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A technique for the extraction of palynomorphs from the Eocene amber

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ABSTRACT

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Amber is fossilized form of resin produced as the metabolic exudates from plants that serve defensive and protective biological purposes as well as other ecological functions. By virtue of excellent preservation potential of the amber, a diverse array of biota in extraordinarily pristine condition was recovered within the fossilized resins from Cambay Basin. In the present study, a new extraction method has been devised to retrieve palynomorphs from the early Eocene Vastan amber, Cambay Basin, Gujarat. The method was found useful for the extraction of organic matter and better yield of spore–pollen. The extracted significant palynomorphs such as *Dipterocarpuspollenites retipilatus*, *Myrtaceidites* spp., *Ctenolophonidites costatus, Polygalacidites* spp. demonstrate excellent preservation and clear morphological features as observed in modern ones. This method has provided a new dimension to study the palynofloral diversity and other ecological associations of the amber produced in tropical rainforest ecosystem in low latitude during the early Paleogene warming.

Key–words—Cambay amber, Early Eocene, Palynomorphs, Extraction method.

आदिनूतन अंबर से प्राप्त परागाणुसंरूपों के निष्कर्षण हेतु एक तकनीक

पूनम वर्मा

सारांश

पादपों से प्राप्त उपापचयी रिसाव के रूप में उत्पादित राल का जीवाश्मित रूप अंबर है जो रक्षात्मक एवं बचाव जैव प्रयोजनों के साथ–साथ अन्य पारिस्थितिकीय प्रकार्यों की आवश्यकता पूर्ण करता है। अंबर की उत्कृष्ट परिरक्षण संभावना की वजह से, कैंबे द्रोणी से जीवाश्मित रालों के अंदर विलक्षण पुरातन दशा में जीव–जात का एक असदृश विन्यास मिला (प्राप्त किया गया) था। मौजूदा अध्ययन में प्रारंभिक आदिनूतन वास्तन अंबर, कैंबे द्रोणी गुजरात से परागाणु संरूपों की पुनः प्राप्ति को एक अभिनव निष्कर्षण विधि आविष्कृत की गई है। कार्बनिक पदार्थ के निष्कर्षण एवं बीजाणु–पराग के बेहतर परिणाम हेतु इसे उपयोगी पाया गया है। निष्कर्षित महत्वपूर्ण परागाणु संरूप उदाहरणार्थ *डिप्टेरोकार्पसलेनाइट्स रेटिपिलेटस, मायरटेसीडाइटस* जातियां, *क्टेनोलोफोनिडाइट्स कॉस्टेटस, पॉलीगेलासीडाइट्स* जातियां उत्कृष्ट परिरक्षण एवं निर्मल (सुस्पष्ट) आकारिकीय लक्षण प्रदर्शित करते हैं जैसा कि नवीन (आधुनिक) अद्वितीय में प्रेक्षित हुआ है। प्रारंभिक पैलियोजीन तपन के दौरान अल्प अक्षांश में अंबर उत्पादित उष्णकटिबंधीय वनवर्षा पारितंत्र की परागाणुपुष्पी विविधता एवं अन्य पारिस्थितिकीय संगुणनों का अध्ययन करने को इस विधि ने अभिनव आयाम प्रदान किया है।

सूचक शब्द—कैंबे अंबर, प्रारंभिक आदिनूतन, परागाणु संरूप, निष्कर्षण विधि।

INTRODUCTION

AMBER is polymerized fossil resin secreted by a wide range of plants, typically found associated with coniferous gymnosperms (Araucariaceae, Pinaceae, Taxodiaceae, Cupressaceae) and the tropical arborescent dicotyledonous angiosperms (Dipterocarpaceae, Caesalpinaceae, Burseraceae). Resins are complex lipid–soluble mixture volatile and/or phenolic or terpenoid compounds which are the product of secondary carbon metabolism of the plant (Langenheim, 1995). They are secreted by specialized structures present internally or on the surface of the plants to perform ecological functions such as structural support, biochemical defense, semi–chemical signaling, defensive mechanics, etc. (Langenheim, 1995, 2003). Terpenoids are genetically programmed and considered to provide some degree of taxonomic specificity (Bohlmann *et al*., 1998). Fresh amber flow makes an excellent medium for the preservation of three–dimensional structure, and ultrastructural cellular details of plant remains, insects and other micro–organism. The records of fossils embedded in amber are available from Mid Carboniferous to sub–recent resins (Sargent Bray & Anderson, 2009). Ambers have also been investigated for the potential preservation of DNA (Cano *et al*., 1994; Greenblatt *et al*., 1999) and also for the original air and water trapped (Cerling, 1989; Berner & Landis, 1998).

In India, the amber is ubiquitously associated with the various early Eocene lignite mines deposited during the extremely warm period. It was the time of global evolution of the tropical and subtropical biomes. A continuum of diverse arthropods and other biota have been reported from the Cambay amber (Alimohammadian *et al*., 2005; Rust *et al*., 2010; Engel *et al*., 2011a, b; Beimforde *et al*., 2011; Grimaldi & Singh, 2012; Sadowski *et al.*, 2012; Henrichs *et al.*, 2016) however, palynological studies are scarce (Singh *et al*., 2014). Though weakly cross linked and polymerized character of the Cambay amber sometimes provide better transparency to get a glimpse of inclusions, but the rough interface surface of the different amber flow lines, dark color, and other inclusions obstruct optical observation of embedded fossils (especially tiny sporomorphs) in the amber chunks.

In the present study an attempt has been made to standardize the procedure for the extraction of the palynomorphs from the amber. Study has been conducted on the samples of the amber collected from Vastan Lignite Mine (latitude 21°25'47" N and longitude 73°07'30" E) situated about 60 km northeast of Surat District (Cambay Basin), Gujarat (Fig. 1). The purpose of the present work is to open fields of retrieval of well preserved palynological entities and their morphographic diagnoses.

PROCEDURE FOR EXTRACTION OF PALYNOMORPHS FROM AMBER

Sample collection and cleaning

Amber pieces were carefully retrieved from the sediment layers with the help of a sharp chisel, needle and brush. In the laboratory, small amber pieces of 2–8 cm in diameter were soaked overnight in water then placed in a standard sieve of 1 mm mesh and washed in tap water to remove the loose sediments or lignite specks. If needed, a soft brush was used to clean the firm sediment attached to the surface of the amber. Reasonably clear amber chunks or pieces with flow lines were chosen for thin section of the amber as well as dissolution for palynomorphs recovery.

Thin section preparation

The thin sections of the cleaned amber pieces were cut by using a very thin diamond blade (1–2 mm). Grinding and polishing was done with a water fed lap wheel with emory papers of different grit size (400–1200) to attain the flat surface. The flat surface of the thin slab is mounted on the glass microscopic slides using Canada balsam. The other side of the amber piece is again cut, ground and polished likewise to make 1–2 mm thick section. Then the cover slip is mounted with Canada balsam, allowing the clear observation of the fossil contents inside the amber pieces.

Dissolution of amber

Despite the early Eocene age, the Cambay amber is soft and sticky similar to copal (recent amber) due to its weak polymerization and cross linking which make its comparatively readily soluble in several solvents such as toluene, xylene, dichloromethane and trichloromethane (Mazur *et al*., 2014). In the present study, toluene, xylene, and dichloromethane were tested for dissolution of the amber. The pieces were dissolved in solvents in a glass jar and were kept in a fume chamber. The jar was maintained air tight in order to minimize evaporation of the solvents. The amber chunks completely dissolved in all the solvents with the help of intermittent stirring with glass rod in 1–2 hrs at slightly different rates. The included organic and inorganic matter flocculated and settled at the bottom as a fine layer. The column of supernatant solvent was carefully removed with the help of a dropper, taking care not to disturb the settled bottom layer. Then samples were transferred to centrifuge tubes and centrifuged repeatedly rinsed with solvent to clear the traces of amber (Fig. 2A–D). The residue of the amber inclusions in solvent was centrifuged at a speed of less than 2000 rpm for 15 min only to avoid breakage of palynomorphs. The final solution was observed under a microscope at low magnification to check the type of inclusions. In case insects or parts of insects, or any other big inclusion were encountered, they were picked at this stage.

Retrieval of palynomorphs

The residue of the amber after dissolution contains several types of inclusions including palynological contents. To retrieve spore–pollen and for their better visibility, a well–established chemical preparation technique, "acetolysis"

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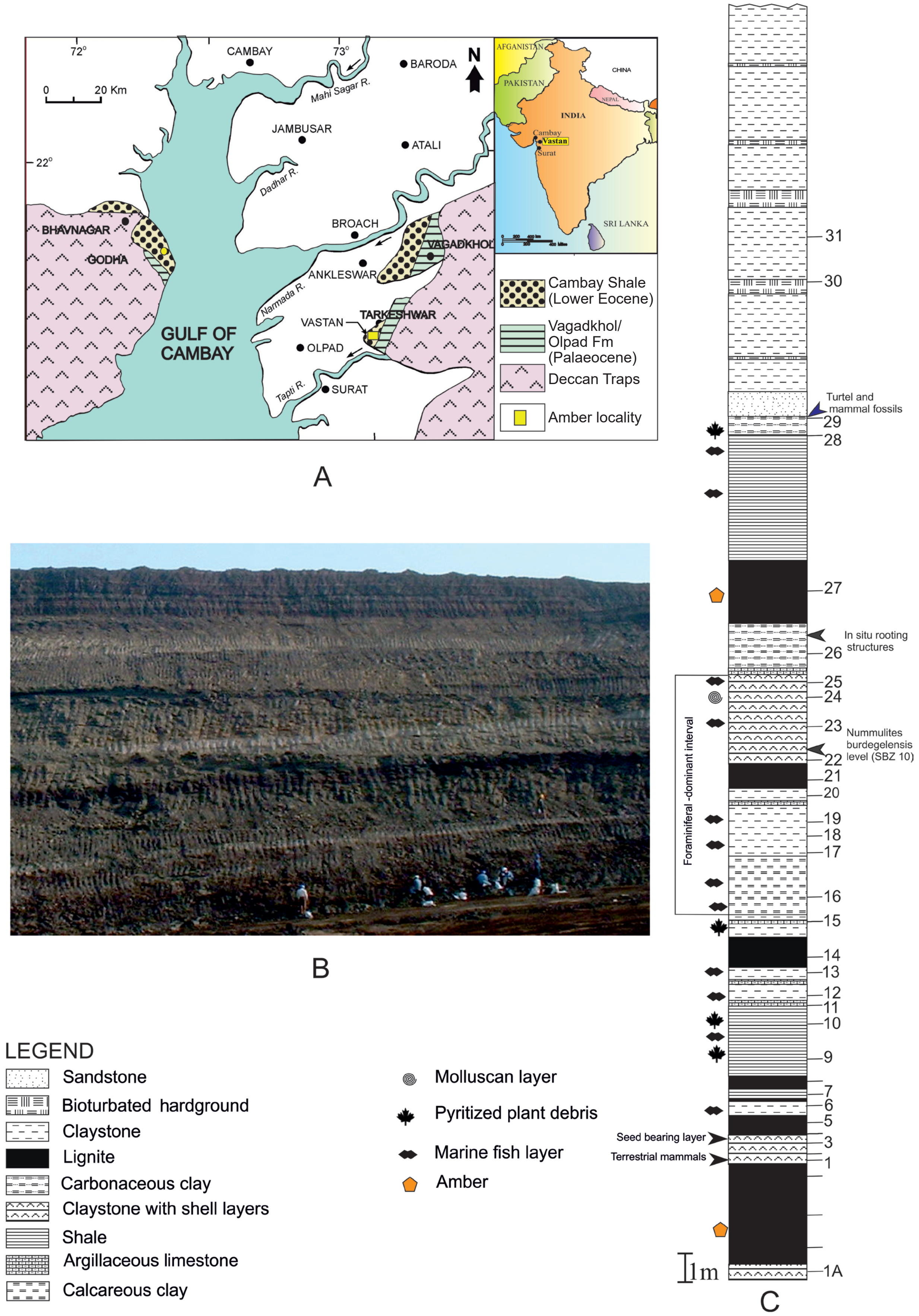
Fig. 1—A. Geological map of the area around Vastan Lignite Mine, Gujarat (after Sahni *et al*., 2006); B. Photograph of Vastan Lignite Mine, Gujarat; C. Litholog of Vastan Lignite Mine, Gujarat (after Sahni *et al.*, 2006).


72°
CAMBAY
73°
BARODA
N
0
20 Km
Mahi Sagar R.
JAMBUSAR
ATALI
22°
Dadhar R.
BROACH
BHAVNAGAR
GODHA
VAGADKHOL
ANKLESWAR
Narmada R.
TARKESHWAR
VASTAN
GULF OF CAMBAY
OLPAD
Tapti R.
SURAT
AFGANISTAN
PAKISTAN
CHINA
NEPAL
INDIA
Cambay
Vastan
Surat
SRI LANKA
Cambay Shale (Lower Eocene)
Vagadkhol/ Olpad Fm (Palaeocene)
Deccan Traps
Amber locality
A
B
LEGEND
Sandstone
Bioturbated hardground
Claystone
Lignite
Carbonaceous clay
Claystone with shell layers
Shale
Argillaceous limestone
Calcareous clay
Molluscan layer
Pyritized plant debris
Marine fish layer
Amber
31
30
Turtel and mammal fossils
29
28
27
In situ rooting structures
26
25
24
23
Nummulites burdegelensis level (SBZ 10)
22
21
20
Foraminiferal -dominant interval
19
18
17
16
15
14
13
12
11
10
9
7
6
5
Seed bearing layer
3
Terrestrial mammals
1
1m
1A
C

(Erdtman, 1943) was adopted. In this technique, the fine dehydrated and shrunken cellulosic matter was removed which hindered light microscopic observations of recovered palynomorphs. For that, the residue from the amber was mixed with absolute glacial acetic acid to dehydrate the residue. After that, the centrifuged residue was mixed with the fresh acetolysis mixture (mixture of acetic anhydride and concentrated sulphuric acid in a ratio of 9: 1). The residue was boiled for 5 minutes in a water bath after adding acetolysis mixture. When the reaction slows down, it was centrifuged and decanted with caution. Finally, the residue was treated with glacial acetic acid and washed with distilled water repeatedly to remove all traces of acid (Fig. 2E).

Slide preparation

After acetolysis, very fine organic and inorganic particles were found needed to be separated from the macerated residue. For that gravity separation method was adopted that was also essential to concentrate the spore/pollen fraction from the residue. Owing to their specific gravity, minerals and organic particles have different settling rate. Spores and pollen possessing lower density than the inorganic mineral particles tend to float in water for a longer time and therefore can easily be separated when swirled. The macerated residue was mixed with 10–15 ml of water in a medium–sized watch glass. The watch glass was rotated in a circular way with hand. This action allows the inorganic mineral particles and bigger organic debris pieces to settle at the bottom in the centre, whereas the lighter particles including spores and pollen remain suspended in the water. The upper layer of water was transferred to another watch glass with the help of a dropper and was allowed to settle for about half an hour. The second watch glass was again rotated allowing the organic matter to collect in the centre of the watch glass. Water from the watch glass was removed with the help of a dropper leaving the organic matter in the watch glass. The collected material containing spores and pollen grains were mounted on slides for observation in the microscope. At this stage stubs for SEM can also be prepared by transferring and drying a drop of material on a small piece of slide.

For permanent slide preparation, the water–free macerated residue was mixed with a few drops of polyvinyl solution and spread uniformly over the cover glass with the help of niddle. The cover glass was dried in an oven for about thirty minutes and was then mounted on the glass slide with Canada balsam. The prepared slides were kept in an oven at 50–60°C for drying.

RESULTS

In the present study, the thin sections of the amber have been prepared for microscopically observations of palynomorphs. Surprisingly, hardly any spore–pollen were noticed, however, organic debris, insects/ insect fragments, and other indiscernible inclusions were present. The possible reason behind the absence of palynomorphs could be the adherence of the spore/pollen on the surface of the amber flow in the form of a thin layer. Multiple layers of the amber flow lines together with other inclusions may possibly have obscured preserved palynomorphs in thin section. However, through dissolution and acetolysis method of extraction of exceptionally well–preserved palynomorphs from the amber were successfully carried out (Pl. 1).

DISCUSSION

Previously, various solvents have been used by different workers for the extraction of the pollen from the amber like, ether (Galippe, 1920), alcohol (Larsson, 1978), etc. but amber was only smoothened and partially dissolved. Azar (1997) used chloroform to dissolve Labenese amber of the early Cretaceous and reported satisfactory results of dissolving amber within two hours but only defragmented articulated insects were recovered. Rust *et al*. (2010) reported that toluene is also a good solvent for the Cambay amber which extracts insect fossils without adversely affecting the inclusions. Later, Mazur *et al*. (2014) examined several solvents to check their ability to dissolve the Cambay amber pieces and reported xylene as preferably best solvent. So far, unavailability of any standard methodology for the retrieval of palynological archives from the amber has been noticed; however, a brief account has been given by Singh *et al*. (2014).

In the present study, monocyclic aromatic hydrocarbons, toluene (toluol, methylbenzene or phenylmethane) and xylene (xylol) both were tested for retrieval of palynomorphs from the amber. The basic structure of these compounds is the benzene ring. However, xylene is proved to be better solvent in contrast to toluene for dissolution of the Cambay amber due to two electron–releasing functional groups which provide a stronger positive mesomeric effect. In contrast, dichloromethane has not been found a suitable solvent due to its high volatility and evaporation rate (Mazur *et al*., 2014). After dissolution, it is very difficult to recognize the pollen type due to masking of the exine by dead cellulose as well as dehydrated and shrunken cell content at the centre. The chemical treatment called acetolysis (Erdtman, 1943) was proven suitable to make the palynomorphs clear for high resolution light microscopy and scanning electron microscopy.

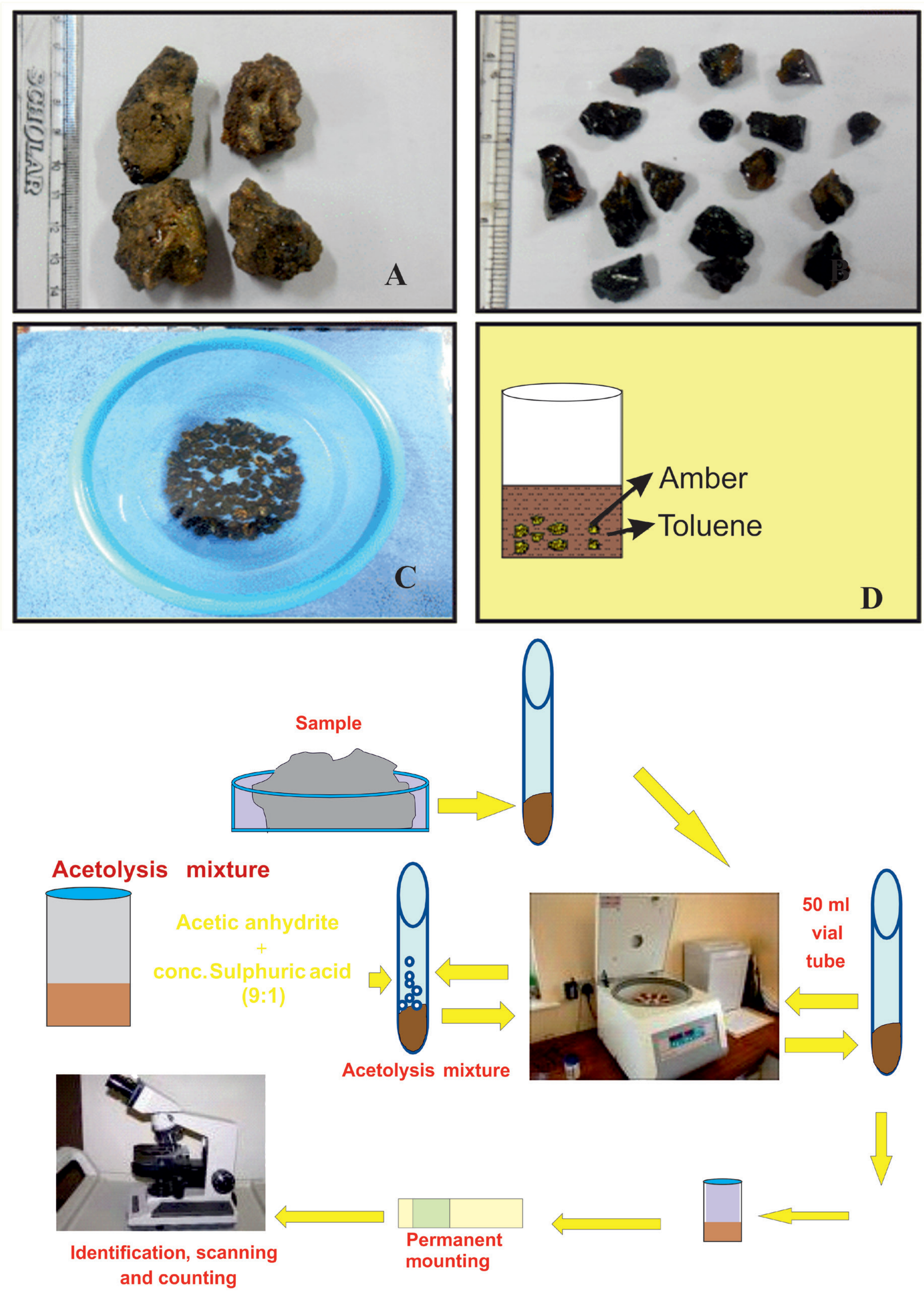


Fig. 2—Pictorial representation, A–D. method of cleaning and dissolution of amber; E. flow chart for acetolysis process.

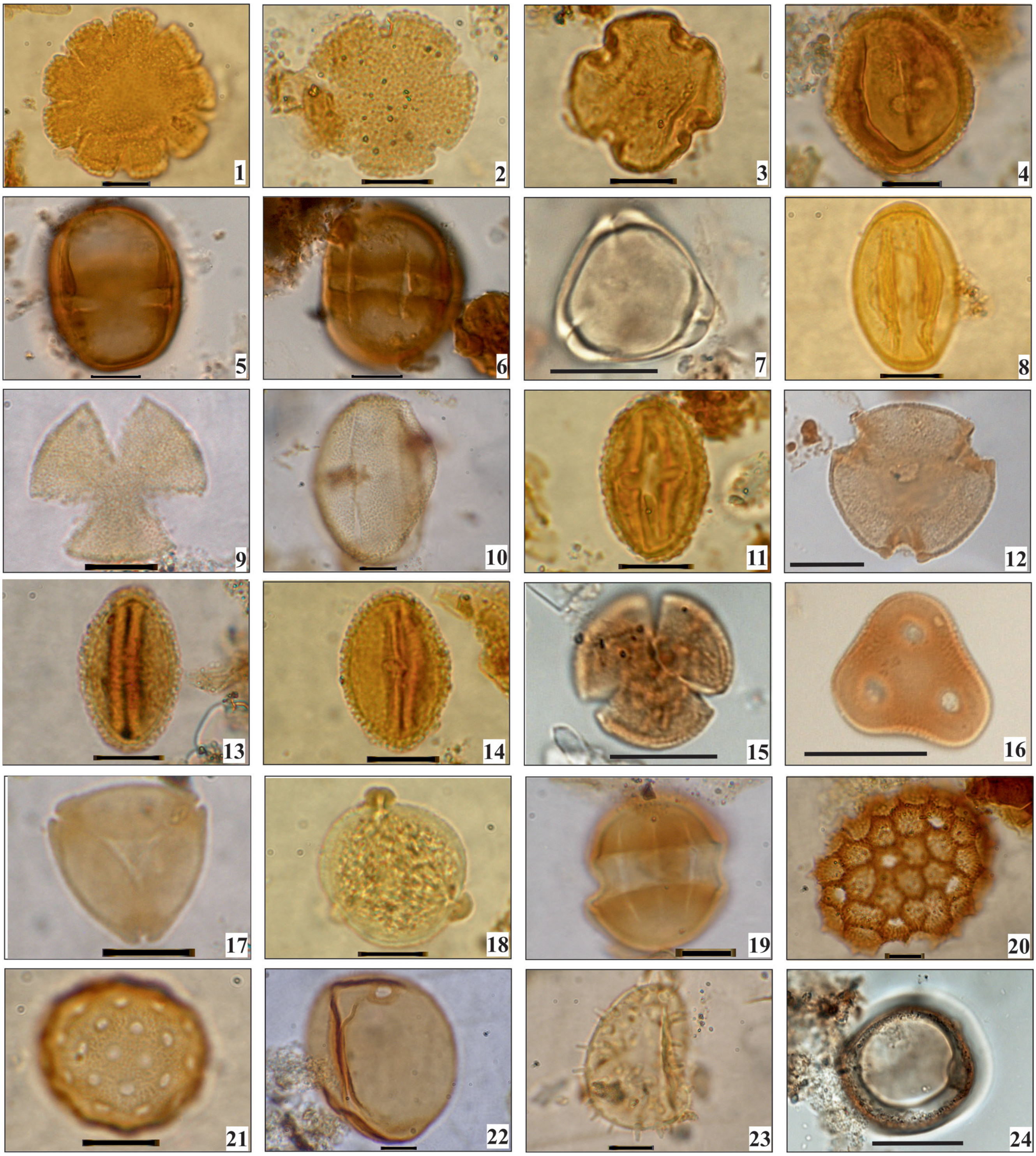


PLATE 1

Previously, palynologically investigated sedimentary succession of Vastan lignite mine associated with the amber flows provided evidence of a well–diversified and luxuriant rain forest community during the early Eocene period (Mandal & Guleria, 2006; Tripathi & Srivastava, 2012; Rao *et al*., 2013). The assemblages were dominated by coastal marshy (*Palmidites, Palmaepollenites, Proxapertites* and *Neocouperipollis*) and mangrove (*Spinizonocolpites*, *Palaeosantalaceaepites*, *Spinomonosulcites* and *Barringtoniapollenites*) palynoflora. The succession was deposited under shallow marine, coastal lagoonal settings under tropical palaeoclimatic regime (Rao *et al*., 2013; Prasad *et al*., 2013). In addition, diverse faunal and floral archives from the Cambay amber have also reported substantial information regarding the source of the amber and palaeoecology of the early Eocene forest where the amber was produced (Rust *et al*., 2010; Dutta *et al*., 2011).

In the present study, the rich and diverse palynomorphs were recovered by using the method developed through dissolution and acetolysis of the amber. The recovered palynoassemblage provided a composition of the tropical inland forest mainly consisted of the family–Dipterocarpaceae, Anacardiaceae, Alangiaceae, Ctenolophonaceae, Thymeliaceae, Myrtaceae, Meliaceae, Sapotaceae, Proteaceae, Polygalaceae, Chenopodiaceae/Amaranthaceae, Poaceae, Asteraceae and Rhizophoraceae. In amber, the copious deposition of spore/pollen can be attributed to a dense canopy of rain forest with slow air flow which adheres pollen in the amber suspended in the understory. The concentration of pollen /spores is more likely to be on the surface of amber flow or at the interface of two amber flows. Moreover, the excellent preservation potential of the amber is also accountable reason for the palynomorphs inclusion in pristine condition.

CONCLUSIONS

Present study provides the critical review of the previously published methodologies focusing particularly on the dissolution of amber for extraction of the fossils embedded in it. The palynoassemblage retrieved from the amber provide unique data regarding composition and diversity of the early Eocene flora of the possibly earliest tropical rain forest of Asia. To my knowledge, the present amber extraction methodology would open a new dimension to study the palynofloral diversity and ecological associations produced in low latitude tropical rain forest ecosystem during the early Palaeogene warming.

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PLATE 1

← (Scale bar = 10 μm)

1. *Ctenolophonidites costatus* van Hoeken–Klinkenberg, 1966, BSIP Slide No. 16370 (K43).
2. *Retistephanocolpites williamsii* Germeraad *et al*., 1968, BSIP Slide No. 16371 (S56).
3. *Pseudonothofagidites kutchensis* Venkatachala & Kar, 1969, BSIP Slide No.16372 (D50/3).
4. *Lanagiopollis microreticulatus* Phadtare & Thakur, 1990, BSIP Slide No. 16373 (T37).
5–6. *Iugopollis tetraporites* Venkatachala & Rawat, 1973, BSIP Slide No.16374 (X52/2).
7. *Myrtaceidites eucalyptoides* Cookson & Pike emend. Martin, 1973, BSIP Slide No. 16370 (G57).
8. *Yeguapollis indicus* Mandal & Guleria, 2006, BSIP Slide No. 16375 (R58).
9–10. *Dipterocarpuspollenites retipilatus* Kar, 1992, BSIP Slide No.16376 (X61/1, N73/3).
11. *Foveotricolpites* sp., BSIP Slide No. 16371 (N57/2).
12. *Pellicieroipollis langenheimii* Sah & Kar, 1970, BSIP Slide No. 16377 (W53/3).
13–14. *Retitrescolpites* sp., BSIP Slide No. 16373 (E65/2).
15. *Sastriipollenites trilobatus* Venkatachala & Kar, 1969, BSIP Slide No. 16378 (H52/2).
16. *Anacolosidites* sp., BSIP Slide No. 16379 (U53).
17. *Myrtaceidites mesonesus* Cookson & Pike, 1954, BSIP Slide No. 16380 (F66/3).
18. *Striatocolporites* sp., BSIP Slide No.16381 (C53/4).
19. *Polygalacidites minutus* Samant & Phadtare, 1997, BSIP Slide No.16382 (L51/2).
20. *Polygonacidites frequens* Sah & Dutta, 1966, BSIP Slide No.16383 (J49/4).
21. *Chenopodipollis* sp., BSIP Slide No.16384 (W67).
22. *Graminidites granulatus* Kar, 1985, BSIP Slide No. 16385 (T38).
23. *Nymphaeacidites* sp., BSIP Slide No.16379 (R54/4).
24. *Arcella* sp., BSIP Slide No.16385 (N61/3).

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