Anadolu University Journal of Science and Technology
*Anadolu Üniversitesi Bilim Ve Teknoloji Dergisi*
Vol. 10, 161-167 (2009)

# 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.).

*Key words: Silene*, SEM analysis, Pleistocene Age, permafrost, fossils

## Özet

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 Tarayıcı 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 $CO_2$ 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.

## 3. RESULTS AND DISCUSSION

The study of ancient plant fossils and remains by *arhaeo/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 (*Archaefructus 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; Süss ,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±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) + $^{14}N_7 \rightarrow {}^{14}C_6$ + 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 $^{14}N_7$ according to the reaction: n (neutron) + $^{14}C_6 \rightarrow {}^{14}N_7$ + $e^-$ (electron) + $\nu_e$ (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; Ruckenbauer, 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).

*Acknowledgement:* The author thanks Prof. L Waters (Auburn) and Prof. L Kovács (Missouri) for their comments on manuscript. The project was supported by grants of EU SOCRATES Student Exchange Program. Ancient seeds were kindly provided by Dr. S.V. Maksimovich.

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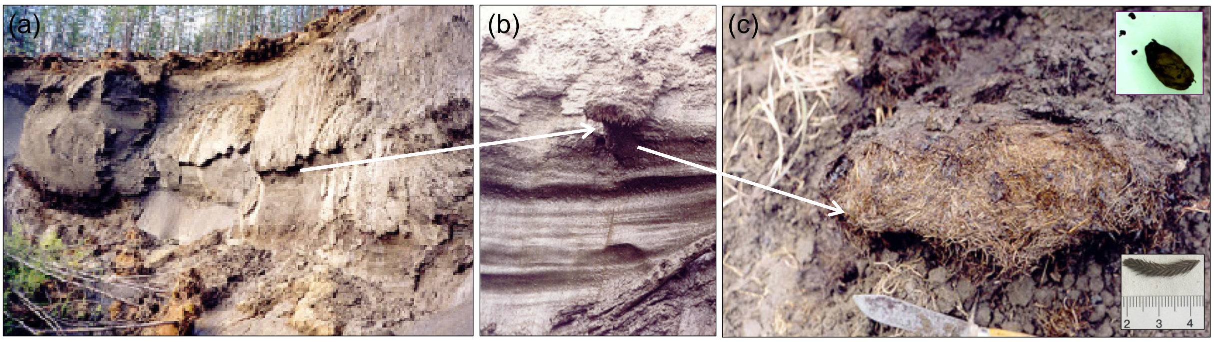


*Figure 1.* Excavation site (a, b) of ancient gopher (*Geomys* ssp.) holes (c) buried under a Pleistocene-age permafrost at a site near Kolyma, Siberia. Ancient *Silene* ball, a cereal spike (cm), and size (by knife) are indicated (c) (Photos by S.V. Gubin)

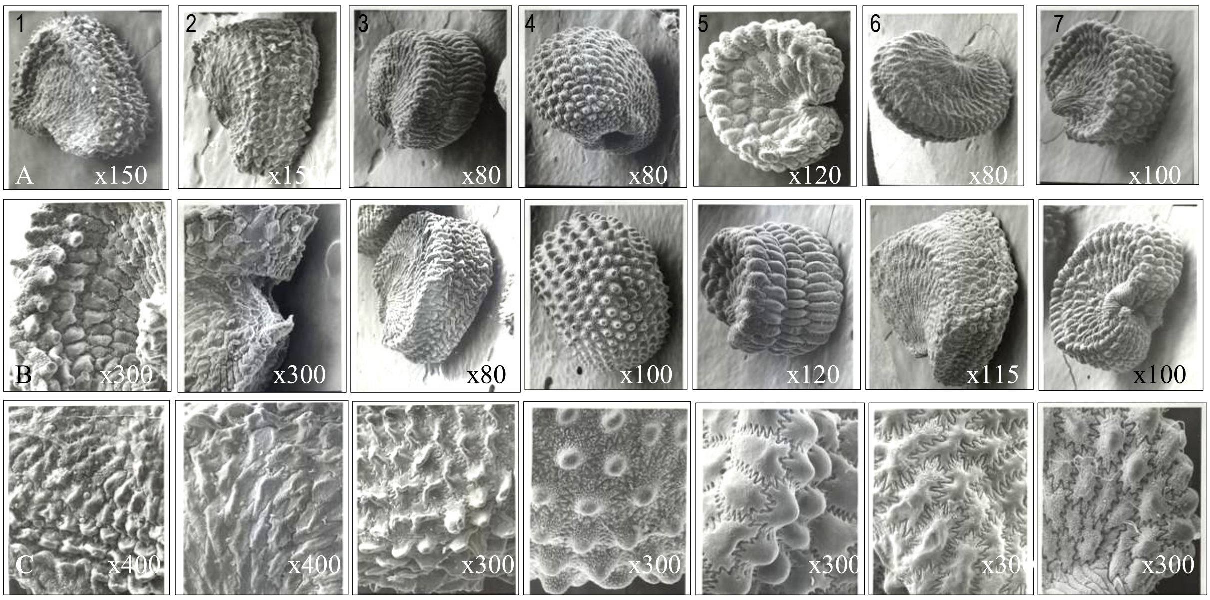


*Figure 2.* Morphology of ancient (**1** #P1075 and **2** #P1300) (provided by S.V. Maksimovich) and current (**3-7**) *Silene* seeds; SEM micrographs: *S. stenophylla* Ledeb. (Kolyma region) (**3**); *S. alba* (Moscow region) (**4**); *S. chlorantha* (Voronyezs region) (**5**); *S. nutans* (Moscow region)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

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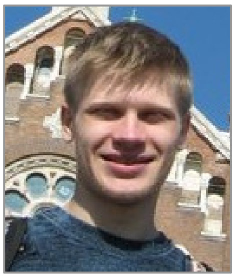

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