

ARAŞTIRMA MAKALESİ /RESEARCH ARTICLE

LIGHT AND SCANNING ELECTRON MICROSCOPIC ANALYSIS OF *SILENE STENOPHYLLA* SEEDS EXCAVATED FROM PLEISTOCENE-AGE (KOLYMA)

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ABSTRACT

We studied the morphology of ancient seeds of the *Silene* species (Caryophyllaceae) excavated from feeding chambers of ancient ground squirrels (*Geomys*, subgenus *Urocitellus*) burrows buried in the Late Pleistocene Age permafrost deposits of Kolyma lowland (Siberia). The ancient seeds were compared to seeds of extant species of *S. alba*, *S. chlorantha*, *S. nutans* and *S. stenophylla* plants presently growing in the same and neighboring regions. Using Light (LM) and Scanning Electron Microscopy (SEM), the ancient seeds were identified to be of *Silene stenophylla* (Ledeb.).

Keywords: *Silene*, Sem Analysis, Pleistocene Age, Permafrost, Fossils.

PLEISTOSEN ÇAĞ KAZILARINDAN (KOLYMA) ELDE EDİLEN *SILENE STENOPHYLLA* TOHUMLARININ IŞIK VE ELEKTRON MİKROSKOP ANALİZLERİ

ÖZ

Bu çalışmada Sibirya, Kolyma bölgesine yakın, Pleistosen döneme ait permafrost tabakada gömülü kalmış sincap yuvasından (*Geomys* sp.) kazılar sonucu çıkarılmış olan *Silene* sp. türünün (Caryophyllaceae) tohumlarının morfolojileri incelenmiştir. Bu tohumlar günümüze kadar ulaşan yine aynı ve yakın bölgelerdeki *S. alba*, *S. chlorantha*, *S. nutans* and *S. stenophylla* türleriyle Işık ve Taramalı Elektron Mikroskop kullanılarak karşılaştırılmış ve bu tohumların *Silene stenophylla* (Ledeb.) olduğu tespit edilmiştir.

Anahtar Kelimeler: *Silene*, Sem, Pleistosen Çağ, Permafrost, Fosil Tohumlar.

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1. INTRODUCTION

The ancient burrows of ground squirrels' (*Geomys*, subgenus *Urocitellus*) buried in permafrost deposits of Late Pleistocene age at the site of Kolyma (Siberia) provided unique seed materials for evolution analysis (*Gubin and Khasanov 1996*). These burrows with seed materials in their feeding chambers have been dated back to 28 – 32.000 years B.P. (before present), determined by radiocarbon (*Stakhov et al., 2008*). It is supposed that burrows have not thawed out from freezing temperature by now.

The Pleistocene has been dated from 1.806 million years (+/- 5.000) to 11,500 years B.P. (before present), expressed in radiocarbon years. The Pleistocene climate was characterized by repeated glacial cycles with a maximum glacial extent when 30% of the Earth's surface (namely permafrost) was covered by ice (today, approximately 20% of the Earth's is covered by permafrost). The mean annual temperature at the edge of the ice was - 6 °C, and at the edge of the permafrost 0°C. Research evidence indicates that humans evolved into their present form during the Pleistocene along with the major extinction events of Neanderthals and large animals such as mammoths, mastodons, saber-toothed cats, etc. The extinctions were especially severe in North America where native horses and camels became extinct.

Archaeological samples preserved under optimal conditions at low (or permafrost) temperature (*Suh et al., 2000; Willerslev et al., 2003; Schlumbaum et al., 2008*) can supply aDNA with amplifiable quality as shown in the

studies of 15-20 thousand year old cereals (rice, wild wheat, barley) (*Suh et al., 2000; Özkan et al., 2002; Piperno et al., 2003*) and medieval samples (*Gyulai et al., 2006; Lágler et al., 2005; Szabó et al., 2005; Tóth et al., 2007*), or, in the case of fossilized samples, the deoxyribose backbone of aDNA as shown in 55 million year old (Lower Eocene) Myrtaceae fossils (*Ozerov et al., 2006*). Ancient DNA analysis of *Silene* seeds of present study are in progress.

2. MATERIALS AND METHODS

The *Silene* seeds in this study were excavated in the Kolyma region (Siberia) at the famous mammoths excavation sites (*Stakhov et al., 2008*) (Figure 1). Radio carbon analysis was carried out according to the basic methodology of *Arnold and Libby (1949)* (*Yashina et al., 2002; Stackhov et al., 2008*). Sediment samples were processed by seed sorting and identification in the laboratory according to *Shermann (1966)* and *Gyulai et al., (2006)*. For SEM (*Scanning Electron Microscopy*) analysis, seeds were air dried, fixed in glutaraldehyde (5% w/v in phosphate buffer 0.07 M, pH 7.2) and washed three times in the same buffer for 10 minutes. Samples were desiccated in an acetone concentration series (10-50-70-90-100%), dehydrated at the CO₂ critical point (*Blazers CDC 020*), and covered with gold (30 nm). Samples were examined and photographed using a TESLA BS-300 scanning electron microscope as described by *Gyulai et al., (1992)*. For LM (*Light Microscopy*) analysis, a Leica microscope (# 301-371.010) was used. For comparative analyses botanical seed samples of extant *Silene* species of *S. alba*, *S. chlorantha*, *S. nutans* and *S. stenophylla* were applied.

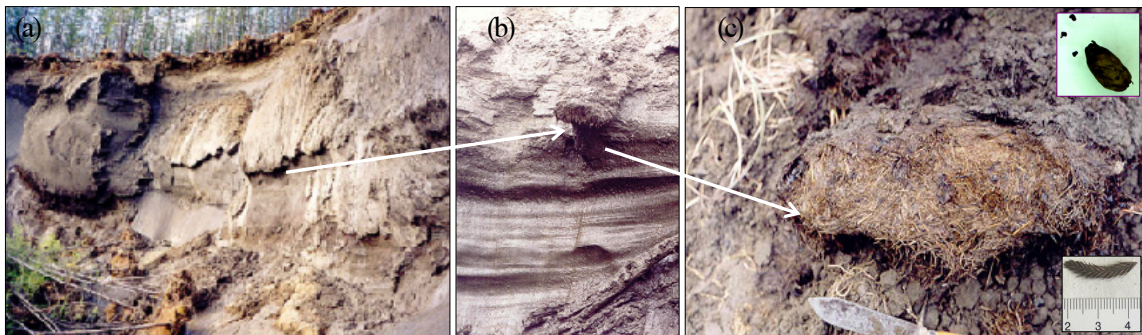


Figure 1. Excavation site (a, b) of gopher (*Geomys* spp.) holes (c) buried under a Pleistocene-age permafrost at a site near Kolyma, Siberia. Ancient *Silene* ball, a cereal spike (cm), and size (by knife) are indicated (c) (Photos by S.V. Gubin)

3. RESULTS AND DISCUSSION

The study of ancient plant fossils and remains by *archaeo/paleo botany*, and the study of aDNA (ancient DNA) by *archaeo/paleo genetics* supplies new data to evaluate changes in genetic variation and domestication (Özkan *et al.*, 2002) that occurred during evolution over the past hundreds or billion years (Gugerli *et al.*, 2005; Gyulai *et al.*, 2006).

Fossilized samples of *Bangiomorphy pubescens* (a red alga) from Canada prove that chloroplasts originated more than 1.2 billion years ago (Butterfield 2000). Fossilization coupled by charcoalification leaved floral morphology of ancient *Nymphaeales* perfectly preserved at a site in Sayreville (NJ, USA) from the earliest Upper Cretaceous time (Turonian, ca. 90 million years B.P.) (Gandolfo *et al.*, 2004; Crepet *et al.*, 2004). Fossils of basal angiosperms (*Archaeofructus sp*) were also discovered from lower early Cretaceous period in China (Zhou *et al.*, 2003). Extinct angiosperm species (e.g. *Pinus tuzsoni* Greguss; *syn. Pinuxylon tarnocziense* Tuzson) were identified from 20 million year old (Lower Miocene) site at Ipolytarnóc (Hungary) (Andreánszky, 1996; Greguss, 1972; Erdei *et al.*, 2005; Hably, 2006; Süs, 2007).

Radiocarbon dating is generally used to determine the age of carbonaceous materials up to about 60,000 years based on the naturally occurring isotope carbon-14 (^{14}C) (Plastino *et al.*, 2001). The technique was developed by Libby (Arnold and Libby, 1949), who was awarded the Nobel Prize in 1960. The methodology of radiocarbon dating is based on the fact that carbon has two stable, nonradioactive isotopes (^{12}C and ^{13}C); and one unstable isotope (^{14}C) with a half-life of $5,568 \pm 30$ years (expressed in Libby half-life) or 5,730 years (in Cambridge half-life). Practically, the small amount of ^{14}C would have vanished from the Earth long ago except for the cosmic rays which enter the atmosphere and continuously generate it from nitrogen molecules (N_2) in the air according to the classical nuclear reaction, as n (neutron) + $^{14}\text{N}_7 \rightarrow ^{14}\text{C}_6 + p$ (proton). The highest rate of ^{14}C production takes place at altitudes of 9 to 15 km but it spreads evenly throughout the atmosphere producing at a constant rate and with the proportion of radioactive to non-radioactive carbon also remaining constant, ca. 1 ^{14}C / 600 billion atoms/mole. As nonradioactive C-isotopes ^{14}C also reacts with oxygen to form CO_2 , which enters plants by photosynthesis, and from plants it is incorporated into animal tissue. When organisms (plants or animals) die, the incorporation of ^{14}C stops, and its content gradually decreases in

the cadaver through radioactive decay by turning back the generative reaction producing $^{14}\text{N}_7$ according to the reaction: n (neutron) + $^{14}\text{C}_6 \rightarrow ^{14}\text{N}_7 + e^-$ (electron) + ν_e (anti neutrino). This decay is used to measure how long ago a piece of once-living material died and this is expressed as years B.P. (before present, and calibrated as 1950 A.D.). The approximate age of the ancient *Silene* (*Caryophyllaceae*) seeds of the present study were determined by radiocarbon method to be 28,000 -32,000 years old.

Ancient *Silene* seeds were compared to seed samples of four recent species growing in the same region (*S. alba*, *S. chlorantha*, *S. nutans* and *S. stenophylla*) and determined to be of *Silene stenophylla* (Ledeb.) by SEM and LM (Figure 2). The ancient *Silene* seeds had morphological features characteristic of those of contemporary *S. stenophylla* seeds, except for smaller size (Figure 2). Interestingly, the ancient seeds had damaged embryos (2B, Figure 2), which might be the result of the gophers' activity and effort to prevent undesired germination (Figure 2). However, a preliminary result about a successful regermination experiment was reported by Yashina *et al.* (2002). As cells of well preserved permafrost seeds might carry intact cells with intact nucleus an experiment for callus initiation for plant regeneration in tissue culture is also in progress similar to the former successful (Aufhammer and Fischbeck, 1964; Ruckebauer, 1971), unsuccessful (Szabó *et al.*, 2005; Lágler *et al.*, 2005) and doubtful ancient seed germination results (Porshild *et al.*, 1967; Quinn, 1999; Shen-Miller, 2002).

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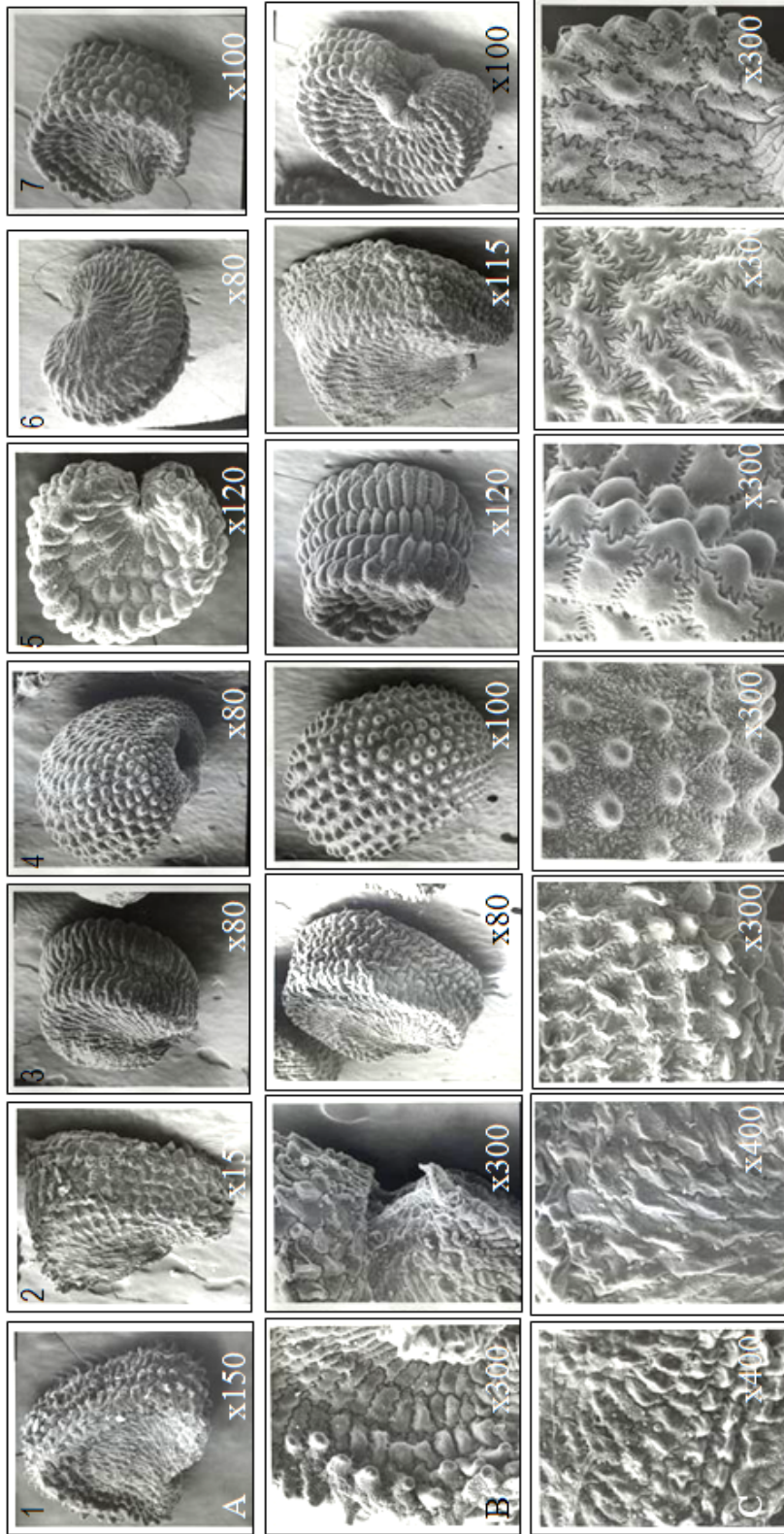


Figure 2. Morphology of ancient (1 #P1075 and 2 #P1300) (provided by S.V. Maksimovich) and current (3-7) *Silene* seeds; SEM micrographs: *S. stenophylla* Ledeb. (Kolyma region) (3); *S. alba* (Moscow region) (4); *S. chlorantha* (Voronyezs region) (5); *S. nutans* (Moscow region) (6); *S. viscosa* (Moscow) (7). Upper (A) and middle (B) rows show seeds morphology, bottom row (C) shows seed coat surfaces. Magnifications are indicated (processed by G. Gyulai).

4. REFERENCES

- Andreánszky, G. (1966). The Upper Oligocene Flora of Hungary. Analysis of the site at the Wind Brickyard, Eger. *Studia Biologica Hungarica* 5, 1–151.
- Aufhammer, G. and Fischbeck, G. (1964). Ergebnisse von Gefass- und Feldversuchen mit dem Nachbau keimfähiger Gersten- und Haferkörner aus dem Grundstein des 1832 errichteten. Nürnberger Stadttheates. *Zeitschrift für Pflanzenzuchtung* 51, 345–378.
- Arnold, J.R. and Libby, W.F. (1949). Age Determinations by Radiocarbon Content: Checks with Samples of Known Age. *Science* 110, 678–680.
- Butterfield, N.J. (2000). *Bangiomorpha pubescens* n. gen., n. sp.: implications for the evolution of sex, multicellularity, and the Mesoproterozoic/Neoproterozoic radiation of eukaryotes. *Paleobiology* 26, 386–404.
- Crepet, W., Nixon, K.C. and Gandolfo, M.A. (2004). Fossil evidence and phylogeny: the age of major angiosperm clades based on mesofossil and macrofossil evidence from cretaceous deposits. *American Journal of Botany* 91, 1666–1682.
- Erdei, B., Hably, L., Kázmér, M., Utescher, T. and Bruch, A.A. (2007). Neogene flora and vegetation development in the Pannonian Basin - relations to palaeoclimate and palaeogeography. – *Palaeogeography, Palaeoclimatology, Palaeoecology* 253, 131–156.
- Gandolfo, M.A., Nixon, K.C. and Crepet, W.L. (2004). Cretaceous flowers of Nymphaeaceae and implications for complex insect entrapment pollination mechanisms in early Angiosperms. *Proceedings of the National Academy of Sciences USA* 101, 8056–8060.
- Greguss, P. (1972). Xylotomy of the living conifers. Pp. 329, Akadémiai Kiadó, Budapest.
- Gubin, S.V. and Khasanov, B.F. (1996) Fossil Burrows of Mammals in the Loess-Ice Deposits of the Kolyma-Indigirka Lowland, *Doklady AN*, 1996, vol. 346, no. 2, pp. 278–279 [Doklady Biol. Sci. (Engl. Transl.), vol. 346, pp. 26–27].
- Gugerli, F., Parducci, L. and Petit, R.J. (2005). Ancient plant DNA: review and prospects. *New Phytologist* 166, 409–418.
- Gyulai, G., Janovszky, J., Kiss, E., Lelik, L., Csillag, A. and Heszky L.E. (1992). Callus initiation and plant regeneration from inflorescence primordia of the intergeneric hybrid *Agropyron repens* (L.) Beauv. x *Bromus inermis* Leys. cv. nanus on a modified nutritive medium. *Plant Cell Report* 11, 266–269.
- Gyulai, G., Humphreys, M., Lágler, R., Szabó, Z., Tóth, Z., Bittsánszky, A., Gyulai, F. and Heszky, L. (2006). Seed remains of common millet from the 4th (Mongolia) and 15th (Hungary) centuries; AFLP, SSR, and mtDNA sequence recoveries. *Seed Science Research* 16, 179–191.
- Hably, L. (2006). Catalogue of the hungarian cenozoic leaf, fruit and seed floras from 1856 to 2005. *Studia Botanica Hungarica* 37, 41–129.
- Lágler, R., Gyulai, G., Humphreys, M., Szabó, Z., Horváth, L., Bittsánszky, A., Kiss, J., Holly, L. and Heszky, L. (2005). Morphological and molecular analysis of common millet (*P. miliaceum*) cultivars compared to an aDNA sample from the 15th century (Hungary). *Euphytica* 146, 77–85.
- Ozerov, I.A., Zhinkina, N.A., Efimov, A.M., Machs, E.M. and Rodionov, A.V. (2006). Feulgen-positive staining of the cell nuclei in fossilized leaf and fruit tissues of the Lower Eocene Myrtaceae. *Botanical Journal of Linnean Society* 150, 315–321.
- Özkan, H., Brandolini, A., Schäfer-Pregl, R. and Salamini, F. (2002). AFLP analysis of a collection of tetraploid wheats indicates the origin of emmer and hard wheat domestication in southeast Turkey. *Molecular Biology and Evolution* 19, 1797–1801.
- Piperno, D.R., Weiss, E., Holst I. and Nadel, D. (2004). Processing of wild cereal grains in

- the Upper Palaeolithic revealed by starch grain analysis. *Nature* 430, 670-673.
- Porsild, A.E., Pharlington, C.R. and Mulligan, G.A. (1967). 'Lupinus arcticus' Wats. grown from seeds of Pleistocene age. *Science* 158, 113-114.
- Plastino, W., Kaihola, L., Bartolomei, P. and Bella, F. (2001). Cosmic background reduction in the radiocarbon measurement by scintillation spectrometry at the underground laboratory of Gran Sasso. *Radiocarbon* 43, 157-161.
- Quinn, R.M. (1999). Kamut: Ancient grain, new cereal. In: *Perspectives on new crops and new uses*. Ed: J. Janick, pp. 182-183, ASHS Press, Alexandria, VA.
- Ruckenbauer, P. von (1971). Keimfähiger Winterweizen aus dem Jahre 1877. Beobachtungen und Versuche. pp. 372-386. Inst. f. Pflanzenbau und Pflanzenzuchtung d. Hochschule f. Bodenkultur in Wien.
- Schermann, Sz. (1966). Seed morphology (Magismeret, in Hungarian), Vol. I (pp. 861) and II (pp. 209) Akadémiai Kiadó, Budapest.
- Schlumbaum, A., Tensen, M. and Jaenicke-Despres, V. (2008). Ancient plant DNA in archaeobotany. *Vegetation History and Archaeobotany* 17, 233-244.
- Shen-Miller, J. (2002). Sacred lotus, the long-living fruits of China Antique. *Seed Science Research* 12, 131-143.
- Suh, H.S., Cho, J.H., Lee, Y.J. and Heu, M.H. (2000). RAPD variation of 13,010 and 17,310 year-old carbonized rice. 4th International Rice Genetics Symposium, Manila, Philippines, Oct. 22-27.
- Stakhov, V.L., Gubin, S.V., Maksimovich, S.V., Rebrikov, D.V., Savilova, A.M., Kochkina, G.A., Ozerskaya, S.M., Ivanushkina, N.E. and Vorob'eva, E.A. (2008). Microbial Communities of Ancient Seeds Derived from Permanently Frozen Pleistocene Deposits. *Microbiology* 77, 348-355.
- Süss, H. (2007). Wood fossils of the morphogenus *Spiroplatanoxydon* gen. nov. from the Tertiary of Europe and the Middle East. *Feddes Repertorium* 118, 1-19.
- Szabó, Z., Gyulai, G., Humphreys, M., Horváth, L., Bittsánszky, A., Lágler, R. and Heszky, L. (2005). Genetic variation of melon (*C. melo*) compared to an extinct landrace from the Middle Ages (Hungary) I. rDNA, SSR and SNP analysis of 47 cultivars. *Euphytica* 146, 87-94.
- Tóth, Z., Gyulai, G., Horváth, L., Szabó, Z. and Heszky, L. (2007). Watermelon (*Citrullus l. lanatus*) production in Hungary from the Middle Ages. *Hungarian Agricultural Research* 2007/4, 14-19.
- Zhou, Z., Barrett, P.M. and Hilton, J. (2003). An exceptionally preserved Lower Cretaceous ecosystem. *Nature* 421, 807-814.
- Willerslev, E., Hansen, A.J., Binladen, J., Brand, T.B., Gilbert, M.T.P., Shapiro, B., Bunce, M., Wiuf, C., Gilichinsky, D.A. and Cooper, A. (2003). Diverse plant and animal genetic records from Holocene and Pleistocene sediments. *Science* 300, 791-795.
- Yashina, S.G., Gubin, S.V., Shabaeva, E.V., Egorova, E.F. and Maksimovich, S.V. (2002). Viability of Higher Plant Seeds of Late Pleistocene Age from Permafrost Deposits as Determined by in vitro Culturing, *Doklady AN*, 2002, Vol. 383, No 5, 714-717 [Doklady Biol. Sci. (Engl. Transl.), Vol. 383, 167-170]



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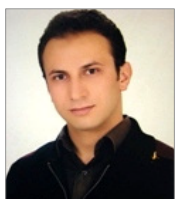
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