp. (Leach) preserved well and therefore easier to be photographed, compared to the other observed Cladocera species (Figs. 1 and 2). The SEM pictures allowed to observe magnificent sculpture of Ceriodaphnia spp. (Dana) and Simpoccephalus ephippia (Schoedler) (Figs. 3 and 4). The delicate structure of Alonella excisa (Fischer) shell and Leydigiosis ornata (Dayad) (Fig. 5), the deep carvings of Graptoleberis testudinaria (Fischer) and Monosipilus dispar (Sars) (Fig. 6), as well as characteristic triangle on the Paralona pigra (Sars) shell are well documented (Fig. 1). The SEM pictures revealed the three-dimensional aspect of the head pores of Alona ossiani (Sinev), Bosmina (E.) coregoni (Baird) and Bosmina (E.) longispina (Leydig) (Fig. 7). On the contrary, the specimens form lake Suchar IV showed high level of degradation, and diminished sculpture of the remains (Fig. 8). This might be possibly due to the fact that these sediment samples were prepared for subfossil Cladocera analysis already five years ago.

DISCUSSION

The simplified procedure of preparing specimens for SEM observation proposed by Saha et al. (2011) was tested on different remains of Cladocera species from several American and European lakes. This procedure is based on the concept developed for the remains of living species (Sinev et al., 2005; Van Damme and Dumont,
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Fig. 1. SEM images of Cladocera remains. A) *Chydorus sphericus* shell, magnification 270x (Lake Comendador, Guatemala). B) *Chydorus sphericus* shell sculpture, magnification 4000x (Lake Comendador, Guatemala). C) *Paralona pigra* shell, magnification 330x (Lake San Martin, Argentina). D) *Paralona pigra* shell, triangular anterior accessory flange on the anterior-ventral margin, magnification 950x (Lake San Martin, Argentina). E) *Chydorus* spp., magnification 500x (Lake Comendador, Guatemala). F) *Chydorus* spp., magnification 270x (Lake Comendador, Guatemala).

Fig. 2. SEM images of Cladocera remains of *Ceriodaphnia* spp. ephippium (Lake Emiliano Zapata, Yucatan Peninsula, Mexico). Magnification: A) 160x; B) 650x; C) 900x; D) 4000x.
The time for drying the remains suggested by Saha et al. (2011) was not long enough in the case of fragmented parts of the cladoceran body found in the studied samples. Since most of them were very small and difficult to pick out from the sediment sample with a needle, they were put on the stage with a pipette, in a fairly large drop of water. Therefore the prolongation of the drying time was necessary.

From all types of examined Cladocera remains, ephippia showed the best reservation of structure and ornamentation, as their chitinous envelope is thick and less prone for mechanic destruction. It was also noted that not all subfossil Cladocera remains were suitable for SEM observation, as some were so thin that the specimens were barely visible. In addition, it was recognized that the subfossil material for SEM observation should be pick out from the freshly prepared sediment sample, as the remains slowly degrade and the chitin structure become less prominent after sediment preparation (Fig. 8). The SEM images clearly showed that the patterns on the shells observed under the light microscope always correspond to three dimensional structures.

CONCLUSIONS

The simplified method of preparing subfossil Cladocera was applied on different samples from several lakes. The presented method resulted to be simple, cheap and allowed to create high quality images of all types of re-

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Fig. 3. SEM images of Cladocera remains of Simocephalus spp. ephippium (Lake Chicabal, Guatemala). Magnification: A) 150x; B) 950x; C) 3500x.

Fig. 4. SEM images of Cladocera remains. A) Alonella excisa shell, magnification 400x (Lake Quezil, Guatemala). B) Alonella excisa shell sculpture, magnification 1300x (Lake Quezil, Guatemala). C) Leidigiopsis ornata head, magnification 230x (Lake Los Negritos, Salvador). D) Leidigiopsis ornata head sculpture, magnification 1600x (Lake Los Negritos, Salvador).
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remains even in fairly high magnifications up to 11,000x. Although the procedure developed for the remains of living species revealed to be effective also for subfossil Cladocera, it appeared necessary to prolong the drying time when working with subfossil remains. Moreover, the samples should be prepared just before the SEM analysis, in order to prevent the degradation of the micro sculpture after the cleaning procedure.

Fig. 5. SEM images of Cladocera remains. A) Graptoleberis testudinaria shell, magnification 300x (Lake Verde, Salvador). B) Graptoleberis testudinaria head, magnification 370x (Lake Verde, Salvador). C) Monospilius dispar head, magnification 400x (Czechowskie Lake, Poland).

Fig. 6. SEM images of Cladocera remains of Alona ossiani head (Lake Madre Vieja, Honduras). Magnification: A) 190x; B) 1600x; C,D) 11,000x.

Fig. 7. SEM images of Cladocera remains from Lake Atnsjøen, Norway. A) Bosmina E.coregoni head, magnification 500x. B) Bosmina E.coregoni head pore, magnification 3300x. C) Bosmina E. longispina head, magnification 800x.

Fig. 8. SEM images of Cladocera decaying remains from sediment from Lake Suchar IV (Poland) prepared for subfossil Cladocera analysis 4 years ago. Magnification: A) 500x; B) 330 x.
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