Double-check in lizard age estimation: use of phalanx bone and keratin claw sheath lamellas

Eduard Galoyan (saxicola@mail.ru)
Severtsov Institute of Ecology and Evolution https://orcid.org/0000-0001-7633-7759
Natalia Sopilko
Lomonosov Moscow State University: Moskovskij gosudarstvennyj universitet imeni M V Lomonosova
Anna Kovalyeva
Lomonosov Moscow State University: Moskovskij gosudarstvennyj universitet imeni M V Lomonosova
Anna Chamkina
Lomonosov Moscow State University: Moskovskij gosudarstvennyj universitet imeni M V Lomonosova

Research Article

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Abstract

Background

Age estimation in vertebrates has always been a tricky and dubious procedure. The method of skeletochronology is commonly used for age estimation in poikilothermic and in some homoiothermic animals. This method is based on counting arrested growth lines (LAGs) within bone structures (tubule bones or teeth) in modern living or fossil animals. However, it fails to reveal the age with necessary precision due to hibernation and aestivation periods, hence, some referent method of age estimation is required for evaluating better precision. During the previous studies of population age structure in several species of rock lizards (*Darevskia*) we prepared microscope slides of finger bones and noticed presence of well-distinguished cornified unguis lamellas of the finger claws. We put forward that the number of these lamellas coincides with the number of LAGs in the periosteum bone of the same finger. To test this hypothesis, we performed special study and compared number of LAGs within each finger bone and cornified claw using traditional skeletochronological technique.

Results

The conducted analysis revealed positive correlation (r_s=0.933, $p < 0.001$) and no significant differences between the number of LAGs in the bone and unguis lamellas of the same fingers of three species of Palearctic rock lizards (*Darevskia portschinskii*, *D. raddei*, *D. valentini*). We found no distinguished cornified lamellas in the claw of the tropical skink *Sphenomoprhus maculatus*.

Conclusion

We assume that the proposed method may be an efficient and reliable means of ecological studies based on toe-clipping and vital age estimation in lizards and, possibly, other poikilothermic vertebrates, as a double-check method based on two independent registration structures present potentially preventing possible mistakes in age estimation.

Background

The problem of age estimation in amphibians and reptiles with annual fluctuations of growth pattern has been considered to be mostly solved after skeletochronological method was introduced back in the middle of 20 century [1]. This method is based on counting the number of registration markers (RM) or lines of arrested growth (LAG), i.e. layers with different optical density within the tubular bones. To date, this method is widely used to estimate age in amphibians [2–6] and reptiles [7–9]. Femoral or humerus bones are normally used as the material for analysis [10], as they are relatively of a large size and contain obvious growing layers. The drawback of this method is that it requires animal euthanasia and, consequently, cannot be considered as humane.

Relying on longitudinal monitoring, ecological and behavioural studies suggest that toe-clipping and distal finger phalanx analysis should be used to collect the material [11–15]. These methods allow the researchers to conduct a life-long study to estimate the growth pattern of individuals. Moreover, this offers a possibility to double-check the obtained results by comparing growth patterns of an individual with the number of the layers within its bone tissue.

It should be noted, though, that in small animals the wintering rings stop occurring after maturation, so when it comes to small lizards it is practically impossible to distinguish between the individuals of six or seven years from much older individuals [15] (Saint Girons et al., 1989). Another problem, which the researchers often face, is that intermediate layers, which can emerge during second annual inactivity – for example, aestivation during summer period in desert or montane species [16, 17] – may hamper the accurate counting of layers [18]. Finally, assessing the exact number of layers may be generally problematic prompting the researchers to use the double-blind method to verify the obtained results [19]. Hence, additional reference data are obviously required to support the results obtained by a skeletochronological method.

In the course of our studies we have noticed that dermal structures on the dorsal part of the claw sheath is multilayered, and suggested that these layers could be useful for age estimation in lizards. We put forward that the proposed procedure might be an effective supplementary method accompanying skeletochronological method, as it may allow using two different structures placed on the same finger to double-check the esteemed age.

To test this hypothesis, we compared the number of RMs (wintering rings) in bone tissue of the finger phalanx with the number of unguis formations of the claw sheath of the same finger in several species of rock lizards belonging to *Darevskia* genus. These lizards inhabit the Caucasus mountains and hibernate under the harsh and cold conditions during a prolonged snowy winter [20]. The species of this genus have undergone skeletochronological analysis in the works of different researchers, which proves the method efficient and reliable. The results obtained by means of this method demonstrate that these small lizards with snout-vent length of about 50–70 mm live over ten years [9, 13, 15, 21, 22].

The tubule bone structure in reptiles includes internal endosteum and external periosteum areas. The endosteum is partly destroyed by osteoclasts and not suitable for the age estimation [17]. The double line in the bone of the phalanx separates periosteum and endosteum and it is called a “juvenile ring” or resorption line [18] (Fig. 1a). Thus, age estimation in reptiles is based on counting the arrested growth rings or the lines of arrested growth within the periosteum bone closer to the center of diaphysis [17].

Mammalian claws include soft internal structures covered by a rigid horn above [23]. The external keratin horn might include the periodically banded structures; this fact proves the presence of annual differences in physiological condition of the animal [24]. The reptile claw comprises bone of distal phalanx and epidermal sheath, with keratinized corneus of the unguis an less keratinized subunguis epidermis [25, 26]. Surface cornification of scales, unguis and subunguis of reptiles is driven by the expression of beta-keratins of epidermis [27]. Growth process in reptiles is often uneven, which is represented in presence of distinguished layers of cornified corneus [27] and suggested to be a good marker of annual growth lines (Fig. 1b).

Results

The structure of cornified unguis in the studied species appeared to be comprised of several distinguishable layers, the number of which were more abundant in the older individuals as compared to younger lizards (Table S1) and coincided with the number of LAGs in the periosteum of the finger phalanx bone (Fig. 1; Table S1).

The number of the unguis cornified lamellas as well as of wintering rings in the periosteum bone of the same finger phalanx exhibited no significant difference in three studied species of *Darevskia* genus (V = 44, p = 0.066, n = 43), yielding a positive correlation between these two methods (r_s=0.933, p < 0.001; Fig. 2). Although, in most cases the number of wintering rings on the finger bone phalanx was calculated to be larger than the age of the individual in comparison with the one in keratinized unguis lamellas (Fig. 2).

The study of the finger phalanx bone showed the absence of any LAGs in bones and in claws of tropical spotted forest skink *Sphenomorphus maculatus* (Fig. 3): subunguis keratin lamellas were unstructured and no distinguished layers can be found within cornified unguis.

Discussion

The obtained results allow us to assume that the number of LAGs in the bones of the finger phalanx correspond with the number of ungular lamellas of the claw in the same individual. It goes without saying, that no method could be flawless, neither the traditional skeletochronological, nor keratinochronological methods are ideal. Age estimation is often debatable as possible errors could occur in LAG counting and bone resorption [18]. This is exactly the case for large and aged individuals [15].

Another difficulty is telling apart the wintering LAGs from the intermediate LAGs; distinguishing between the two may become quite a tricky task especially for the period with low food intake and/or aestivation [18]. The method of bone resorption might explain why the samples analysed in our study differ in size: a number of unguis LAGs appeared to be slightly smaller than those in bone LAGs (Fig. 2). It was also revealed that keratinized structures are normally larger, thicker, and easier to be seen under the microscope than wintering rings in the periosteum of the finger phalanx (Fig. 1).

Finally, the proposed method allows to double-check the number of registration markers on the same finger phalanx belonging to the same individual, which significantly facilitates age estimating in the studied poikilothermic individuals and makes the results more accurate and valid. Moreover, this method allows to work with the same individuals through a long study period.

The elaborated method might be promising for conducting ecological and behavioural studies on reptiles and amphibians. The procedure should undergo further large-scale testing in amphibians and other poikilothermic animals with annual growth fluctuations.

Conclusions

To conclude, the proposed method might contribute to age estimation making it considerably more precise and hence reliable.

Methods

Histology

We used amputated digits prepared according to the methods developed by Castanet (1994) and Smirina (1974). We place the phalanx bone tissue in the 5% nitric acid for the decalcination and prepared microscopic sections (10–15 μm) on the Reichert rotary microtome. After that we stained them with Erlich's haematoxylin, fixed them in the Canadian balsam and covered with cover glass [11, 15]. The study of the microscope slides was obtained using the Zeiss Primo Star microscope. To reduce the chance on erroneous LAGs counting each phalanx has been studied by two researchers (the first two authors) independently.

Study Subjects and Data Analysis

For the present study we used finger phalanxes of rock lizards (*Darevskia* Arribas, 1997) from Armenia. The total number of age-estimated individuals using skeletochronological method (including those considered in the present paper) was 43, among which there were 13 individuals of *Darevskia portschinskii* (Kessler, 1878); 16 – *D. raddei* (Uzzel & Darevsky, 1793); 14 – *D. valentini* (Boettger, 1892).

The samples of *Darevskia portschinskii* and *D. raddei* were collected in the vicinity of Zuar Village (N40.0692; E46.2433, WGS 84) in the south-eastern region of the Lesser Caucasus Mountains at 1500 m a.s.l. during May–July 2017; 2018; 2019. This allowed us to double-check the lifespan of residential individuals by recapturing them in adjustment years. The data on *D. valentini* was obtained during the field study near the Mets Sepasar village (area of Ashotsk City, Shirak Province, 2 030 m a.s.l., (N41.0319; E43.8296, WGS 84) in 2014 and 2016.

To ensure that keratinized claw sheath structures grow in distinguished layers we also made histological slices of the spotted forest skink *Sphenomorphus maculatus* (Blyth, 1853) (Scincidae) from a tropical rainforest in Vietnam, Dong Nai province, Tanh Phu distr., Cat Tien National Park (N11.4227; E107.4275, WGS 84) in March 2009. This is a short-lived animal with the 1.5–2-year lifespan, which has no aestivation period [28].

We used paired Wilcoxon test to compare the figures of the esteemed age by counting the LAGs on the finger bone and the claw sheath of the same finger and performed Spearmen's rank correlation test (r_s) to compare these two data sets. The significance level was set up at $p = 0.05$ in each test. The calculations and graphs have been performed in R 4.1.2 stat software using “ggplot2” package [29].

Declarations

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Authors’ Contributions

Eduard Galoyan championed the hypotheses that keratin structures in the claws may correspond to wintering lines in the bone; he conducted the analysis of microtomic slides and reported on the results having written the better part of the manuscript. Natalia Sopilko contributed to the study of microtomic slices having conducted a significant number of items; she also took part in reporting on the results I the present paper. Anna Kovalyeva and Anna Chamkina prepared most microscope slides. All authors contributed considerably to the drafts and gave the final approval for publication.

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Ethics approval and consent to participate

All applicable international, national and institutional guidelines for the care and use of animals were followed during the work. All the manipulations with animals were approved by the Armenian Ministry of Nature Protection (N5/22.1/51043) and the Department of Forestry, Ministry of Agriculture and Rural Development of Vietnam (permits No. 170/ TCLN–BTTN and 831/TCLN–BTTN).

Consent to publish

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

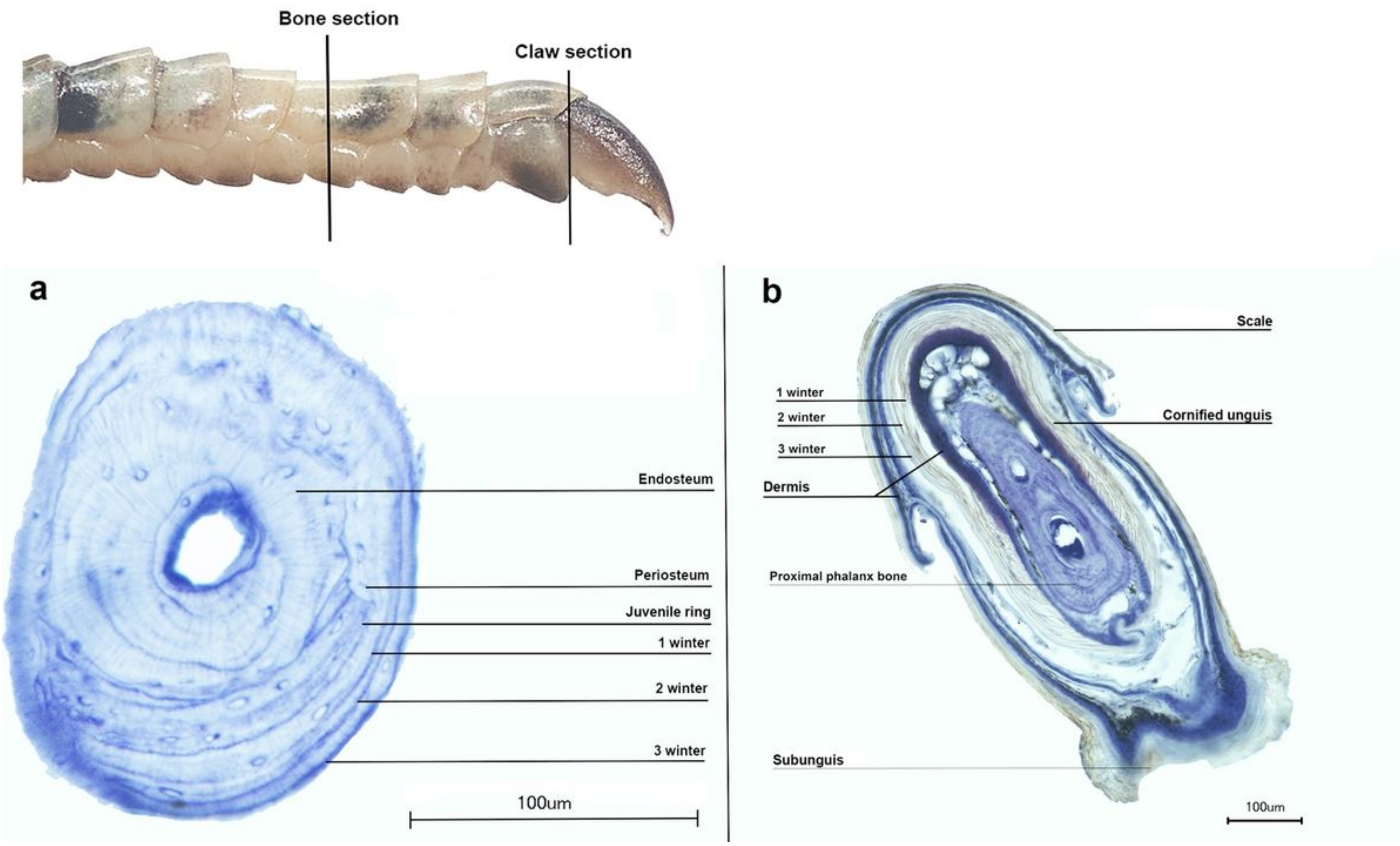

Figure 1

Microscope slides of bone finger phalanx (a) and distal phalanx of the same finger with claw sheath (b) in a 3-year-old male of *Darevskia portschinskii* (Ind. N 20, May 2017 year from Artsakh area)

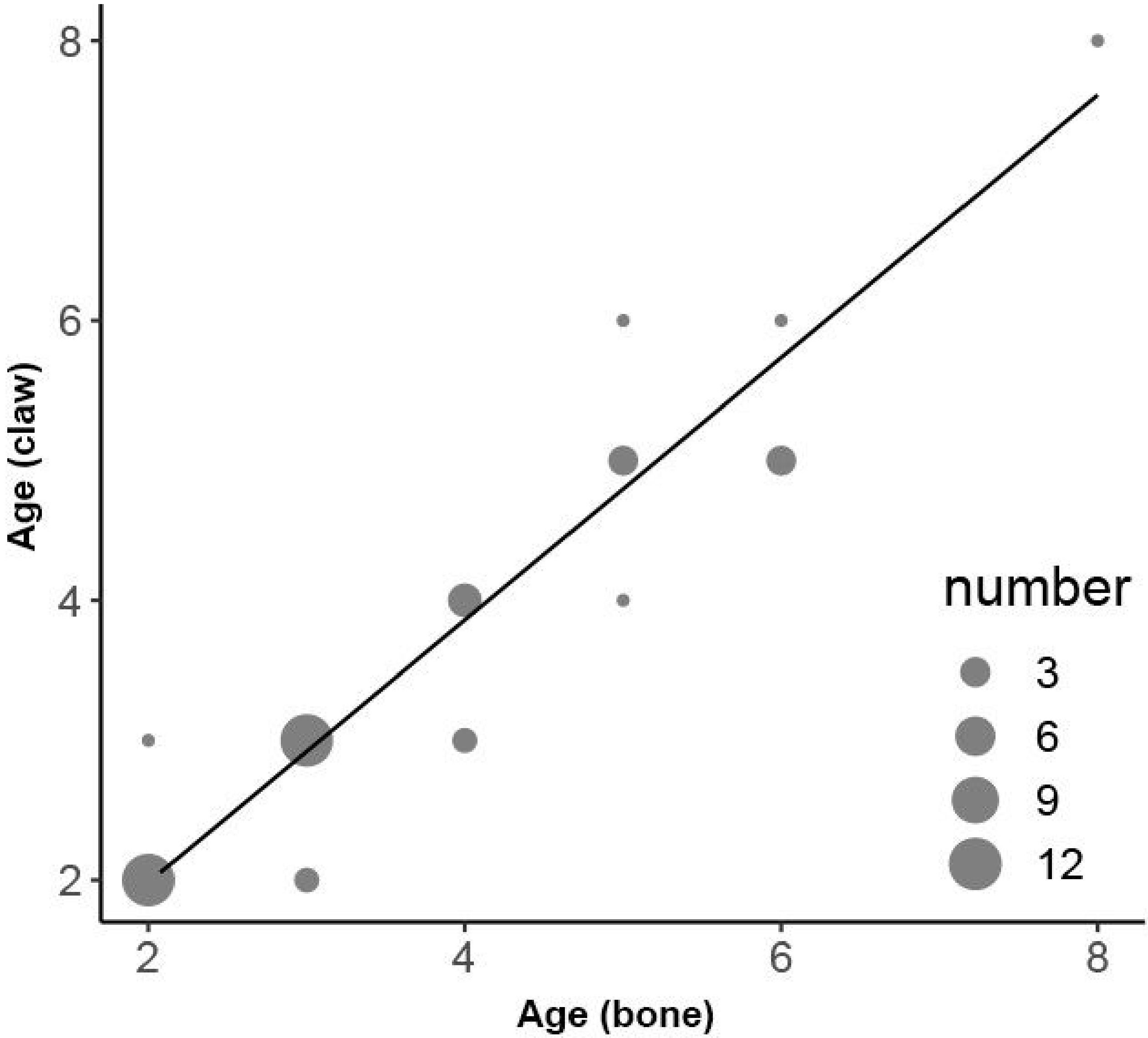

Figure 2

Bubble plot of relation between the estimated age by skeletochronological age estimation in bone structure and claw sheath of the same digit in three species of rock lizards (*Darevskia portschinskii*, *n*=13; *D. raddei*, *n*=16; *D. valentini*, *n*=14). ‘number’ stands for the number of lizards per estimated age

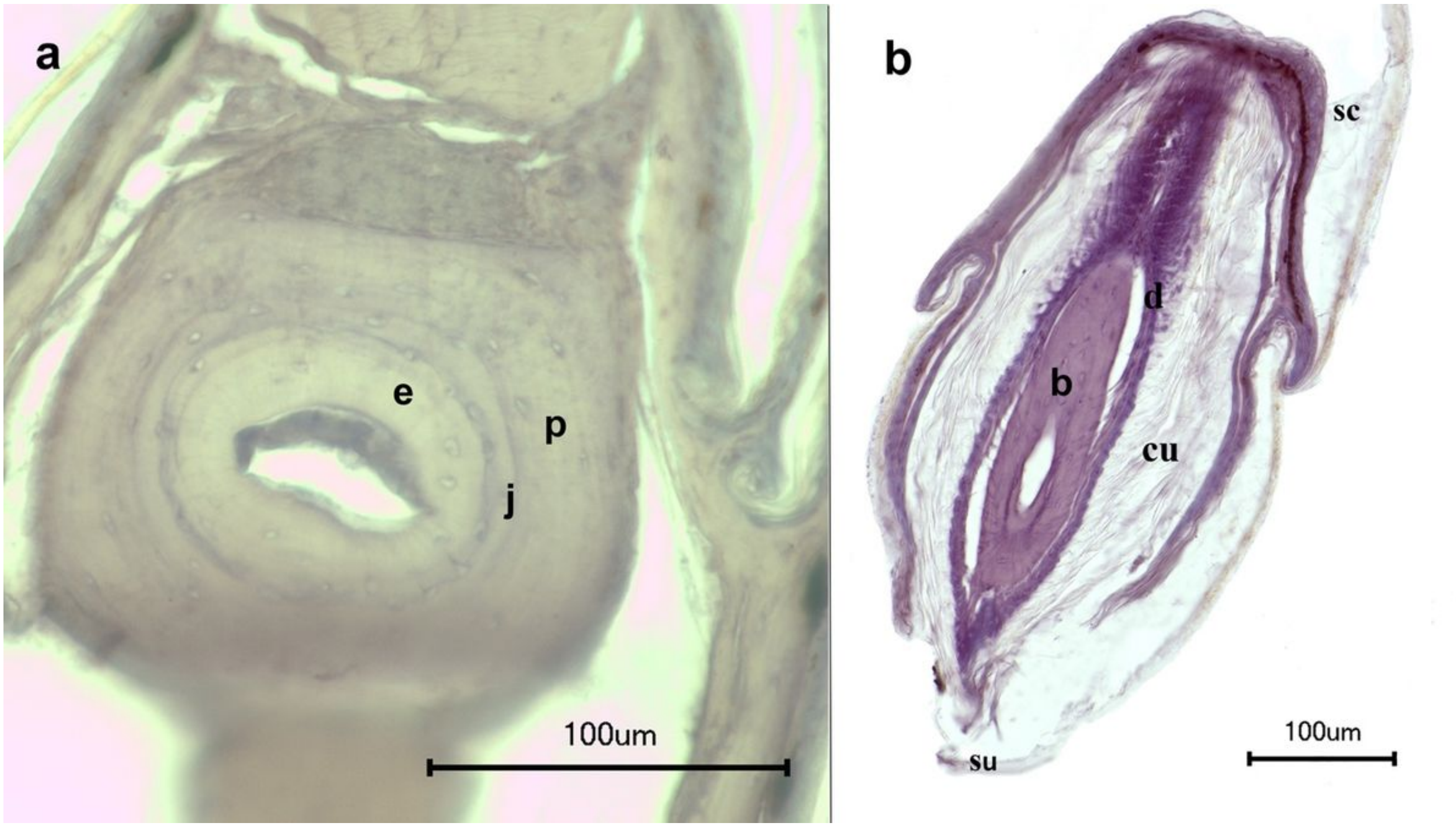

Figure 3

Microscope slides of bone finger phalanx (a) and distal phalanx of the same finger with claw sheath section (b) in *S. maculatus* N7 (March 2009) from Cat Tien National park, Vietnam. **e** – endosteum; **p** – periosteum; **j** – juvenile ring; **b** – bone of the last phalanx; **d** – derma; **cu** – cornified unguis; **sc** – scale; **su** – subunguis

Supplementary Files

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- AgeDarevskia.xlsx