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Review Article

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Paleontological Approaches for The Study of Fossils

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Abstract

Fossil inclusions are found in amber, fossiliferous limestones and fossil bon beds. The review focuses on paleontological approaches to study fossils including morphological and molecular analyses of life evolution based on scientific evidence. These approaches include tools for morphological characterization of amber inclusions and fossil limestones, molecular paleontology with paleogenomics and paleoproteomics, and the implication of fossil commercialization and collections. The integration of multiple interdisciplinary approaches provides information for a better description and analysis of the origin and evolution of modern organisms and their environments.

Keywords: Amber; Evolution; Fossil; Paleontology; Paleoproteomics; Tick

1. Introduction

Fossils defined as the "evidence of life preserved in a geologic context" include bones, footprints, petrified wood, ichnofossils trilobites and remains of microscopic organisms or grains of pollen to organic molecules or chemical signatures left by ancient life. Fossils in amber include inclusions such as arthropods, plants and animal remains. The study of fossils with multiple paleontological approaches provides information about the history of life on Earth, and its evolution through time (e.g., Creatures of other days. Popular studies in Paleontology by Rev. H.N. Hutchinson, author of "Extinct Monsters", edition in English, published by Chapman and Hall, LD., London in 1896 with numerous illustrations by J. Smith and others) (Fig. 1).

Paleontology is the scientific study fossils to characterize the origins and evolution of ancient life on Earth [1]. Paleontological approaches include field excavation, high-tech imaging, and geology with key approaches combining morphological and molecular analyses to reconstruct evolutionary history, behaviors, and past environments from fossilized remains.

Limestone fossil inclusions and remains mainly contain preserved seafloor skeletal remains and waste products of marine organisms such as shells, corals, ammonites and fish skeletons, but also other organisms such as plants and insects. The main source of fossil limestones is marine life such as algae, clams, corals, snails, starfish, sea urchins, and microscopic organisms and are mainly composed of calcium carbonate from their shells and skeletons in compacted hard rocks. Amber originates from sticky terpene resin exuded by coniferous trees in which organisms (mainly arthropods but also plants and vertebrate parts) get trapped in the fresh resin. Then, the resin with inclusions falls to the ground and gets covered by sediment in environments such as riverbeds and lagoons. At copal stage, the resin polymerizes with heat and pressure and harden into copal. From resin origin to copal stage, other inclusions may appear such as microorganisms (e.g., environmental bacteria) and aquatic organisms. Fossilization of the copal then occurs during more than 40,000 but mostly during millions of years by pressure, heat, oxidation and removal of essential oils to produce the hard and stable amber with inclusions.

Fossils have also a significant role in artistic representations and in traditional medicine as illustrated by Leonardo da Vinci (1452-1519), a reference in art and science, who made important contributions to paleontology with the study of body and trace fossils, evolution and connection with art [2].

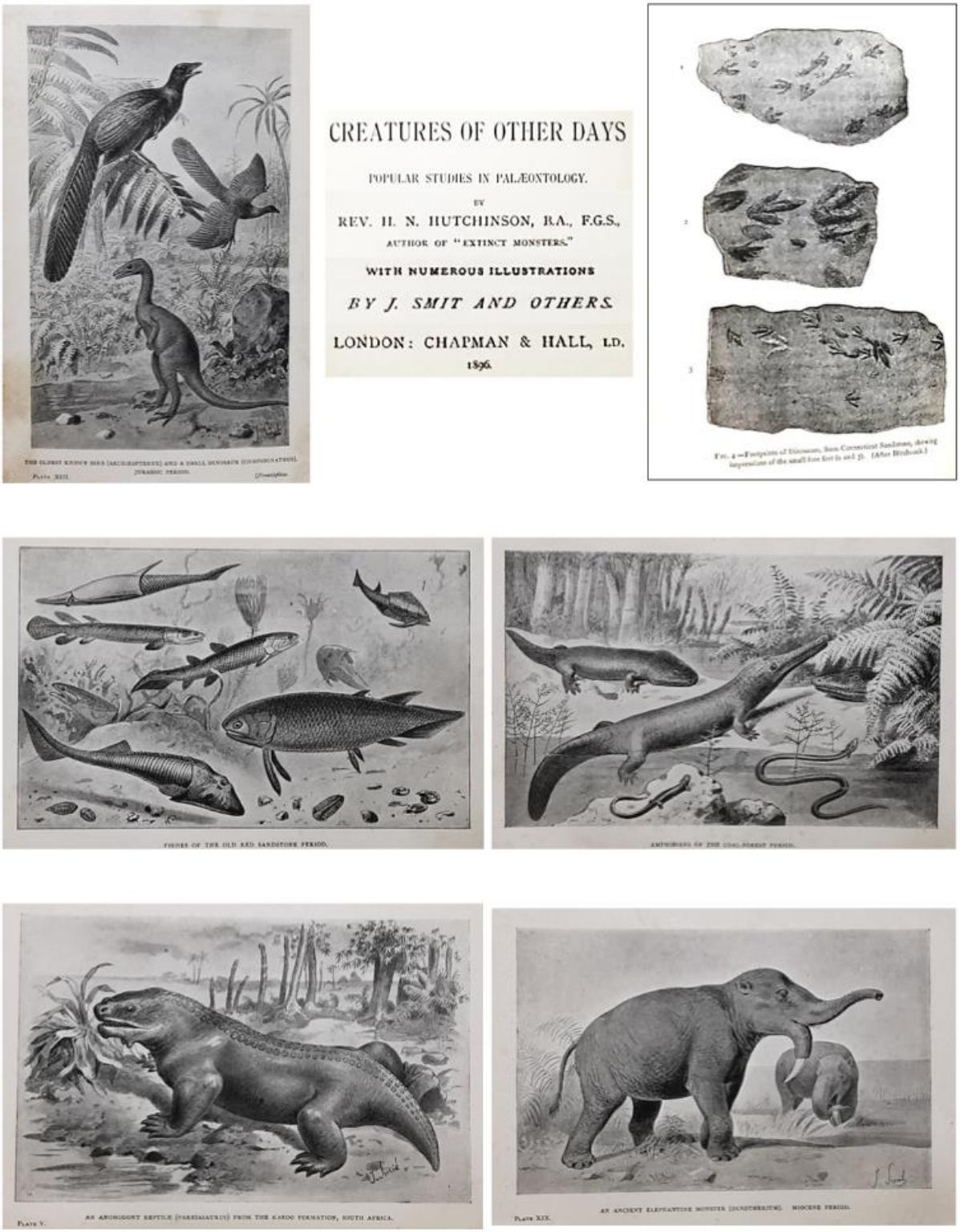

Figure 1: Illustrations of fossil organisms and remains. Images by J. Smith and others from H. N. Hutchinson, Popular studies in Paleontology, Chapman and Hall, LD., London, 1896.

2. Authenticity of fossil remains and inclusions

The main considerations to authenticate fossil remains and inclusions are to follow well authenticated references [3].

Fossil limestones

(e.g., https://thespacestore.com/blogs/blog/how-to-tell-if-a-fossil-is-authentic?srsltid=AfmBOoqfecZJiaTOs-Jbn3yYL1COJBW8b5jPb-JnaEuXeyHDHcglsrjg).

Trusted age and provenance. Density, permeabilization (authentic fossils are heavier than pure rock or bone). Surface texture and porosity ("lick test" shows that fossilized bone or shell tends to stick to the tongue due to natural pores). Natural wear, like hairline cracks or chips. Authentic fossils usually show uneven, natural coloration. Under UV light, modern materials may fluoresce, while real fossils generally do not.

Additional information in the Fossil Identifier (https://www.identifyrock.net/fossil-identifier).

Fossil amber [4]. Trusted age and provenance (Declarations of legal origin, DLO and Certificate of authenticity, COA; Appendix 1). Amber pieces are certified as authentic when tested with UV light, saltwater floating, sinks in fresh water, acetone resistant, and heat – smell of pine resin smell. Dated to fossil epoch by radiometric analysis (Shi et al. 2012). Other sources provide a Certificate of Amber Jewelry Identification (CAJI) and/or Inspection Certificate (IC) by recognized institutions (Appendix 1). Applications such as Insect Identifier (https://insect-identifier.netlify.app) may be used for the complementary morphological analysis of some amber inclusions. Nevertheless, as approached in Story 10 "Fossil ambiguity", it is sometimes difficult to identify amber fossil inclusions.

3. Morphological characterization of fossil amber inclusions

Multiple approaches use tools to capture fossil images for morphological analysis (Figs. 2A-2C and 3). These approaches include among other stereomicroscope image capture and analysis using Image J program (https://imagej.net/ij/), interpretative camera drawings generated with Befunky application (https://www.befunky.com/features/photo-to-sketch/) [5], micro-computed tomography (micro-CT) scanning (https://www.cenieh.es/en/infrastructure/laboratories/micro-computed-tomography) and drawings prepared with a camera lucida attachment on a Leica M205C stereomicroscope (Leica Microsystems, Wetzlar, Germany) (Chitimia-Dobler et al. 2023) (Appendix 2). Reference databases are then used for classification of the fossil inclusions. High resolution micro-CT scanning allows the identification of structures such as mite setae that are used as sensors (Fig. 3).

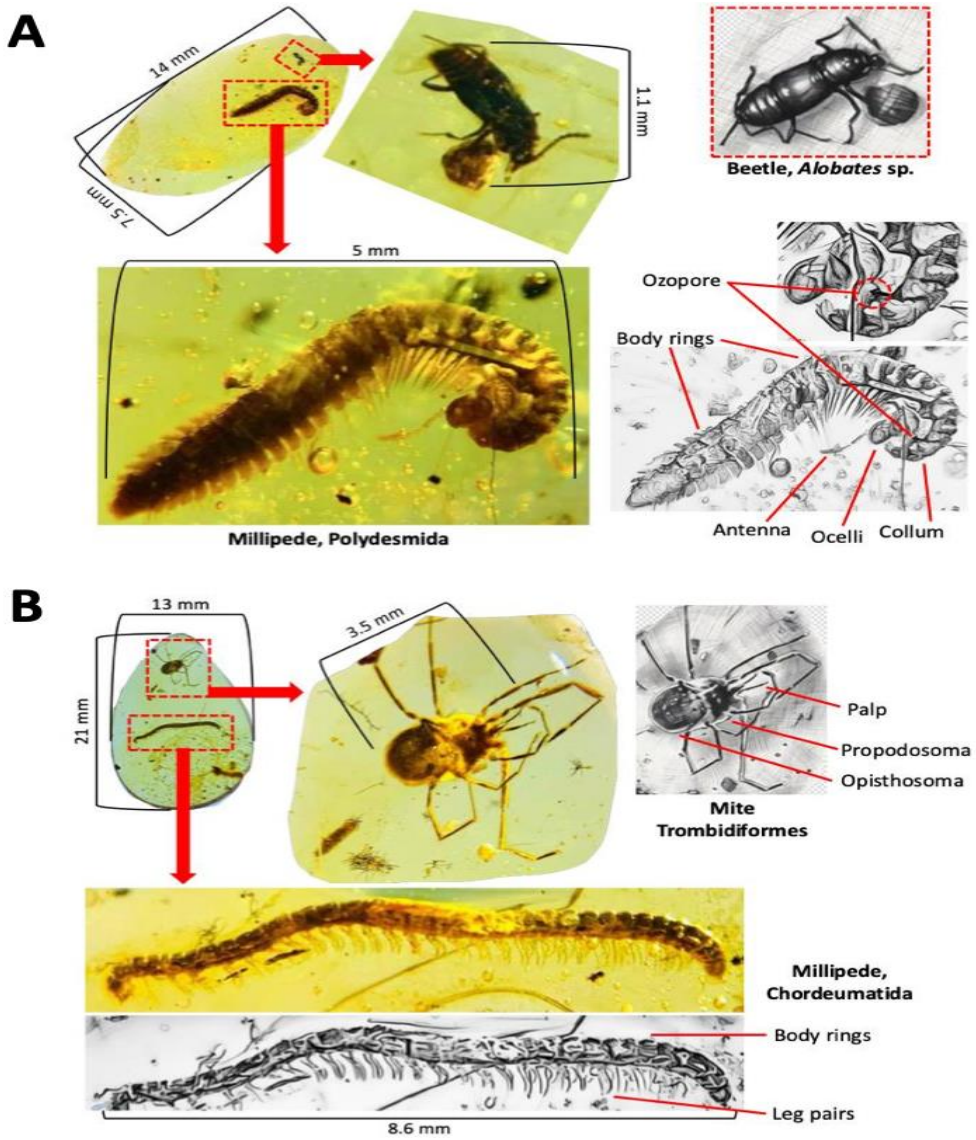

Figure 2: Analysis and classification of amber arthropod syninclusions in Burmese amber (Cretaceous, ca. 99 Mya). (A) Millipede, Polydesmida and beetle (*Alobates* sp.). (B) Millipede, Chordeumatida and mite, Acari:Trombidiformes. Amber pieces are shown, and the arthropod inclusions are highlighted with the body parts and interpretative camera drawings morphological patterns used for classification.

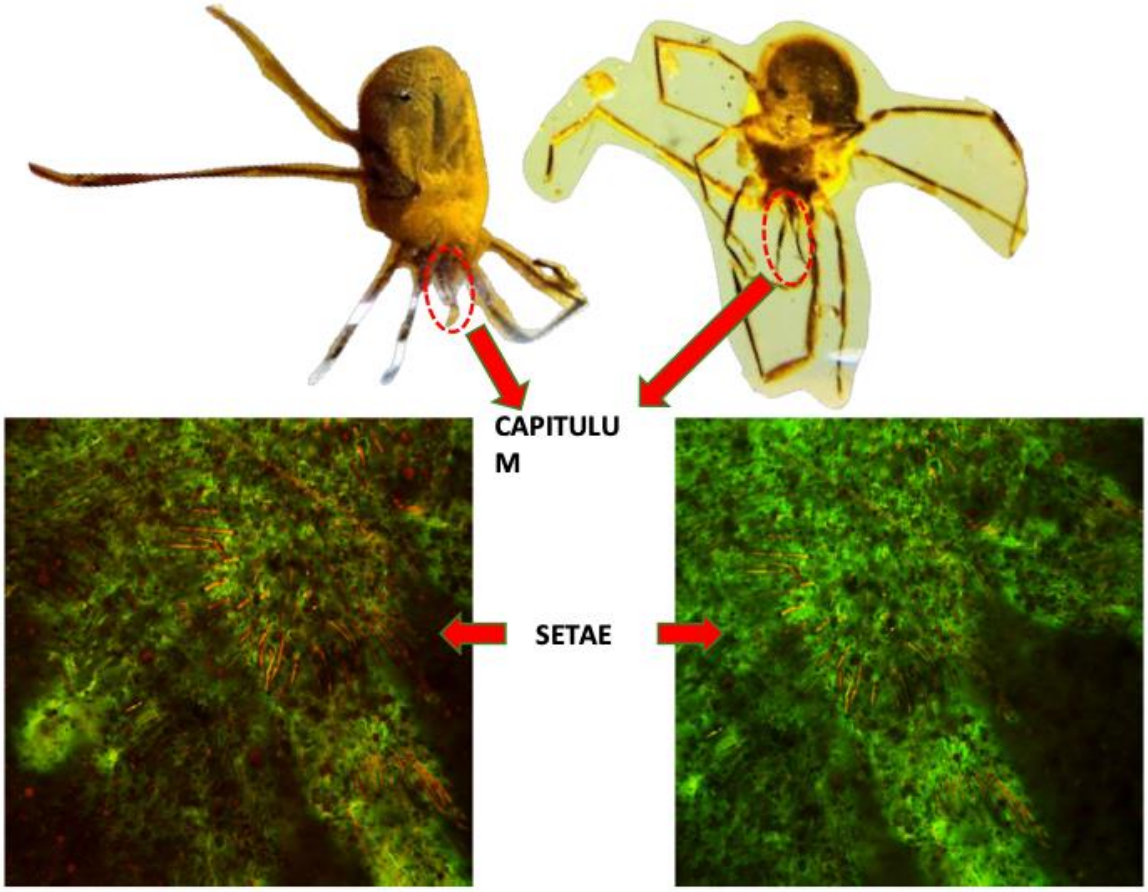

Figure 3: Morphological analysis of mite capitulum with high resolution micro-computed tomography (micro-CT) scanning. Mites are from inclusions in Burmese amber (Cretaceous, ca. 99 Mya).

4. Unique fossil inclusions

Some fossil inclusions are unique and require special attention for their key information on ancient environments and evolution. For example, well preserved insects in fossil limestone such as a Grillo (Fig. 4A) and a Coleoptera beetle (Fig. 4B) are rare and provide information about past environments and evolution of these organisms. Interestingly, beetles are found in both Cretaceous amber (Fig. 2A) and fossil limestone (Fig. 4B). In amber, rare inclusions include Orthoptera insect grasshopper and feather rachis from birds or dinosaurs, scorpion with possible predation of an insect, a feather parasitic acari mite (Figs. 4B-4D), and nematodes (Figs. 4C and 4D). These fossils found in Cretaceous (ca. 99 Mya) Burmite from Hukawng Valley, Myanmar (Burma) reveal the evolution of parasitism [6]. Aenictopecheidae (commonly known as enicocephalomorphan unique-headed bugs) are rare and poorly understood family of predatory unique-headed true bugs (Hemiptera: Enicocephalomorpha) (Fig. 4E).

The fish *Cyclobatis* (Egerton 1844) is a stingray-like skate extinct genus from Hakel, Lebanon marine environment (Cenomanian Cretaceous, ca. 98 Mya), which is difficult to find due to its scarcity in Lebanese limestone deposits. Accordingly, this genus is valued for its unique circular shape and exceptional preservation, often appearing as highly detailed specimens (Egerton 1845). Most recognized species include *C. oligodactylus* (short-tailed) and *C. major* (long-tailed) (Egerton 1845), thus suggesting that the specimen in Figure 5A is a *C. oligodactylus*.

The well preserved Conulariida, *Eoconularia loculata* (Wiman 1895) from Fezouata formation, Bou Chrebeb, near Zagora, Morocco is an extinct species of the Phylum Cnidaria, which includes modern corals [7] (Fig. 5B). They are almost exclusively known for their hard external structures, also called theca, periderm or test, which has a pyramidal shape and were composed of numerous lamellae. They are considered sessile animals that grew with the narrower tip anchored to the seafloor with an array of tentacles in the wider end used to ensnare preys. This specimen is extremely rare and completely intact even down to the tip of its attachment.

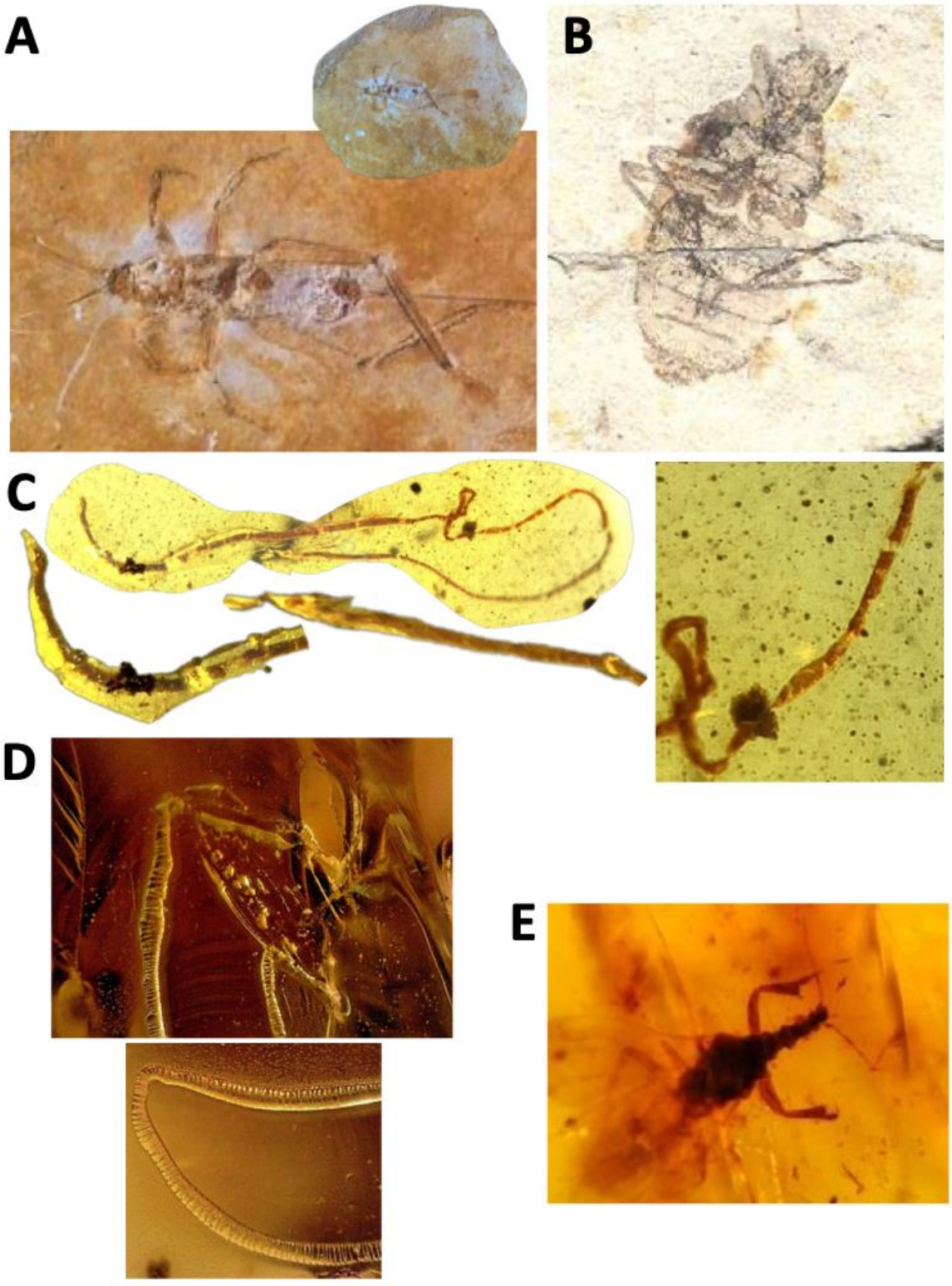

Figure 4: Unique fossil inclusions in limestone and amber. (A) Grillo from Brazil, Formation Santa Ana (Cretaceous, ca. 92-120 Mya; fossil insect, 65 mm). (B) Coleoptera beetle in limestone from China (Jurassic, ca. 208 Mya; fossil insect, 1.3 cm). (C) and (D) nematodes in Burmite from Hukawng Valley, Myanmar (Burma, Cretaceous, ca. 99 Mya; fossil nematodes, ca. 1.2 cm). (E) Aenictopecheidae true bug (Burma, Cretaceous, ca. 92-120 Mya; amber piece, 25 x 12 mm).

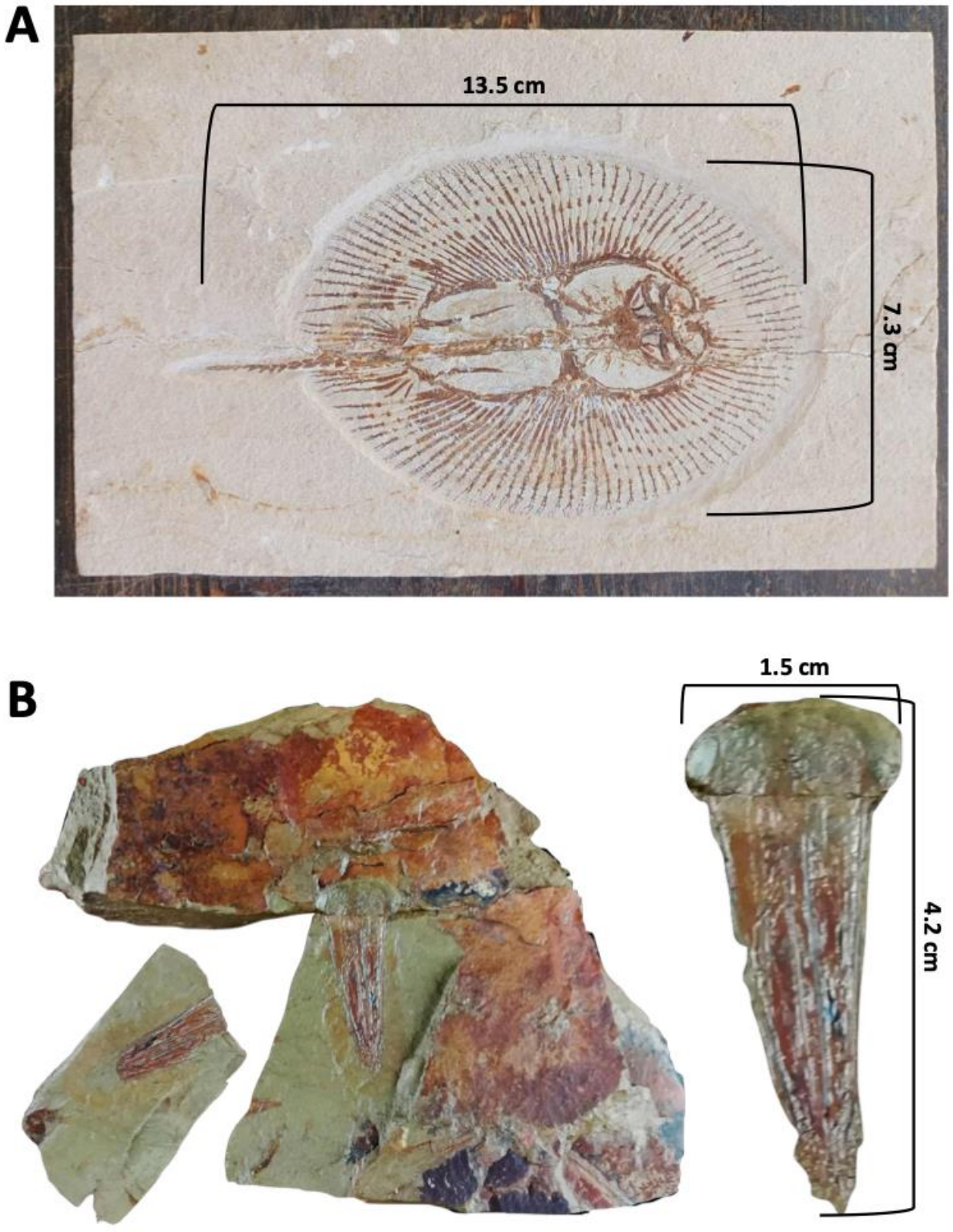

Figure 5: Unique fossil inclusions in limestone. (A) Fossil limestone with a difficult to find stingray-like skate extinct genus, *Cyclobatis* (Egerton 1844) from Hakel, Lebanon marine environment (Cenomanian Cretaceous, ca. 98 Mya). (B) Extinct Conulariida, *Eoconularia loculata* (Wiman 1895) from Fezouata formation, Bou Chrebeb, Morocco (Ordovician, 472-480 Mya).

5. Tick fossil inclusions in Cretaceous Burmese amber

Ticks are also rare fossil inclusions in amber and particularly in Cretaceous (ca. 99 Mya) Burmite (Table 1). Ticks are considered to originate in the Afrotropics with extant families composed of Argasidae, Ixodidae and Nuttalliellidae and extinct genera *Deinocroton*, *Legionaris* nov. gen. and *Nuttalliella* of family Nuttalliellidae [8].

Table 1: Description of tick in Burmite fossil inclusions.

Tick genus-species	Classification	References
Deinocroton bicornis sp. nov.	Nuttalliellidae	Chitimia-Dobler et al. 2024a [8]
Deinocroton lacrimus sp. nov.		
Nuttalliella gratae sp. nov.,		
Nuttalliella tuberculata sp. nov.		
Nuttalliella odyssea sp. nov.		
Nuttalliella tropicasylvae sp. nov		
Nuttalliella placaventrala sp. nov.		
Legionaris robustus sp. nov.		
Deinocroton copia sp. nov. *Deinocroton draculi* gen. et sp. nov.	Nuttalliellidae	Chitimia-Dobler et al. 2022 [9] Poinar and Brown 2003 [10] Peñalver et al. 2018 [14]
Cornupalpatum burmanicum sp. nov.	Metastriata:Ixodidae	
Bothriocroton muelleri sp. nov.	Metastriata:Ixodidae	Chitimia-Dobler et al. 2023 [12]
Archaeocroton kaufmani sp. nov.		
Ixodes antiquorum sp. nov.	Prostriata:Ixodidae	Chitimia-Dobler et al. 2022 [9]
Khimaira fossus fam., gen. et sp. nov.	Khimairidae	
Haemaphysalis cretacea	Metastriata:Ixodidae	Chitimia-Dobler et al. 2018 [13]
Dominican amber *Amblyomma* sp. with *Borrelia*-type spirochetes	Metastriata:Ixodidae	Poinar Jr., 2015 [6]
Amblyomma birmitum sp. nov	Metastriata:Ixodidae	Chitimia-Dobler et al. 2017 [14] Figures 5E, 5F
Compluriscutula vetulum	Metastriata:Ixodidae	Poinar and Buckley 2008 [15] Chitimia-Dobler et al. 2024b [16] de la Fuente et al. 2024 [17]
Under investigation	Ixodidae	Figures 5C, 5D
Carios sp.	Argasidae	Klompen and Grimaldi 2001 [18]

The preservation of fossil ticks in amber allows the identification of extant and extinct species illustrating the evolution and diversity of these organisms (Figs. 6A-6F and Fig. 7). These ticks represent *Cornupalpatum* sp. (Metastriata:Ixodidae) (Figs. 6A, 6B, 6E, 6F) and Ixodidae (Prostriata) (Figs. 6C and 6D).

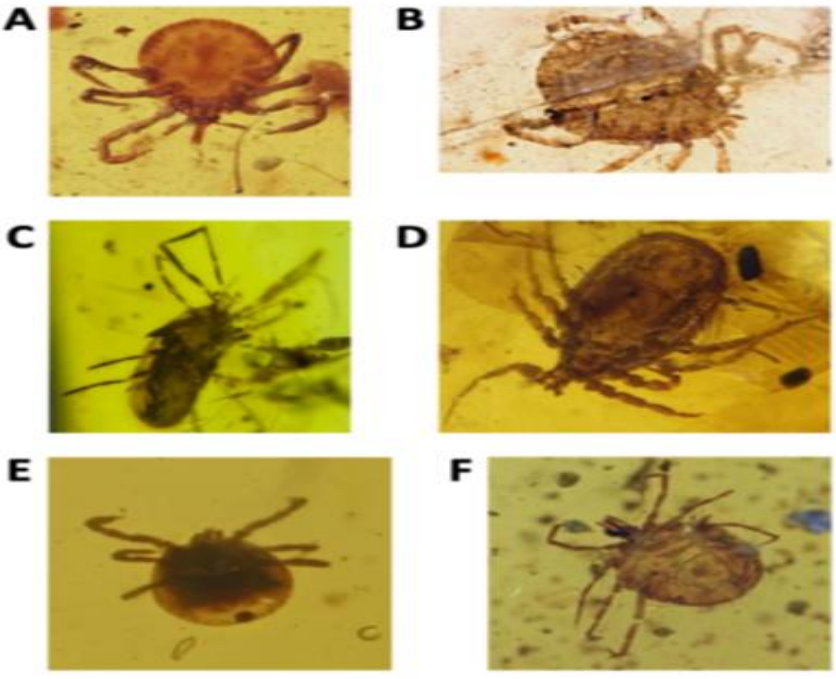

Figure 6: Tick fossil inclusions in Burmese amber. Examples of fossil ticks to illustrate these organisms in the Cretaceous (ca. 99 Mya) period. (A) *Compluriscutula vetulum* larva. (B) *Cornupalpatum* sp. (C) Tick Ixodidae inclusion under investigation. (D) Tick putative *Ixodes antiquorum* sp. nov. (Chitimia-Dobler et al., 2022). (E, F) Hard round ticks, *Amblyomma birmitum* sp. nov., (E) nymph and adult (F).

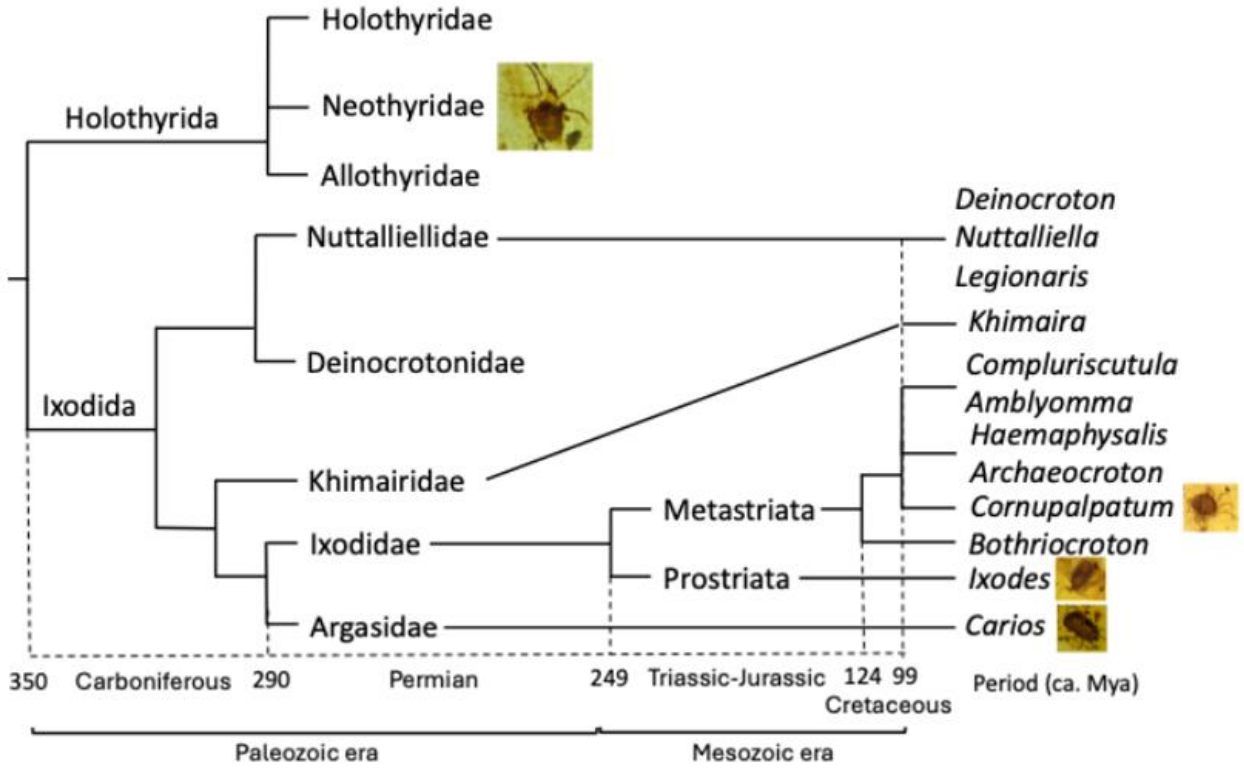

Figure 7: Phylogenetic tree of tick (Ixodida) and mite (Holothyrida) evolution. Example of organisms identified in fossil amber inclusions are illustrated.

6. Quality of fossil amber inclusions

Conservation, preparation and imaging of amber inclusions are a challenge due to deterioration from environmental factors [19]. Due to these factors, although fossil amber inclusions are well preserved, some pieces contain organisms that are difficult to classify due to low quality of the different parts of the body (e.g., Figs. 8 and 9). Nevertheless, in these cases it is possible to use different tools (e.g., shown in Figures 2 and 3) to provide some information about the fossil inclusion even if not possible at species level (Fig. 8). These challenges inspire to explore the fossil natural environment of millions of years ago (Fig. 9).

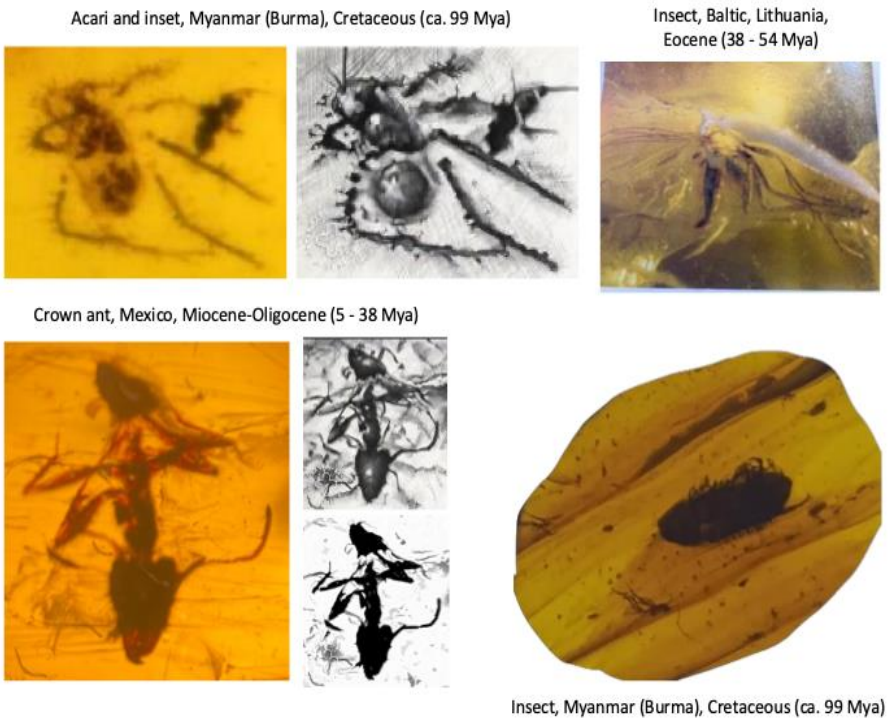

Figure 8: Fossil amber inclusions with low preservation quality. Interpretative camera drawings were generated with Befunky application (https://www.befunky.com/features/photo-to-sketch/) [5].

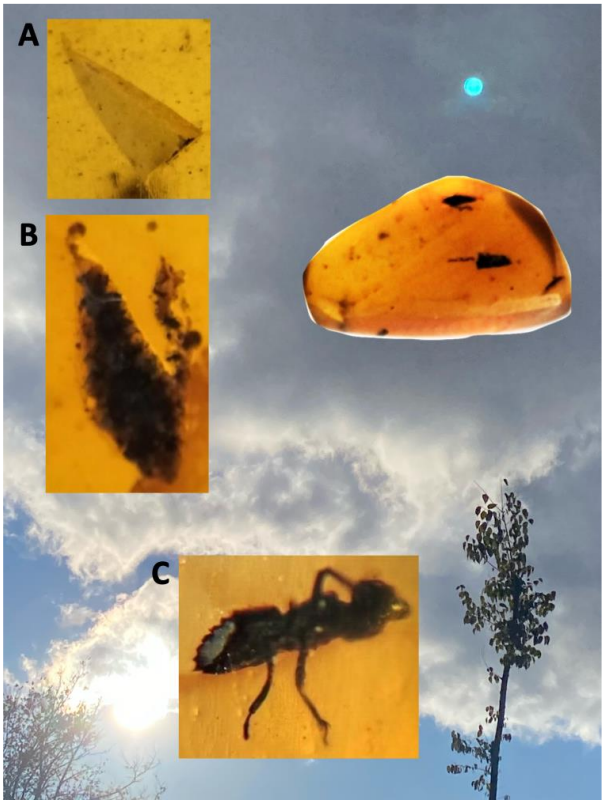

Figure 9: Not classified insect and another fossil amber syninclusions. Addressing questions related to some fossil inclusions is a challenge. Example, fossil syinclusions in amber from Myanmar, Burma (Cretaceous, ca. 99 Mya, Dimensions amber piece: 19 x 10 mm). (A) Unknown. (B) Plant remains, putative leaf (C) Insect, putative ant.

7. Molecular paleontology: paleogenomics and paleoproteomics

Paleogenomics and paleorpoteomics are the study of ancient DNA and proteins of archaeological and paleontological specimens, respectively to reconstruct and analyze the genomes and proteomes of extinct organisms [20,21,4]. These approaches have been mainly used for the analysis of ancient bone samples and the identification of source organisms and also allow the taxonomic identification of archeological bones and fossil amber inclusions (e.g., Lleonart et al. 1999, Lleonart et al. 2000, Warinner et al. 2022, de la Fuente and Toirac 2024, Sheng et al. 2025) [22,23,20,5,21].

Research of ancient proteins was first applied to determine blood groups in mummies by Boyd and Boyd (1934) [24]. Paleoproteomics analysis of fossil mineralized remains have been applied to bone, teeth, dental calculus and coprolites collected from historical glues, paints, leather, parchment, or hair and other samples could be eggshells, corals, mollusk shells, and plants [25,26,20,4,27].

Focusing on paleoproteomics analysis of amber fossil inclusions, it flows from microscopic and tomographic morphological classification to peptide/protein analysis by mass spectrometry, phylogeny and protein structure (Fig. 10, Appendix 2). Without the identification of peptide/proteins or only associated with environmental bacteria in control amber without inclusions, identified validated proteins provide molecular support for morphological classification (Fig. 10). In fossil amber inclusions, paleoproteomics has been applied to identify yeast proteins [28] and arthropods [4], but future studies may include plants, bones, snails and theropod feathers. In fossil mineralized remains, paleoproteomics has been used in bone, teeth, dental calculus, and coprolites [20,21]. Molecular paleontology in fossil inclusions may also provide information on the evolution of host-vector-pathogen interactions and the synthesis of alpha-Gal (carbohydrate molecule, galactose-α-1,3-galactose) associated with allergic pathologies such as the Alpha-gal Syndrome [29], and for drug discovery [30].

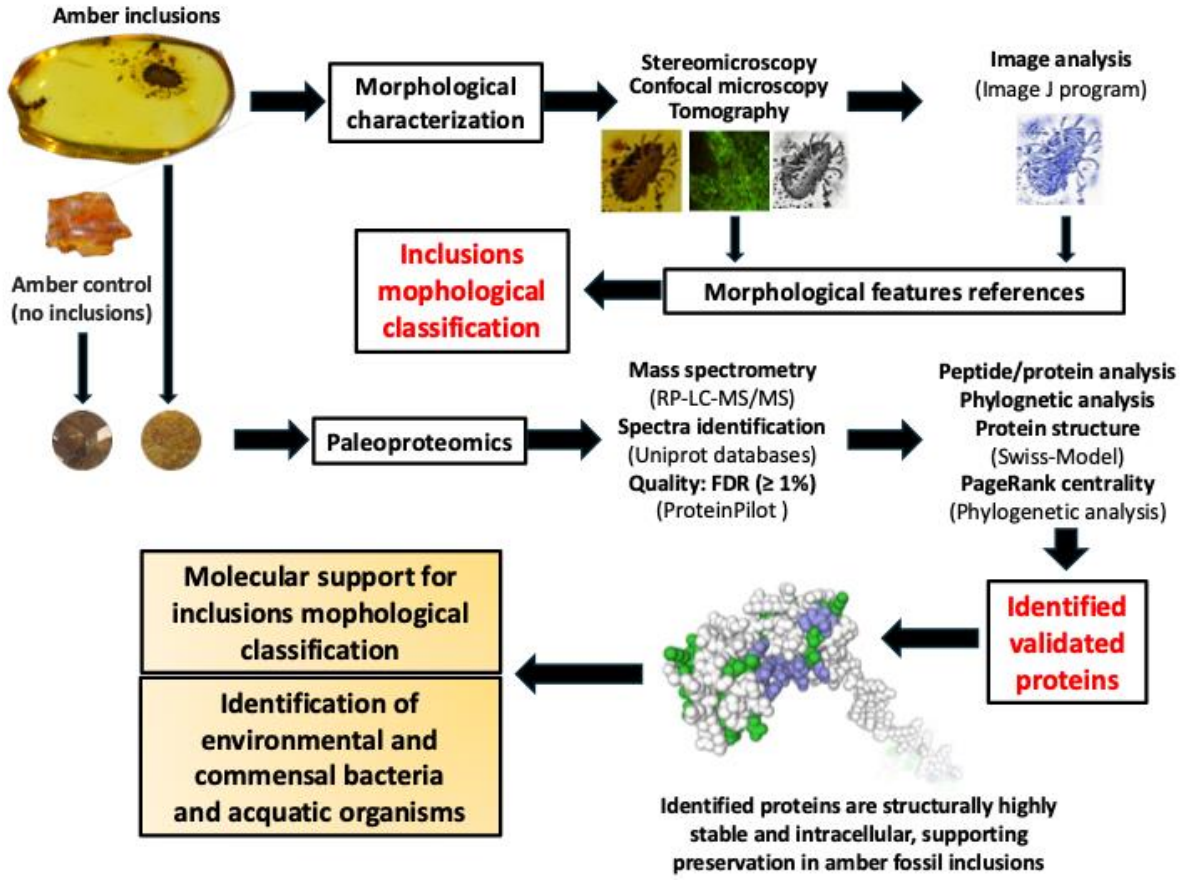

Figure 10: Paleoproteomics characterization of amber fossil inclusions. Summary of methodological approaches and results. Detailed paleoproteomics methodology is described in Appendix 2.

Despite recent advances with modern genomics and proteomics technologies, challenges and main limitations in paleogenomics and paleoproteomics are associated with the molecular degradation of ancient DNA and proteins and possible contamination from modern sources that can affect their quality and quantity reducing the certainty of the results [31]. Nevertheless, paleoproteomics has been applied not only to study samples from fossil bones but also amber inclusions [28,4], and paleogenomics has been used only in paleontology [21].

The simultaneous analysis of multiple amber fossil insect and acari syninclusions by paleoproteomics analysis may require using a single compiled Uniprot database with all sequences from Acari, Insecta and Enterobacterales together with contaminants database and human keratins and bovine trypsin to provide higher certainty for protein identification (Appendix 2). With this approach, proteins identified with one peptide of 1% FDR are not selected for analysis to provide a higher confidence for results. However, the analysis of individual inclusions can be conducted with a target species-specific Uniprot database in agreement with morphological classification [4] and applied to complement the analysis conducted with the single compiled database (Appendix 2).

The paleoproteomics analysis of Burmese amber syninclusions with putative lacewing larva (Neoptera: Chrysopidae) and thrip, *Frankliniella* sp. using a single compiled Uniprot database identified an Actin protein with stable structure and highly conserved in insects (> 97% identity and E-value = 0.0) supporting the thrip morphological classification [4]. Then, the analysis using a Uniprot target species-specific Insecta database provided additional evidence of the fossil thrip inclusion also with the identification of an Actin protein [4] (Figs. 11A and 11B).

Additionally, the paleoproteomics analysis can identifies environmental bacteria (e.g., *Serratia* sp. and *Kluyvera* sp.; Figs. 12 and 13), and aquatic organisms (e.g., East China marine sediment bacterium, *Shimia sediminis* sp. nov. and Pacific coast of Asia oyster, *Magallana gigas*). These organisms were probably introduced during fossil amber formation [22].

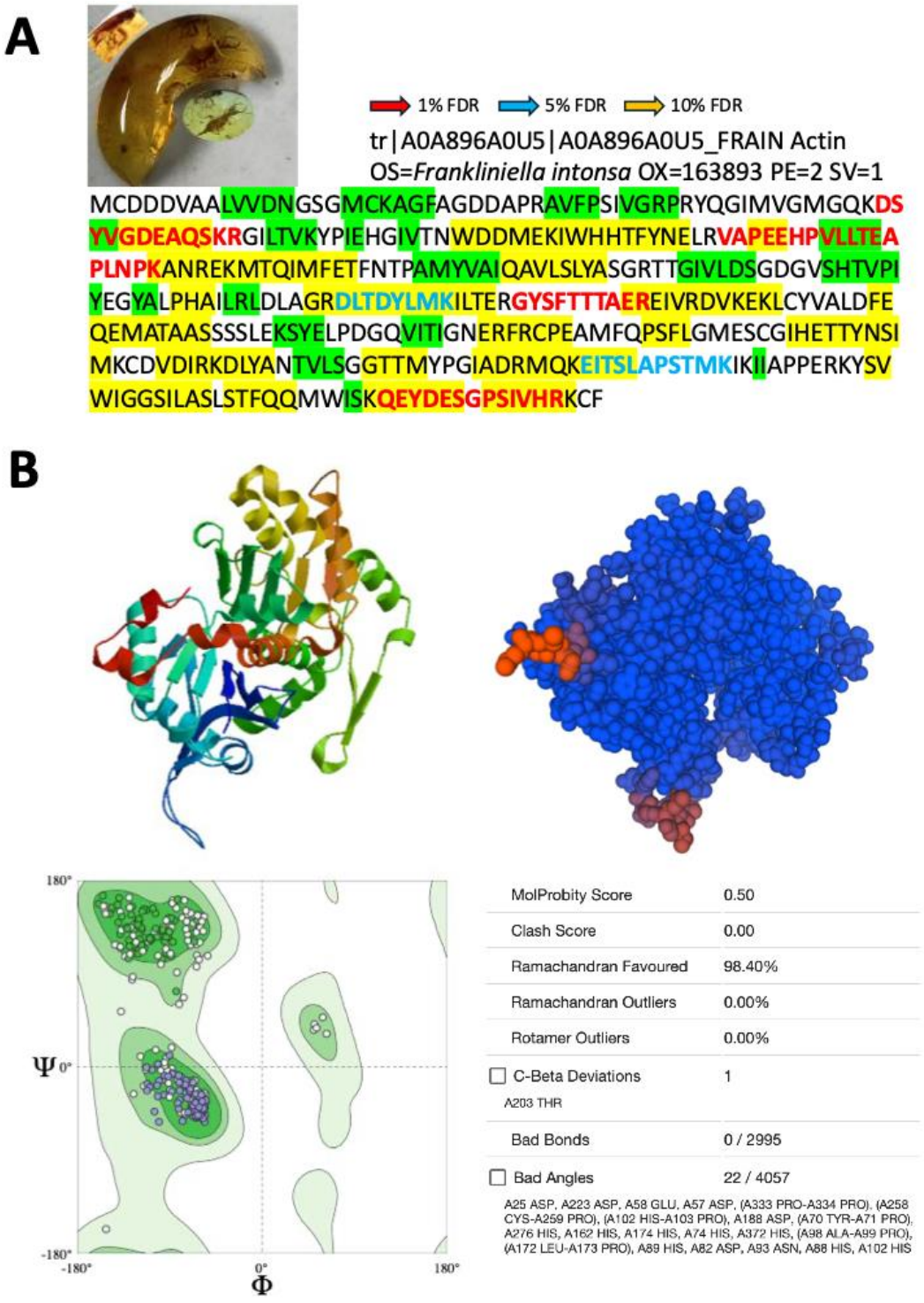

Figure 11: *Frankliniella intonsa* Actin protein identified in insect amber inclusions. (A) Burmese amber syninclusions with putative lacewing larva and thrip, *Frankliniella* sp. The analysis of paleoproteomics data was conducted with Uniprot target species-specific Insecta database (accessed on January 20, 2025 UniProtKB 6,898,282 entries; https://www.uniprot.org/uniprotkb?query=taxonomy_name:insecta). In protein sequence, letter colors are associated with peptide FDR while letter highlights with protein structure. (B) The SWISS-MODEL template library (SMTL version 2025-11-05, PDB release 2025-10-31) was searched with BLAST and HHblits for evolutionary related structures matching the target sequence (https://swissmodel.expasy.org).

For example, in the paleoproteomics analysis of fossil arthropod parasitiformes in Burmese amber inclusions [4], two peptides (VNNEITLTK and VDQLSNDVNAIR, 1% FDR) were not assigned to any organism. The peptides were then combined (VNNEITLTKVDQLSNDVNAIR) to improve the identification of protein sequences and associated species. A Basic Local Alignment Search Tool (BLAST) analysis of NCBI non-redundant protein sequences (nr) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome; November 8, 2025) resulted in the identification of major outer membrane lipoprotein (94% sequence identity, E-value 1e-04)

of *Kluyvera* sp. Kluyver 1938 (Kluyver and Niel 1936) (Bacteria; Pseudomonadati; Pseudomonadota; Gammaproteobacteria; Enterobacterales; Enterobacteriaceae; *Kluyvera*) (WP_438614644.1;https://www.ncbi.nlm.nih.gov/protein/WP_438614644.1?report=genbank&log$=protalign&blast_rank=1&RID=H1MX6RH6014) (Fig. 12).

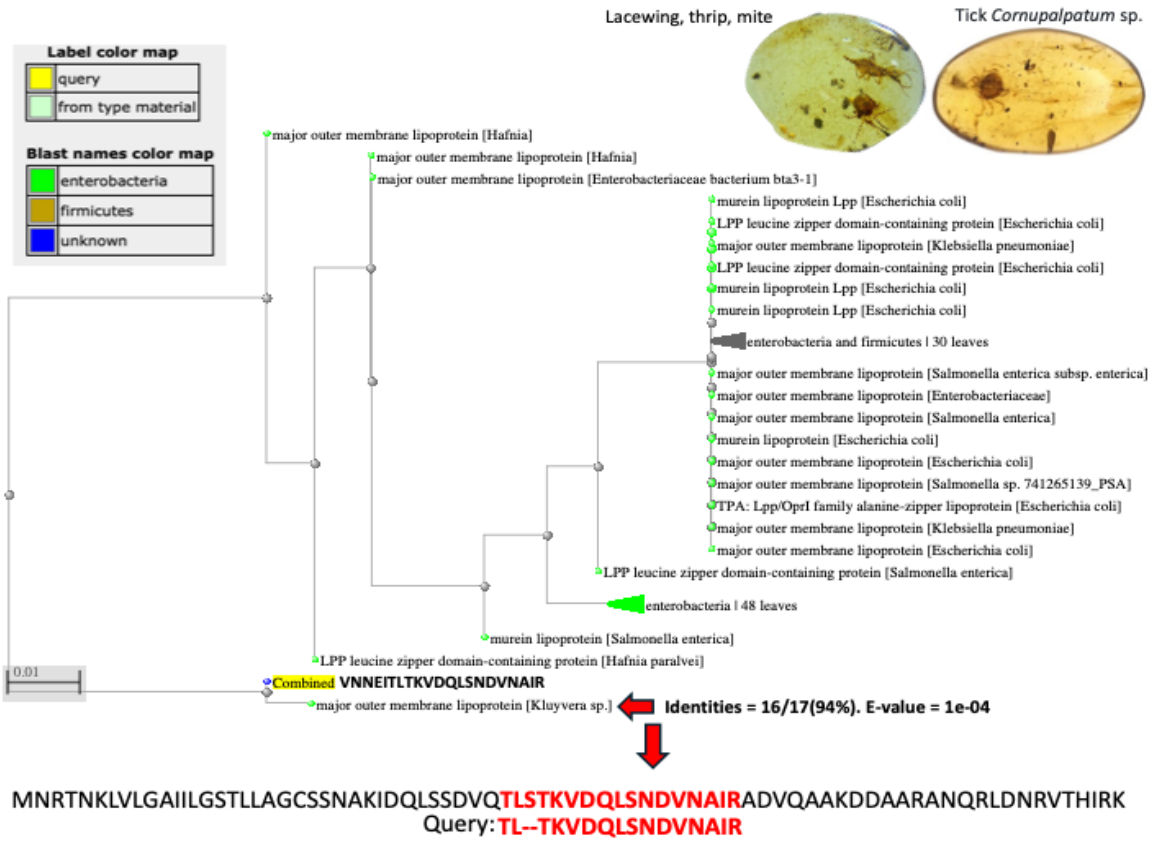

Figure 12: Phylogenetic analysis. The analysis was conducted with identified proteins derived from the hybrid combined peptide designed with the two sequences identified in Burmese amber (ca. 99 Mya) with arthropod inclusions and not assigned to any organism. Most of the organisms associated with the hybrid peptide are Enterobacteriaceae.

The major outer membrane lipoprotein identified in *Kluyvera* sp. with the hybrid combined peptide showed a highly conserved primary structure in Enterobacteriaceae (Fig. 12). The analysis of protein secondary structure with the SWISS-MODEL supports a highly stable structure for this protein with the identified hybrid peptide located in the most stable protein region (Fig. 13). These results support preservation of the protein in fossil amber inclusions.

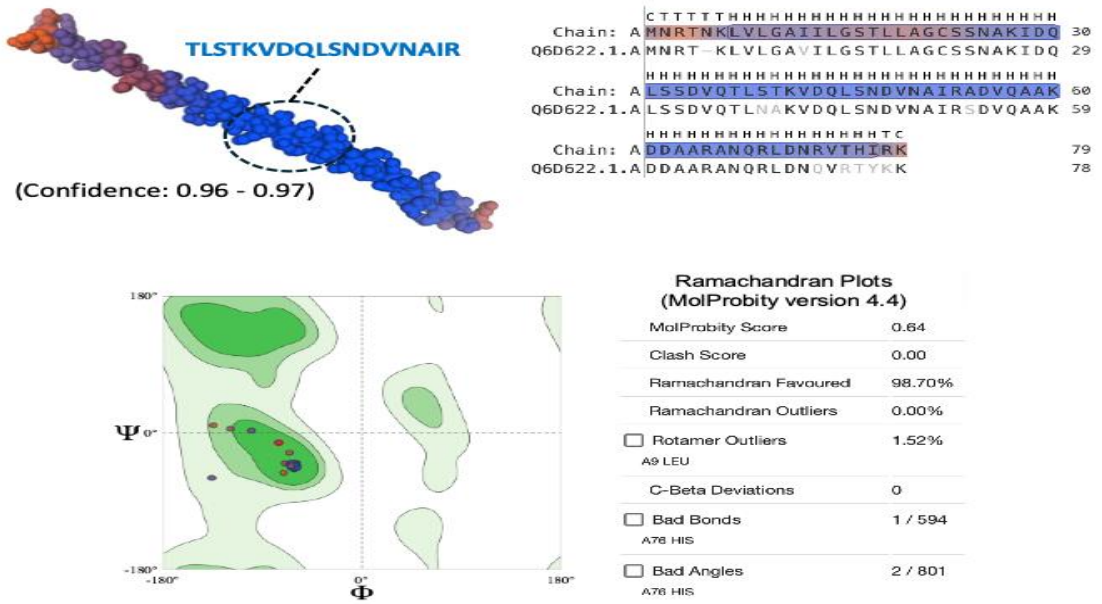

Figure 13: Model building of the major outer membrane lipoprotein of *Kluyvera* sp. (WP_438614644.1). The SWISS-MODEL template library (SMTL version 2025-11-05, PDB release 2025-10-31) was searched with BLAST and HHblits for evolutionary related structures matching the target sequence (https://swissmodel.expasy.org).

Using similar approaches in the paleoproteomics analysis of mite Prostigmata (Acari:Trombidiformes:Tetranychidae) syninclusions in Cretaceous Burmite, proteins supporting morphological characterization were identified using both single compiled Uniprot database and targeted databases for Mite, Soft Ticks and Acari. Then, to expand these results, a hybrid protein was designed with all identified peptides (1-10% FDR; TITLEVEPSDTIENVKVLDELTALRLEYEDEIGRGKQITV NDLPVGRIQDKEGIPPDQQRTLSDYNIQKNLSNSLSYGV YR) and used for search in the UniProt (https://www.uniprot.org/) nr (All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects) database (867,712,132 sequences, updated January 31, 2025) and phylogenetic analysis (Fig. 14). Random sequences are used to evaluate the significance of the results of sequence analysis (Appendix 2). The results supported the identification of Ubiquitin-like proteins in mite, tick and bird species, and provided the first finding of a parasitic nematode, *Trichinella zimbabwensis* (Fig. 14).

The results of the study not only provided additional support for the paleoproteomics methodological approach developed for the study of fossil amber inclusions to expand morphological analysis and molecular evolution of parasitiformes [4] but also expanded the possibility to characterize fossil holobiont components in acari-host-parasite interactions (Fig. 15). In support to the findings of this study, birds have been also characterized in Asian Cretaceous period fossils [32,33] and nematodes in Burmese amber [34,25] (Fig. 14). The interaction between birds and mites has been documented for both Astigmata [36] and Mesostigmata (Knee 2008) species with mites acting as ectoparasites and ectosymbionts of birds. Furthermore, feather parasitic Acari Parasitiformes inclusions in Burmite amber support ancient interactions between these species [11] (Fig. 15). Additionally, the presence of *Trichinella* nematodes has been documented in both mite [37,38,39] and bird [40] hosts. This evidence suggests ancient interactions between these species' components of the mite holobiont.

The analysis of negative control sample of amber without inclusions can identify environmental bacteria when using the single compiled Uniprot database including Enterobacterales together with contaminants. With a target species-specific Uniprot database, protein sequences appearing as "REVERSED", meaning "FALSE" arise from "target-decoy approach" generated by ProteinPilot by inverting the real sequences. These false proteins are considered an error and included to calculate the False Discovery Rate (FDR). Additionally, in negative control amber analysis against all databases the results with between one and three peptides with lowest FDR = 10%.

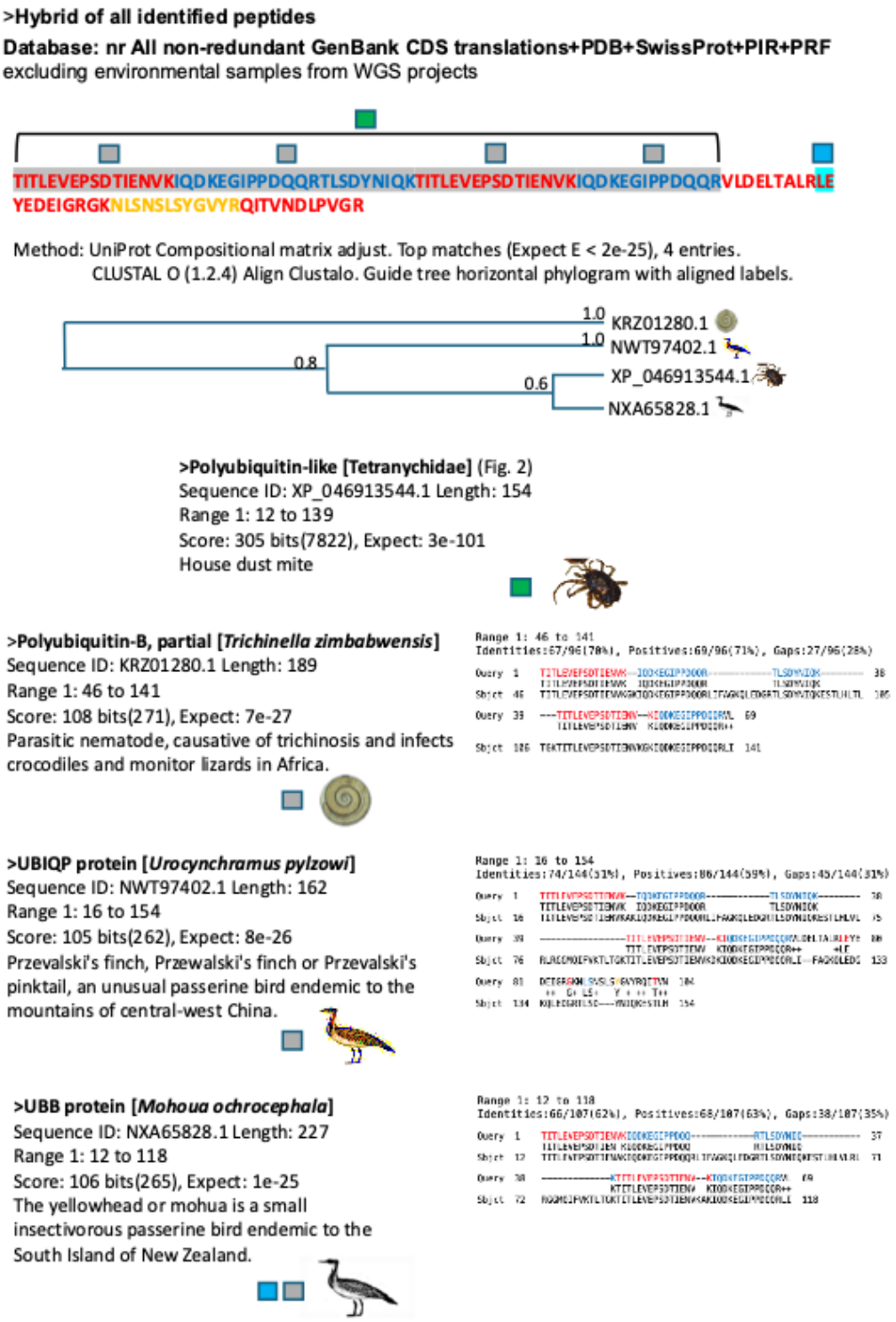

Figure 14: Analysis of a hybrid protein constructed with all identified peptides in mite syninclusions. The hybrid protein sequence was used for analysis in nr (All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from Whole Genome Shotgun WGS projects; https://www.ncbi.nlm.nih.gov/genbank/wgs/) database with compositional matrix adjust top matches (E < 2e-25). Matched proteins and species are shown. CLUSTAL O (1.2.4) multiple sequence alignment (Align Clustalo with guide tree horizontal phylogram with aligned labels, https://www.uniprot.org/align/clustalo) analysis of identified proteins showed a high betweenness and PageRank centrality range for all proteins.

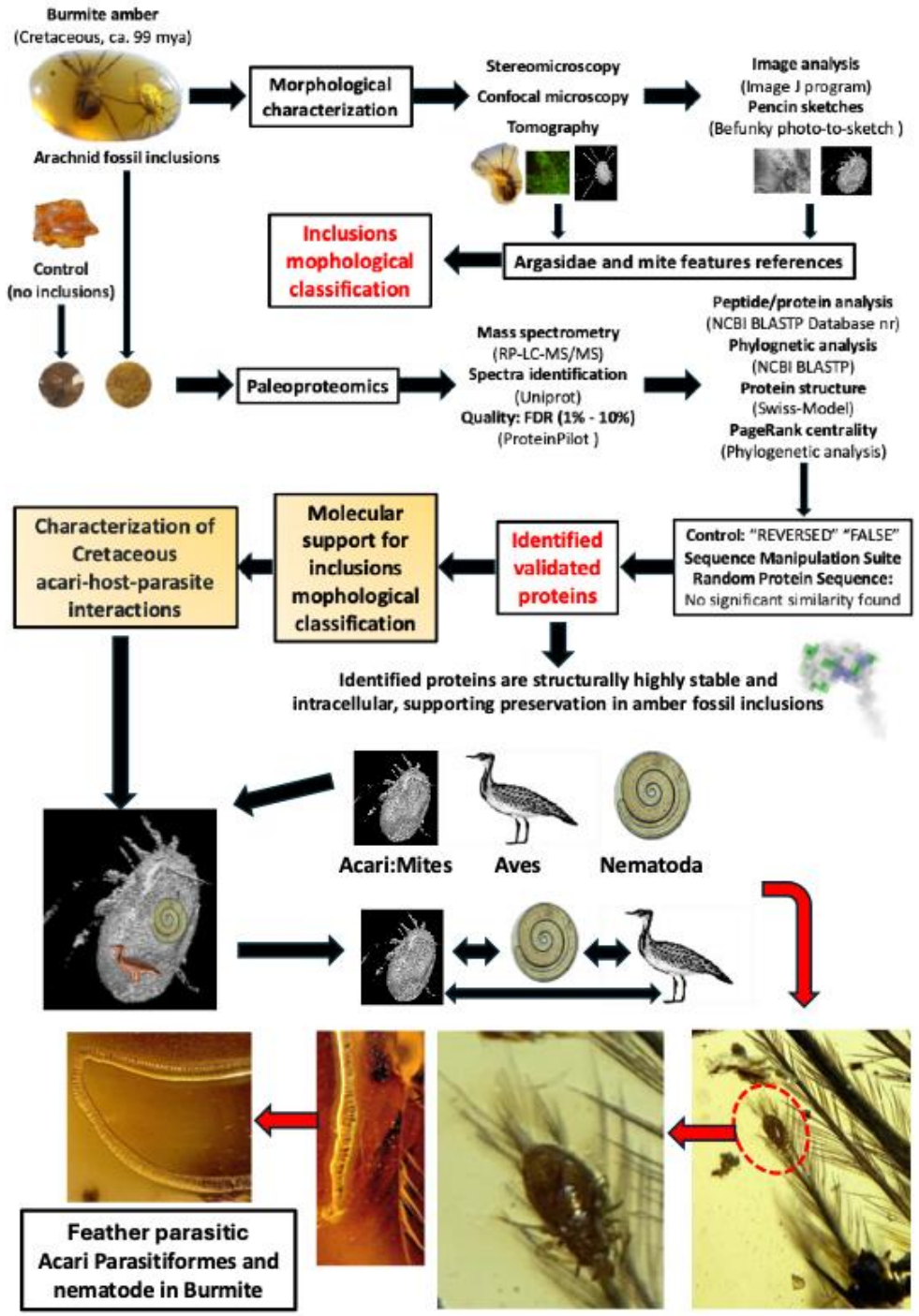

Figure 15: Paleoproteomics characterization of acari-host-parasite interactions: Summary of methodological approach and results. Paleoproteomics analysis of Arachnid fossil inclusions in Burmite amber flew from microscopic and tomographic morphological classification as mites Tetranychidae to peptide/protein analysis by mass spectrometry, phylogeny and protein structure. Without identification of peptide/proteins in amber without inclusions and after control reverse/false analysis, identified validated proteins provided molecular support for mite morphological classification on paleo holobiont with interactions between mites, Aves and nematodes. These results are supported by the identification of putative feather parasitic Acari Parasitiformes larvae and nematode in Burmese amber.

8. Bibliometric analysis

Bibliometric analysis can be conducted with different databases such as PubMed (https://pubmed.ncbi.nlm.nih.gov/), Scopus (https://www.elsevier.com/products/scopus) and Google Scholar (https://scholar.google.com) [41]. Bibliometric analysis includes as shown in Figure 16 the quantitative evaluation of scientific papers using terms of interest to study publication patterns. Accordingly, an example of recent bibliometric analysis showed that the number of publications about fossil ants (n = 60) is similar to those on fossil mites (n = 63). These results support that ant inclusions are common in amber. However, a limited number of published studies have addressed the co-finding of fossil ants with other organisms such as wasps (n = 3), mites (n = 2) or spiders (n = 2), and without articles with millipede, land snail, oak or moss as recently reported.

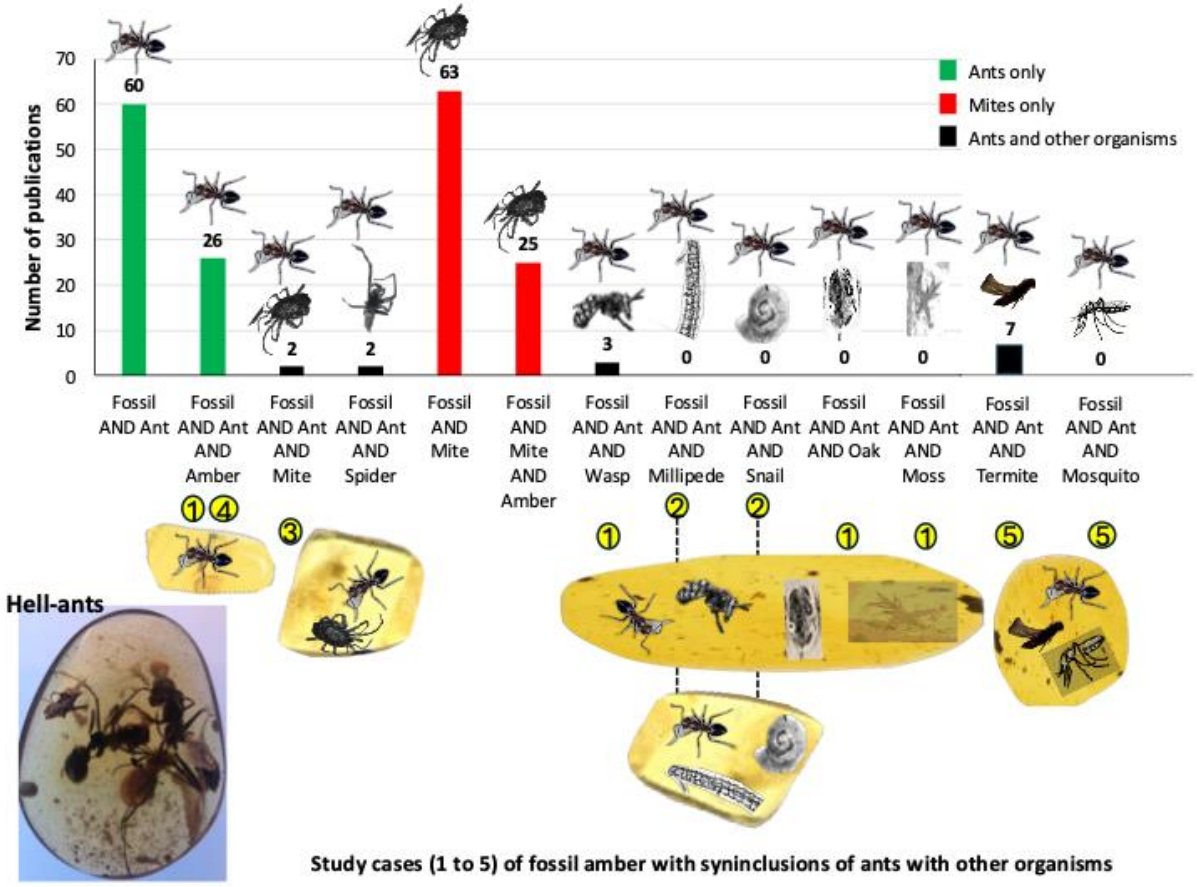

Figure 16: Results of bibliometric analysis. Bibliometric analysis was conducted in PubMed (https://pubmed.ncbi.nlm.nih.gov/; May 30, 2025) with terms disclosed for each column. Extinct hell-ants of the genus *Sphecomyrminae* in amber from Myanmar, Burma (Cretaceous, ca. 99 Mya, Dimensions amber piece: 16 x 7 mm) are shown as example of ant inclusions.

Despite these findings the question is, are ant syninclusions a random process reflecting only coexistence or a reflection of interactions between different organisms? [42]. For example, based on bibliometric analysis, the absence of previous reports on fossil amber syninclusions of ants and millipede or snail may suggest that these are only evidence of coexistence of these organisms from Cretaceous epoch. However, previous results do support possible interactions between ants and mites, spiders, wasps and plants.

9. Fossil commercialization and collections

Fossils are attractive to collectors and scientists, and commercialization operates with both legal and illegal purchases (Raja et al. 2023). Legal operations can provide funds and donate specimens for scientific studies and education, but some pieces may not reach museums and thus are not accessible unless private collectors collaborate with science [5]. The market for fossils is expanding since the early 19th Century with online platforms for professional e-market providing COA and/or DLO for fossil pieces (Appendix 1). Dinosaur fossils are the most unique and expensive with a record of a 150-million-year-old stegosaurus sold for $44.6 millions at Sotheby´s in 2024 (Antique Collecting Magazine 2025). Even smaller dinosaur remains are also rare and expensive.

Ethical concerns raise questions about fossils origin and destructive sampling that may be considered affordable when multiple specimens of a given taxon/species are available [4]. Scientists claim regulatory and ethical practices to protect scientific studies and heritage [43]. To face these challenges, authors agree with Larson and Russell (2014) [44] proposal that "Greatest challenge to paleontology of the 21st century, is finding a way for amateurs, commercial fossil dealers and academic paleontologists to work together and do what is best for the public and the fossils as the only way the science will thrive".

To build a fossil collection, personal criteria are used together with key factors such as (a) legal origin for fossils (e.g., official statement "Seller knows that in line with the laws and regulations in his country it is allowed to sell/export this object"), (b) finding and buying authentic specimens with support to type, location and date (i.e., COA, DLO, CAJI and/or IC in Appendix 1 and amber authentication in Appendix 2), (c) prepare and clean specimens with basic and professional tools to preserve the original fossil inclusions [45], (d) for selected pieces prepare high resolution images for study, morphological classification based on references and communication, (e) organize the collection with proper documentation and ID, and (f) display pieces with proper cases and stands with protection from light and humidity particularly for amber fossil inclusions.

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Supplementary Information

Appendix 1. Certificates for fossil amber

Examples of Certificate of authenticity (COA) and Declarations of legal origin (DLO) for amber piece disclosed in Figure 18D.

THEROPODA HOUSE

CERTIFICATE OF AUTHENTICITY

I hereby certify and guarantee the authenticity of this fossil piece, as well as the accuracy of its description.

The information provided in this certificate is given to the best of my knowledge and expertise, and considering the paleontologic data available to the present date.

Should further scientific discoveries be made in the future, this document may be reissued with the novel relevant findings with regards to its description and classification.

Scientific Name : Ixodida in amber
Age : Cretaceous, Upper (100.5 - 66 million years)
Location : Myanmar
Size : 20*16*5 mm

Wang You Chen
THEROPODA HOUSE person in charge

DECLARATION of LEGAL ORIGIN

Object Information

Item	amber	**Culture**	Burma
Dimensions	20 x 16 x 5 mm	**Century TimeFrame**	Cretaceous

Provenance Information

Acquired in (year)	2021	**From** Hukawng Valley, Kachin State Myanmar (Burma)	☐ **Private** ☒ **Fair** ☐ **Gallery/Antique shop** ☐ **Auction** ☐ **Other** .....
Acquired in (city/country)	Taiwan	**Previous Seller/Owner**	Amber Exhibition

Previous ownership history

Acquired in	☒ **Private collection** ☐ **Excavated** ☐ **Gallery/Antique shop** ☐ **Auction** ☐ **Other**	**From** Confuciusornis Therapoda House	☐ **Private** ☒ **Fair** ☐ **Gallery/Antique shop** ☐ **Auction** ☐ **Other** .....
In collection since (year)	2020	**In collection since**	2020

Documents that corroborate this ownership or provenance

I am able to demonstrate the legal origin with	☐ **Invoice** ☐ **COA** ☐ **Provenance statement** ☒ **Photos** ☐ **Export license** ☐ **Other**	**Copy of it will be sent to the buyer**	☒ **Yes** ☐ **No**
Description	Ant behavior and forest floor ecology. Dated by radiometric analysis, 99.3 ± 1.2 mya.		

The seller ensures

☒	Seller knows that in line with the laws and regulations in his country it is allowed to sell/export this object.
☒	Seller ensures that any necessary permits are/will be arranged.
☒	Seller will give to the buyer all provenance information known about the piece.

Place: Taiwan ..... **Date:** 2025 .....

Name: Wang You Chen ..... **Signature:** Wang You Chen .....

Wang You Chen

Examples of Certificate of Amber Jewelry Identification (CAJI) and/or Inspection Certificate (IC) by recognized institutions for amber piece disclosed in Figure 38.

Appendix 2. Paleoproteomics methodology for the analysis of fossil amber inclusions

1. Fossil amber inclusions

1.1. Amber pieces are certified as authentic as tested with UV light, saltwater floating, sinks in fresh water, acetone resistant, and heat – smell of pine resin.

1.2. Amber pieces are dated to fossil epoch by radiometric analysis [1].

2. Morphological analysis of fossil amber inclusions

2.1. Morphological analysis is conducted based on references for the different organisms [2-5]. For example, Argasidae and mite morphological references [6-8] and ITP Identification Technology Program (Mite morphology; https://idtools.org/bee_mite/index.cfm?pageID=1783) are used for analysis.

3. Image capture and morphological analysis

3.1. High-resolution images are captured with confocal microscopy and Ct scan tomography. **3.1.1.** For confocal microscopy, the amber inclusions are imaged using a Leica TCS SPE DM 5500 CSQ V-Vis (Mannheim, D-68165, Germany). The images are acquired with a solid-state laser operating at 488 nm, a 10X eye piece, HCX PL FLUOTAR 5X/0.15, ACS APO 10X/0.3 dry objectives and the Leica Application Suite Advanced Fluorescence software (Leica MM AF 1.4). Fluorescence emissions are collected from approximately 10 nm above the excitation wavelength up to 800 nm. Laser power for acquisition is set by viewing the fluorescence emission and increasing the power until the rate of increase in fluorescence appeared to have slowed. The photomultiplier gain for acquisition is then set by viewing the image and increasing the gain until signal overload was detected, at which point the gain was reduced slightly. The 532 nm laser is used for general visions and the 635 nm for selected parts. Pixels matrices of 1024 × 1024, two-dimensional mode, with speed of 400 Hz, and frame average of 4 are acquired for each Z-step of 0.68 micros at a zoom setting of 1.5. An Airy unit setting of 1 is routinely used for the observation pinhole.

3.1.2. Tomography is applied sing a Nikon XTH 160 micro-CT X-ray scanner to obtain high-quality 3D images of the inclusion. The images are generated with a molybdenum target and an X-ray voltage of 117 kV and 55 µA, obtaining a voxel size of 3.6 µm. During tomography, projections are collected every 0.12 degrees in duplicate, thus improving the signal/noise ratio and with an integration time of 708 msec.

3.2. Low-resolution images are captured with a Leica M80 routine stereo microscope using a 1X PLAN objective and a 2X-6X zoom (https://www.leica-microsystems.com/products/light-microscopes/stereo-microscopes/p/leica-m80/) and a Carl Zeiss stereomicroscope (SteREO Discovery V12, Munich, Germany) using the ZEN 2 pro software. Microscope images are analyzed using Image J program (https://imagej.net/ij/) and pencil sketch of images using IOimageonline.co (https://pencilsketch.imageonline.co/index.php).

3. Processing amber inclusions for paleoproteomics analysis

3.1. Processing of amber pieces is conducted in the level 2 laboratory (BSL-2) under laminar flow hood conditions.

3.2. Amber pieces are scrubbed in 5% SDS with a brush and abundantly rinsed first in water and then in 100% methanol.

3.3. The amber is covered with liquid nitrogen and fractured with a sterile ceramic pestle. Amber fragments without inclusions are discarded and arthropod inclusions are triturated to appearance of powder.

3.4. Triturates are extracted in 50 µl Laemmli sample buffer by applying 10 cycles of sonication followed by vortex. Subsequently, samples are heated to 90 ºC for 5 min, centrifuged at 12,000 x g for 5 min and supernatants collected, concentrated on-gel and trypsin digested [9].

3.5. The resulting tryptic peptides are desalted onto OMIX Pipette tips C18 (Agilent Technologies, Santa Clara, CA, USA), dried down and stored at -20 °C until mass spectrometry analysis.

4. Proteomics analysis of amber inclusions by mass spectrometry

4.1. Samples are resuspended in 10 µl of 2% acetonitrile - 5% acetic acid in water and analyzed by reverse-phase liquid chromatography coupled online to mass spectrometry (RP-LC-MS/MS) using an Ekspert^{TM} nLC 415 system coupled with a high-resolution mass spectrometer (HRMS) (e.g., 6600 TripleTOF, AB Sciex, Framingham, MA, USA) through Information-Dependent Acquisition (IDA).

4.2. The peptides are concentrated in a 0.1 × 20 mm C18 RP precolumn (Thermo Scientific, Waltham, MA, USA) with a flow rate of 5 µl/min during 10 min in solvent A.

4.3. Peptides are then separated in a 0.075 × 150 mm C18 RP column (e.g., Eksigent, part of AB Sciex) with a flow rate of 300 nl/min.

Peptides elution is done in a 60-min gradient from 5% to 30% solvent A followed by a 10-min gradient from 30% to 60% solvent B (Solvent A: 0.1% formic acid in water, solvent B: 0.1% formic acid in acetonitrile) and directly injected into the mass spectrometer for analysis. Two µl of each independent sample are analyzed by duplicate.

4.5. The mass spectrometer is set to scanning full spectra from 350 m/z to 1400 m/z (250 ms accumulation time) followed by up to 50 MS/MS scans (100-1500 m/z).

4.6. Candidate ions with a charge state between +2 and +5 and counts per second above a minimum threshold of 100 are isolated for fragmentation.

4.7. One MS/MS spectrum is collected for 100 ms, before adding those precursor ions to the exclusion list during 15 s (e.g., mass spectrometer operated by Analyst R TF 1.7, AB Sciex).
4.8. Dynamic background subtraction is turned off.
4.9. Data are acquired in high sensitivity mode with rolling collision energy on and a collision energy spread of 5.

5. Data analysis using a single compiled Uniprot database

5.1. The IDA MS raw files for each sample are combined (2 runs) and subjected to database search in unison using ProteinPilot software v. 5.0.1 (AB Sciex) with the Paragon algorithm. **5.2.** Spectra identification is performed by searching against the compiled Uniprot database with all sequences from inclusion organisms (e.g., Acari, Insecta) and Enterobacterales taxonomies together with contaminants DB from Hendy et al. [10] supplemented with human keratins and trypsin bovine.
5.3. The search parameters are: iodoacetamide cysteine alkylation, gel-based ID as special factor, identification focus on biological modifications, variants: evolutionary and amino acid substitutions and thorough ID as search effort.
5.4. The detected protein threshold is set at 0.05.
5.5. An independent False Discovery Rate (FDR) analysis with the target-decoy approach provided by ProteinPilot™ is used to assess the quality of identifications.
5.6. Two independent searches are performed selecting "trypsin digestion" and "no digestion" parameters, respectively, to identify tryptic and non-tryptic peptides.
5.7. Positive identifications are considered when identified proteins reach a 1% global FDR.
5.8. For the final analysis, proteins with two or more peptides per protein with at least one peptide with 1% FDR are used. Non-tryptic peptides are considered in proteins with at least one peptide with 1% FDR.

6. Data analysis using a target species-specific Uniprot databases

6.1. The IDA MS raw files for each sample are combined (2 runs) and subjected to database search in unison using ProteinPilot software v. 5.0.1 (AB Sciex) with the Paragon algorithm. **6.2.** Spectra identification is performed by searching against the corresponding Uniprot databases (e.g., for insects, protein identification is performed by searching against the Insecta Uniprot database (https://www.uniprot.org/uniprotkb?query=taxonomy_name:insecta).
6.3. The search parameters are: iodoacetamide cysteine alkylation, trypsin digestion and gel-based ID as special factor, identification focus on biological modification and thorough ID as search effort.
6.4. The detected protein threshold is set at 0.05.
6.5. An independent FDR analysis is used with the target-decoy approach provided by ProteinPilot™ to assess the quality of identifications.
6.6. Positive identifications are considered when identified proteins reach a 1% global FDR.

7. Analysis of identified proteins

7.1. Protein Blast (Blastp), conserved domains, phylogenetic and taxonomic analyses are conducted at National Center for Biotechnological Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome; https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=2762511) and UniProt (https://www.uniprot.org/blast).
7.2. Protein structure is modelled using Swiss-Model (https://swissmodel.expasy.org).

8. Analysis of negative control amber

8.1. As negative control, a piece of amber with the same origin and without inclusions is processed in the same manner.
8.2. The analysis is conducted by searching against all Uniprot databases used for compiled and target species-specific data with the same parameters.

9. Notes

(a) These protocols were used and validated in previous studies (e.g., [11]).
(b) The MS results are deposited in public access databases such as ProteomeXchange Consortium (https://www.proteomexchange.org) via the PRIDE (https://www.ebi.ac.uk/pride/) [12] partner repository.
(c) Data of Blastp and phylogenetic analyses are submitted as Supplementary Information.

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