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Letter to the editor Open Access

Phylogenetic context of a deep-sea clam (Bivalvia: Vesicomyidae) revealed by DNA from 1 500-year-old shells

DEAR EDITOR,

Ancient DNA (aDNA) from mollusc shells is considered a potential archive of historical biodiversity and evolution. However, such information is currently lacking for mollusc shells from the deep ocean, especially those from acidic chemosynthetic environments theoretically unsuitable for long-term DNA preservation. Here, we report on the recovery of mitochondrial and nuclear gene markers by Illumina sequencing of aDNA from three shells of *Archivesica nanshaensis* – a hydrocarbon-seep vesicomyid clam previously known only from a pair of empty shells collected at a depth of 2 626 m in the South China Sea. Carbon-14 analysis showed that the shells collected here from a depth of 3 003 m were about 1 500 years old. Sequence analysis indicated that *A. nanshaensis* was distinct from other vesicomyids with available molecular evidence and was sister to *A. marissinica* with a K2P distance of 3.4% in the mitochondrial cytochrome c oxidase I (*COI*) gene. Fossil-calibrated molecular dating indicated that *A. nanshaensis* and *A. marissinica* diverged approximately 8.5 million years ago (Ma) (4.8–13.1 Ma, 95% highest posterior density (HPD)) in the middle Miocene. This study demonstrates the potential of high-throughput sequencing of DNA from ancient shells to unravel the evolution and historical diversification of deep-sea molluscs, especially for those species described based solely on empty shells.

The use of aDNA in the study of biodiversity and ecology has increased in recent decades, especially since the widespread application of high-throughput sequencing (HTS) technologies to recover millions of short DNA fragments from the genomes of ancient remains. However, HTS aDNA technology has not been widely applied to invertebrate aDNA material such as mollusc shells. Mollusc shells are primarily composed of calcium carbonate and chitin, but other organic molecules such as proteins and DNA are also incorporated during shell growth. Although various studies on mollusc shell DNA have been conducted since the 2000s (Ferreira et al., 2020), only four such studies have employed HTS techniques (Der Sarkissian et al., 2017, 2020; Psonis et al., 2022; Sullivan et al., 2021). Der Sarkissian et al. (2020) recovered DNA from mussel shells dated ~10 000 years before present (BP). Sullivan et al. (2021) reported that successful recovery of nuclear DNA from temperate marine gastropods declines with shell age, from 4.57 thousand Illumina reads per sample in modern discarded shells and 12.1 thousand reads in archaeological shells (984–1 258 years BP) to only 114 reads in paleontological shells (5 711–7 187 years BP). These studies highlight the potential applicability of shell DNA as an archive of ancient biodiversity but indicate that environmental conditions may dictate the degradation patterns of shell DNA.

Here, we aimed to extend the aDNA HTS approach to deep-sea molluscs, whose diversity is poorly known. We determined the phylogenetic position of the deep-sea clam *Archivesica nanshaensis* (Xu & Shen, 1991) by extracting and sequencing shell DNA. This species belongs to Vesicomyidae, a family of clams widely used to study adaptation to extreme deep-sea environments (Decker et al., 2012). The taxonomic history of *A. nanshaensis* illustrates some of the problems associated with classifying deep-sea molluscs, which has traditionally relied on the morphology of a limited number of shells (Linse et al., 2020; Xu & Shen, 1991). Although studies based on DNA sequences from live-collected specimens have established a robust phylogenetic framework for Vesicomyidae (Johnson et al., 2017), there is a lack of genetic data for species known from modern or subfossil empty shells only, such as *A. nanshaensis*, *A. angulata*, *A. garuda*, *A. shikamai*, and *Phreagena ochotensis*. This has hindered our determination of their phylogenetic position and understanding of their evolutionary history. Prior to this study, *A. nanshaensis* was only known from a pair of shells collected in 1985 (Xu & Shen, 1991).

This study was conducted using three pairs of *A. nanshaensis* shells newly collected from a depth of approximately 3 000 m in the South China Sea (Figure 1; Supplementary Figure S1; see Supplementary Material for further study details). We dated two valves and found that their ^{14}C age (~2 000 years BP) and calibrated calendar age (~1 480 years BP) were very similar (Supplementary Table S1). We also used three shell valves to extract DNA and perform Illumina sequencing (details in Supplementary Notes). We generated 75.2–89.0 million raw reads and assembled the reads using *de novo* and reference-guided assembly. We mapped the clean reads to the reference mitochondrial and nuclear genomes of *A. marissinica* – a species closely related to *A. nanshaensis*. Mapping success varied markedly between the mitochondrial and nuclear reads, with only 7–20 mitochondrial reads but as many as 20 000 nuclear reads per sample (Supplementary Table S2). Sullivan et al. (2021)

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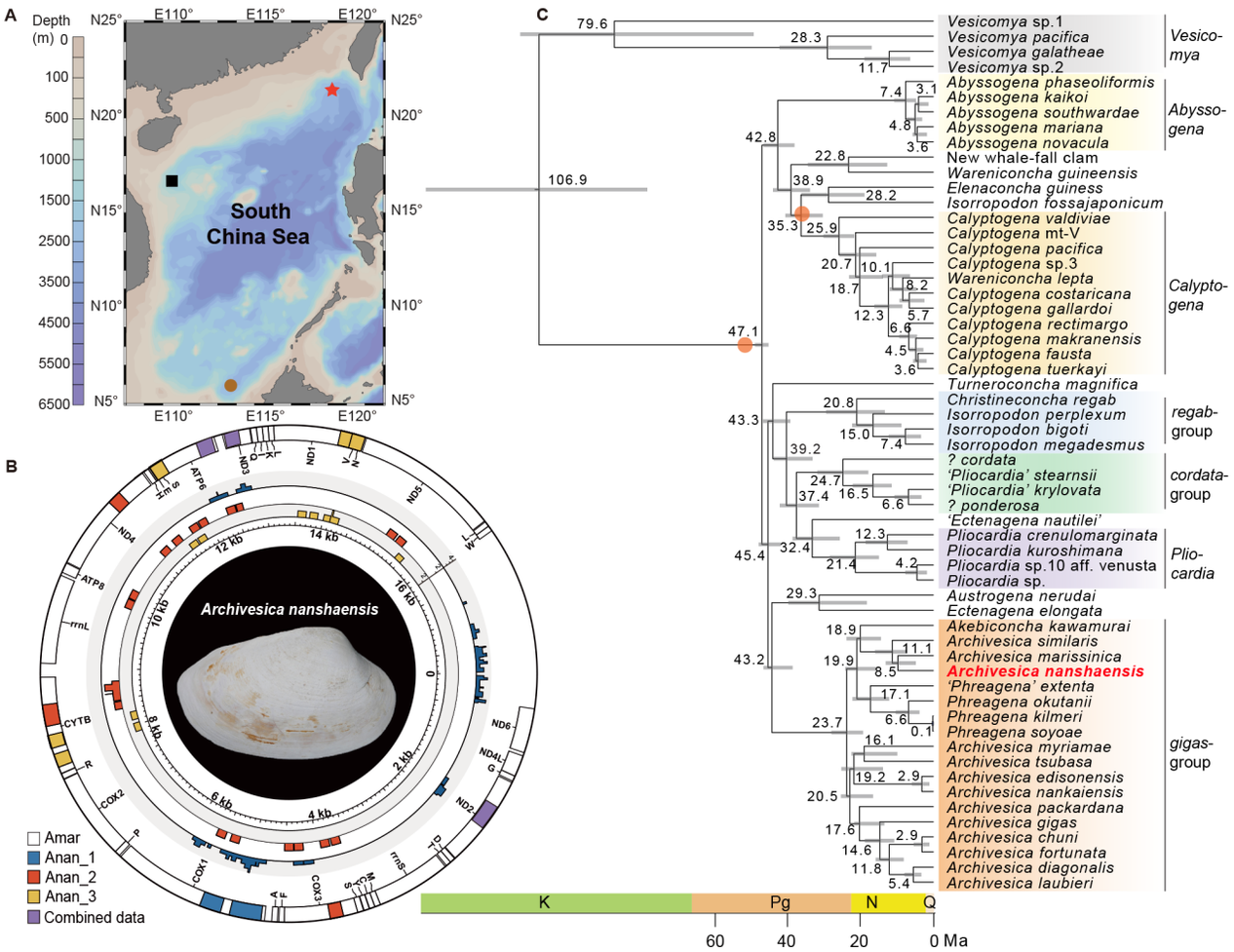

Figure 1 Distribution, mitochondrial gene fragments, and phylogenetic information of *Archivesica nanshaensis*

A: Map showing type locality (brown circle) and collection site (red asterisk) of *A. nanshaensis* shells used in this study, as well as type locality of *A. marissinica* used for comparison (black rectangle). Map was generated using Ocean Data View (ODV) v.5.0 (https://odv.awi.de). B: Visualization of arrangement of mitochondrial gene fragments recovered from shell samples of *A. nanshaensis* (Anan_1, 2, 3) compared with mitochondrial genome of *A. marissinica* (Amar). Mapping results of Anan_1, 2, 3 are indicated in different circles, with bin height indicating base depth. Additional details on Blast results are included in Supplementary Table S3. Photographs of the holotype of *A. nanshaensis* and all shells used in this study are shown in Supplementary Figure S1. C: Bayesian inferred fossil-calibrated time tree based on a 4 525 bp concatenated dataset from four gene fragments (*COI-H3-18S-28S*). Each number at a node represents the median value of estimated age in million years (Ma). Most taxon names follow those used in Johnson et al. (2017) and Linse et al. (2020). Some names were updated based on the World of Registered Marine Species (WoRMS) website. Single quotes and question marks indicate dubious generic assignments. Orange dots indicate fossil-calibrated records. Abbreviations of geologic time scale along axis: K, Cretaceous; Pg, Paleogene; N, Neogene; Q, Quaternary.

reported similar results for DNA extracted from gastropod shells (dated 1 000–7 000 years BP), with only 0–11 mitochondrial reads but tens to thousands of nuclear reads recovered from their HTS data.

To obtain gene sequences of *A. nanshaensis* for phylogenetic analysis and determination of genetic distances, the assembled contigs were searched against the reference database (Supplementary Note for details). We recovered 12 mitochondrial gene fragments with a total length of 2 473 bp (Figure 1; Supplementary Table S3), as well as *18S* and *28S* ribosomal RNA (rRNA) gene fragments with a total length of 857 bp and 960 bp, respectively (Supplementary Table S4). The success of sequence recovery was highly variable among the three samples. Using sequences from the individual samples, we only recovered two, four, and four mitochondrial contigs from Anan_1, Anan_2, and Anan_3, respectively (Supplementary Table S3). Among the recovered contigs, only *CYTB* and *trnE-trnS-ATP6* recovered from Anan_2 and Anan_3 showed some overlap. Using combined data from the three samples, we recovered three further mitochondrial gene fragments. These results highlight the stochastic nature of shell DNA preservation in historical shell samples and the benefits of using multiple samples for DNA recovery for multi-gene phylogenetic analysis. We also annotated the protein-coding genes from the assembled contigs and searched these genes for other nuclear gene markers previously used in phylogenetic analysis of molluscs. A total of 17 435 non-redundant protein-coding nuclear reads were found, corresponding to 859 annotated proteins (Supplementary Table S5). Furthermore, eight eukaryotic coding contigs were found among the 2 913 *A. nanshaensis* contigs assembled using clean reads mapped against the transcripts of *A. marissinica* and *P. okutanii* (Supplementary Table S6). From these sequences, we identified one repeat sequence (*C482*) and additional nuclear genes previously used for phylogenetic analysis of molluscs, including *ITS1*, *H3*, and *ACTB*.

Analysis of the sequencing data using mapDamage v.2 revealed no discernable fragmentation. However, there was a low level of nucleotide misincorporation induced by cytosine deamination characteristic of postmortem DNA damage (Supplementary Figure S2), with a mean read length of 165.1 bp, C-to-T misincorporation rate at the 5′ end of 0.87%, and G-to-A misincorporation rate at the 3′ end of 1.34%. The highly fragmented aDNA (mostly <100 bp) from shallow-water mollusc shells (Der Sarkissian et al., 2017, 2020; Sullivan et al., 2021) was not observed in the current study (Supplementary Figure S2A) but minor damage at the 5′ and 3′ ends was observed for the collapsed mapped reads (Supplementary Figure S2B). Nevertheless, compared to shallow-water mollusc shells of similar age (~1 000–7 000 years) with C-to-T misincorporation rates at the 5′ end of 16.8%–20%, the postmortem DNA damage in the deep-sea vesicomyid shells was relatively minor. Severe DNA damage in shallow-water shells may result from more dramatic environmental changes in the intertidal regions. With a stable cold environment, permafrost is considered ideal for aDNA preservation (Murchie et al., 2022). Indeed, DNA recovered from ~100 000-year-old shells in frozen permafrost showed less severe damage, with a C-to-T misincorporation rate at the 5′-read end of only 6.4% (Der Sarkissian et al., 2020). The deep-sea environment where the *A. nanshaensis* shells were found (Supplementary Figure S3) may be better for shell DNA preservation than shallow-water environments (except in high latitude regions). This highlights the potential of deep-sea mollusc shells as archives of historical genetic diversity. Nevertheless, as our data filtering was conducted against an *A. nanshaensis* assembly with an unknown level of contamination, further studies are required to test postmortem shell DNA damage using living specimens.

Based on the *COI* dataset, *A. nanshaensis* showed 3.4% divergence from *A. marissinica* (K2P distance, Supplementary Table S7). Other pliocardiine species were more divergent from *A. nanshaensis*, with a K2P distance of at least 5.8% (*A. similaris*) between pairs. The K2P distance between *A. nanshaensis* and *A. marissinica* was larger than that between several pairs of vesicomyid species, such as *Calyptogena fausta* and *C. makranensis* (1.1%) and *A. diagonalis* and *A. laubieri* (2.3%). These findings indicate that *A. nanshaensis* is genetically distinct from other compared vesicomyid species, confirming its distinctness from *A. marissinica* based on morphology and distribution (Chen et al., 2018).

In the phylogenetic trees reconstructed using the 4 525 bp mitochondrial-nuclear gene dataset (Figure 1; Supplementary Figure S4), *A. nanshaensis* was most closely related to *A. marissinica*; these two species are part of a monophyletic clade containing *A. similaris* and *Akebiconcha kawamurai*, corresponding to the "g1c" clade within the complex "*gigas*-group" in Johnson et al. (2017). The complex relationships among the *Archivesica*, *Phreagena*, and *Akebiconcha* genera in the "*gigas*-group" have not yet been resolved in morphological and molecular analyses (Johnson et al., 2017). Here, the "*gigas*-group" showed a clear internal structure containing four main clades, similar to Johnson et al. (2017) but with different placement of the "g1d" clade and inconsistent position of *Akebiconcha kawamurai* based on the two phylogenetic methods. The sister relationships among the four clades were not fully resolved based on the low Bayesian posterior probability (BPP) values between "g1a" and "g1d" (BPP=0.27) and "g1b" and "g1c" (BPP=0.57). Nevertheless, sequence divergences among the clades were substantial, with K2P distances ranging from 8.4% to 10.1% (Supplementary Table S8), although these divergences are lower than those among pliocardiine genera (11.1%–15.0%). The K2P distances within the four clades (4.5%–7.5%) were lower than between the clades, indicating closer relationships among these clades. In addition, the placement of most pliocardiine species, especially those in *Abyssogena*, *Calyptogena*, and *Pliocardia*, was consistent with Johnson et al. (2017). Molecular dating using BEAST recovered a mid-Miocene divergence time of 8.5 Ma (4.8–13.1 Ma, 95% HPD) between *A. nanshaensis* and *A. marissinica* and 11.1 Ma (7.2–16.0 Ma, 95% HPD) between the clade of (*A. marissinica*, *A. nanshaensis*) and *A. similaris* (Figure 1). Within the "*gigas*-group", the (*Akebiconcha kawamurai* (*A. similaris* (*A. marissinica*, *A. nanshaensis*))) clade diverged from the clade containing *Phreagena* ("g1c") 19.9 Ma (15.7–24.3 Ma, 95% HPD), close to the divergence time between "g1a" and "g1d". Thus, the Miocene appears to be a period of rapid divergence of clades within *Archivesica*, during which most extant lineage diverged (Johnson et al., 2017). The earliest known fossil records of *Archivesica* species occur in the Miocene (Amano et al., 2022), consistent with our molecular results. The cause of this radiation event is unknown, but intense methane emissions between 13 and 6 Ma coinciding with a decrease in sea level (Oppo et al., 2020) may have provided opportunities for such speciation events.

In the present study, we applied Illumina sequencing to capture DNA sequences from ~1 500-year-old *A. nanshaensis* shells. We found low levels of postmortem DNA damage in these historical deep-sea shells. Using the mitochondrial and ribosomal gene fragments recovered from the shell DNA, we successfully reconstructed the phylogenetic relationship of this enigmatic species within Vesicomyidae, confirming its placement in the genus *Archivesica*, and estimated its time of divergence from other congeners. The main purpose of this study was to provide a proof-of-concept for the feasibility of extracting molecular markers from deep-sea mollusc shells to reconstruct phylogenetic relationships. To avoid introducing taxonomic confusion, we marked problematic taxa in quotation and question marks in our tree of Vesicomyidae tree, as in Johnson et al. (2017). We found that high-throughput sequencing DNA from historical shells produced many microbial reads, some of which may have originated from past environmental microbial communities or may be symbiotic bacteria of vesicomyids (Supplementary Table S9), indicating the potential of this approach to unravel the interactions between these bivalves and their surrounding bacterial communities.

DATA AVAILABILITY

The raw sequencing data of *Archivesica nanshaensis* shell DNA were deposited in the National Center for Biotechnology Information Sequence Read Archive (BioProjectID PRJNA834558), Science Data Bank (DOI: 10.57760/sciencedb.06902), and National Genomics Data Center (accession No. GSA: CRA009856).

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Y.X.L.: Data analysis, writing original draft. Y.Z.: DNA extraction. J.C.H.I.: Data analysis. J.L.: Sample comparison. C.C. and C.T.S.L.: Conceptualization, reviewing and editing. Y.Y. and M.Y.: Isotope analysis. J.W.Q.: Conceptualization, project design, sampling, reviewing, and editing. All authors read and approved the final version of the manuscript.

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REFERENCES

Amano K, Kiel S, Hryniewicz K, et al. 2022. Bivalvia in ancient hydrocarbon seeps. *In*: Kaim A, Cochran JK, Landman NH. Ancient Hydrocarbon Seeps. Cham: Springer.

Chen C, Okutani T, Liang QY, et al. 2018. A noteworthy new species of the family Vesicomyidae from the South China Sea (Bivalvia: Glossoidea). *Venus* (*Journal of the Malacological Society of Japan*), **76**(1–4): 29–37.

Decker C, Olu K, Cunha RL, et al. 2012. Phylogeny and diversification patterns among vesicomyid bivalves. *PLoS One*, **7**(4): e33359.

Der Sarkissian C, Pichereau V, Dupont C, et al. 2017. Ancient DNA analysis identifies marine mollusc shells as new metagenomic archives of the past. *Molecular Ecology Resources*, **17**(5): 835−853.

Der Sarkissian C, Möller P, Hofman CA, et al. 2020. Unveiling the ecological applications of ancient DNA from mollusk shells. *Frontiers in Ecology and Evolution*, **8**: 37.

Ferreira S, Ashby R, Jeunen GJ, et al. 2020. DNA from mollusc shell: a valuable and underutilised substrate for genetic analyses. *PeerJ*, **8**: e9420.

Johnson SB, Krylova EM, Audzijonyte A, et al. 2017. Phylogeny and origins of chemosynthetic vesicomyid clams. *Systematics and Biodiversity*, **15**(4): 346−360.

Linse K, Sigwart JD, Chen C, et al. 2020. Ecophysiology and ecological limits of symbiotrophic vesicomyid bivalves (Pliocardiinae) in the Southern Ocean. *Polar Biology*, **43**(10): 1423−1437.

Murchie TJ, Karpinski E, Eaton K, et al. 2022. Pleistocene mitogenomes reconstructed from the environmental DNA of permafrost sediments. *Current Biology*, **32**(4): 851−860.e7.

Oppo D, De Siena L, Kemp DB. 2020. A record of seafloor methane seepage across the last 150 million years. *Scientific Reports*, **10**(1): 2562.

Psonis N, Vardinoyannis K, Poulakakis N. 2022. High-throughput degraded DNA sequencing of subfossil shells of a critically endangered stenoendemic land snail in the Aegean. *Molecular Phylogenetics and Evolution*, **175**: 107561.

Sullivan AP, Marciniak S, O'Dea A, et al. 2021. Modern, archaeological, and paleontological DNA analysis of a human‐harvested marine gastropod (*Strombus pugilis*) from Caribbean Panama. *Molecular Ecology Resources*, **21**(5): 1517−1528.

Xu FS, Shen SP. 1991. A new species of Vesicomyidae from Nansha Islands waters. *Marine Biology in Nansha Islands Adjacent Seas*: 164−166 (in Chinese)