

Metapopulation of *Ellobium chinense* Through The Late-Middle and Late Pleistocene Expansions: Four Covariate COI Hotspots Linked to G-Quadruplex Conformation

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Research Article

Keywords: Ellobium chinense, COI, microsatellite markers, metapopulation, G-quadruplex, Pleistocene expansion

Posted Date: January 27th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-152844/v1>

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Abstract

The land snail *Ellobium chinense* (Pulmonata, Ellobiida, Ellobiidae), which inhabits the salt marshes along the coastal areas of northwestern Pacific, is an endangered species on the IUCN Red List. Over recent decades, the population size of *E. chinense* has consistently decreased due to environmental interference caused by natural disasters and human activities. Here, we provide the first assessment of the genetic diversity and population genetic structures of northwestern Pacific *E. chinense*. The results analyzed with *COI* and microsatellites revealed that *E. chinense* population exhibit metapopulation characteristics, retaining under the influence of the Kuroshio warm currents through expansion of the Late-Middle and Late Pleistocene. We also found four phylogenetic groups, regardless of geographical distributions, which were easily distinguishable by four unidirectional and stepwise adenine-to-guanine transitions in *COI* (sites 207-282-354-420: A-A-A-A, A-A-G-A, G-A-G-A, and G-G-G-G). Additionally, the four *COI* hotspots were robustly connected with a high degree of covariance between them. We discuss the role of these covariate guanines which link to form four consecutive G-quadruplexes, and their possible beneficial effects under positive selection pressure.

Introduction

*Ellobium chinense*¹, of the family Ellobiidae (Gastropoda, Pulmonata, Ellobiida), is a small, air-breathing, land snail that dwells between-stones in freshwater tidelands near the shoreline and on halophytes such as *Zoysia sinica*, as shown in Figs 1a and S1. Its distribution range is confined only to the northwestern Pacific coasts including the coastlines of South Korea, Japan, and China². Importantly, *E. chinense* is a key indicator of the environmental health of the intertidal zone of its habitat. However, over recent decades, its natural habitat and the number of populations and individuals have rapidly declined due to environmental stresses, such as typhoons and tsunamis, and human activities including land reclamation and coastal development; thus, *E. chinense* has been designated as an endangered species on the South Korean Regional Red List³, as a vulnerable species on the Japanese Regional Red List, and as an endangered species on the International Union for Conservation of Nature (IUCN; Association of Wildlife Research and Envision, 2007).

Sufficient genetic diversity is important for ensuring the persistence of species, increasing their adaptive potential in the face of environmental change, and avoiding inbreeding depression⁴. On the other hand, the loss of intraspecific genetic diversity dramatically reduces the capacity for species to respond to novel selection pressure and thereby increases their extinction risk⁵. In recent decades, research on the population genetics of endangered metazoan species has been increasingly conducted because of its central importance in planning both in-situ and ex-situ conservation efforts⁶. In such research, molecular markers, including specific mitochondrial genes [mainly cytochrome *c* oxidase I (*COI*)]⁷, whole mitochondrial genomes⁸, and microsatellites⁹ are consistently used. Given the importance of determining the genetic diversity and population genetic structure of an endangered species in conservation planning, it is surprising that such research has rarely been northwestern Pacific *E. chinense*.

In the present study, therefore, we provide the first assessment of the genetic diversity and the pattern of genetic structures among populations of northwestern Pacific *E. chinense* (sampled in South Korea and Japan). Our analyses were based on the popular DNA barcode region of the mitochondrial gene *COI* and 10 selected microsatellite markers. In total, 140 *COI* data (consisting of 139 South Korean samples and one Japanese sample), and 54 microsatellite data (from South Korea only) were analyzed. In addition, we employed four distinct covariate adenine/guanine hotspots observed on *COI* which are distinct key sequences to easily classify phylogenetic groups of *E. chinense*; these sites were closely associated with G-quadruplex structure conformation. G-quadruplexes, i.e., four-stranded noncanonical DNA structures, can be spontaneously formed by Hoogsteen bonds between stacked sets of four guanines from each of four separated runs of two, three, or four guanines¹⁰. A stable G-quadruplex within the coding sequences (CDS) causes ribosome stalling; protein expression can be enhanced through silent mutations that affect its stability¹¹. Herein, by determining the genetic diversity and structures of populations of *E. chinense*, we were able to discuss not only the roles of the four covariate hotspots on the CDS of *COI* in relation to G-quadruplex structure conformation but also the associated beneficial and resistant effects against environmental perturbation such as oxidative stress.

Methods

Sample collection and DNA extraction

In total, 103 individuals of *E. chinense* were collected from eight sampling sites across the western and southern coastal areas of South Korea; these covered the entire remaining habitats of *E. chinense* on the Korean Peninsula (Figs 1b and S1; Table 1). Since this species is protected as an endangered species in South Korean government and the IUCN Red List, samples were collected after gaining permission from the Ministry of Environment, South Korea. The collected samples were immediately preserved in 95% ethanol immediately. Total genomic DNA was extracted from the foot tissue of *E. chinense* with the DNeasy Blood and Tissue Kit (Qiagen Co., USA) following the manufacturer's protocol and then maintained at -20°C until further use. The efficiency of the genomic DNA extraction and the purity of the extracted DNA were examined with a spectrophotometer (Nanodrop Technologies, USA).

PCR amplification and sequencing of COI

To amplify a partial fragment of mitochondrial *COI* gene of *E. chinense*, PCR was conducted using two universal primers: LC01490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HC02198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3'). These primers, which were developed for invertebrates by Folmer et al. (1994)³⁴, can be used for PCR amplification of a partial mitochondrial *COI* fragment, i.e. a DNA barcoding region, prior to sequencing. The PCR mixtures were prepared to a total volume of 50 μl containing 2 μl of 10 pM of each primer, 1 μl of 10 mM dNTP mix, 5 μl of $10 \times$ *Taq* DNA polymerase reaction buffer, 0.25 μl of 5U/ μl DiaSter *Taq* DNA polymerase, and 2 μl of total genomic DNA. Thermal cycling conditions were as follows: denaturation at 94°C for 2 min, 35 cycles of 94°C for 40 s, 52°C for 1 min, and 72°C for 1 min, and a final extension step of 72°C for 7 min, which followed a cool down step at

4°C. The PCR products were electrophoresed on 1% agarose gel including eco-dye and examined under UV light; they were purified using a QIAquick PCR Purification Kit (Qiagen Co., USA) and then sequenced using the primers applied in PCR and an ABI Prism 3730 DNA sequencer (PerkinElmer, USA) with a BigDye Termination Sequencing Kit (PerkinElmer, USA). The determined DNA sequences were manipulated in BioEdit v7.2.5³⁵, and finally verified by visual inspection.

PCR amplification and genotyping of microsatellite markers

A total of 30 microsatellite markers that have recently been developed for *E. chinense*³⁶; these were tested using some representative *E. chinense* samples collected in the present study. The initial screenings discovered ten primer pairs showing clear and reproducible profiles; these were selected for subsequent genetic analyses of *E. chinense* populations (Table S1). A tailed PCR primer was produced by adding a M13 (-21) universal sequence “tag” to the 5' end of each forward primer as described by Schuelke (2000)³⁷. The M13-forward oligonucleotide was labeled with the 6-FAM fluorescent dyes. PCRs were performed in a total volume of 20 µl consisting of 20 ng of template (gDNA), 10 mM dNTPs, 10 pmol of M13 modified forward primer, 10 pmol of each reverse primer and M13 primer fluorescently labeled with 6-FAM (Eurofins Genomics, France), with 10 × incubation mix including MgCl₂, 5 units HANLAB *Taq* DNA polymerase, and filled with distilled water. PCR cycling conditions comprised an initial denaturation phase at 94°C for 5 min, followed by 34 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s, and finally an extension step at 72°C for 5 min. The tagged PCR product (0.2 µl) was added to 9.8 µl of Hi-Di formamide loading buffer, sized precisely using 0.2 µl of GeneScan 500 LIZ Size Standard (Applied Biosystems, USA) and subsequently run on an ABI 3730x1 automated sequencer (Applied Biosystems, USA). Allele size calling and genotypic analyses were performed using GeneMapper Software ver. 4.1 (Applied Biosystems, USA).

Analyses of population genetic diversity and structure

To elucidate the level of genetic diversity and structure at the interpopulation and/or intrapopulation levels in *E. chinense*, the 140 obtained *COI* sequences were aligned with Clustal X³⁸ (Data S1). The numbers of polymorphic sites and haplotypes, haplotype diversity, and nucleotide diversity were estimated for each population using the program DnaSP v.6.0³⁹. AMOVA (analysis of molecular variance) was conducted in ARLEQUIN v.3.5⁴⁰ to partition the genetic variance within and among populations. Pairwise F_{ST} values were calculated to assess the pattern of population differentiation. A neutrality test was performed by calculating Tajima's D^{41} and Fu's F_s^{42} via ARLEQUIN v.3.5⁴⁰ to detect the historical existence of demographic expansion. To examine whether *E. chinense* has passed through recent expansion or frequent migration among neighbor demes, a mismatched distribution analysis was conducted based on the distribution of the observed pairwise nucleotide site differences and the expected values obtained assuming the populations of constant size or under the growth¹². Based on the sequence alignment set of 58 *COI*/haplotypes (Fig. S2; Data S2), unrooted and outgroup-rooted maximum likelihood (ML) trees were reconstructed using the IQ Tree web server (<http://iqtree.cibiv.univie.ac.at>) with

the GTR+I+G model and 1,000 bootstrapping replicates. Additionally, a phylogenetic network was generated via the neighbor-net algorithm⁴³. Furthermore, a statistical parsimony haplotype network was constructed with a 95% connection limit using TCS v.1.2.1⁴⁴; this was used to assess the genealogical relationship at the intraspecific level and to infer biogeography by constructing a *COI* haplotype network. To evaluate and visualize the geographic genetic structure among populations, principal coordinates analyses (PCoA) were conducted via DARwin v.6.0.9⁴⁵, which ordinated genetic distance estimates calculated with the haplotype data used in this study.

Using the 10 microsatellite loci (Table S1) selected from the 30 microsatellite markers previously developed by Hyun et al. (2017)³⁶, we reexamined the genetic diversity and structure of *E.chinense* populations in comparison to the results inferred from *COI* haplotype data. The PCR primers selected as microsatellite markers in the present study exhibited highly informative values of polymorphism information content ($PIC > 0.5$). Based on these microsatellite markers, genetic variability was estimated in terms of the number of alleles averaged over 10 loci (N_A), observed heterozygosity (H_O), and expected heterozygosity (H_E) across populations and across loci using GenALEx v.6.5⁴⁶. Furthermore, F_{IS} was calculated for each population and for each locus, based on Wright's F-statistics⁴⁷. The genetic differentiation coefficient (F_{ST}) was estimated using GenALEx v.6.5; additionally, tests for deviation from the Hardy-Weinberg Equilibrium between the microsatellite loci were performed using this software. To characterize the overall genetic structure, we applied a Bayesian clustering approach implemented in STRUCTURE v.2.3.4⁴⁸ which identifies the number of potential genetic clusters (K) in the dataset without a priori information on geographical or population identifiers. An admixture model was used with correlated allele frequencies applying burn-ins of 2×10^5 and runs of 10^5 repetitions for each value of K varying from 1 to 8. Three iterations were completed for each tested K value. The true number of K was identified based on the approach of Evanno et al. (2005)¹⁶ by applying Structure Harvester v.0.6.94⁴⁹. In addition, we investigated isolation by distance (IBD) was examined by correlating the matrix of pairwise geographic distances between sampling locations with two correspondent matrices of genetic differentiation estimates: F_{ST} (estimated based on haplotype frequency) and ϕ_{ST} (considering the level of distance among haplotypes)⁵⁰. Geographic distances were calculated as the shortest pathways along the coastal line.

Covariance calculation and screening of possible G-quadruplex motifs in COI

From the multiple sequence alignment results of the *COI* haplotypes (Fig. S2), we searched for cases of concerted patterns of variation between different alignment positions. In performing this covariance calculation, we made pairwise gene sequence comparisons for every position. The base was checked for covariance with the frequency ratio in the multiple sequence alignment results, and this result was displayed on the network.

To screen for possible G-quadruplex-forming sequence motifs in *COI* (Fig. S1), we used the QGRS-Conserve program⁵¹, considering new types of G-quadruplex in addition to the canonical forms. The

frequencies of nonoverlapping G-quadruplex-forming sequence motifs in the *COI* barcoding region were calculated using uninterrupted or interrupted by non-G-rich fragments not exceeding the maximum loop length (20 bp). This calculation process was repeated up to 10 times.

Divergence time estimation based on COI

Divergence time estimation of the nodes on the phylogeny of *E. chinense* was conducted based on *COI* haplotype sequences via the BEAST 2.6.0.⁵² The divergence time was estimated using the strict molecular clock algorithm under the calibrated-Yule tree prior. For the calibration point, the previously estimated age of the subfamily Carychiinae (from the family Ellobidae) was used as the basal node (60 mya; normal distribution). The age of Carychiinae was given by Weigand et al. (2013)⁵³, who among others reported that it originated no earlier than the beginning of the early Cenozoic (about 65 mya)^{53,54}. The best-fit nucleotide substitution model was selected by jModelTest⁵⁵ as GTR+I+G. The posterior distributions of parameters were estimated using 1,000,000 MCMC generations with sampling every 1,000 generations. Additionally, an effective population size value was determined in Tracer 1.7⁵⁶. TreeAnnotator 2.6.0.⁵⁷ was used to produce a maximum clade credibility tree with a median height after removing the initial 25% of trees as burn-in. FigTree 1.4.2.⁵⁸ was used to visualize the topology of the resultant consensus tree.

Results

Genetic diversity and phylogeny of E. chinense based on COI

The partial fragment of *COI*, 595bp in length, was sequenced from 103 *E. chinense* individuals collected from the eight sites in South Korea (Table 1). These sequences were aligned together with 37 *COI* haplotype sequences retrieved from the NCBI GenBank. Hence, 140 *COI* haplotype sequences of *E. chinense* were analyzed, representing 18 collection sites at the nine populations in South Korea and Japan (Table 1). Based on the alignment set (no indels) of these 140 *COI* sequences (Data S1), we obtained a total of 58 *COI* haplotypes, of which 43 were singleton, appeared in only a single site (Table 2). The novel 58 *COI* haplotype sequences obtained were registered under the GenBank accession nos. MW265437–MW265477. According to the sequence alignment of the 58 *COI* haplotypes (Fig. S2; Data S2), there were 71 polymorphic sites and 31 parsimoniously informative sites (Fig. 1c), among which four adenine/guanine hotspots at 207, 282, 354, and 420 were ascertained to articulately divide the haplotypes of *E. chinense* into four meaningful phylogenetic groups: (a) A(207)-A(282)-A(354)-A(420), (b) A-A-G-A, (c) G-A-G-A, and (d) G-G-G-G.

Based on the *COI* haplotype sequence alignment (Fig. S2; Data S2), we reconstructed a ML tree using *Ellobium aurisjudae* as an outgroup. In the resultant tree topology (Fig. S3), it was confirmed that *E. chinense* appeared as a monophyletic group, but no distinction between the haplotypes from each geographical population was observed. To define detailed relationships among the *COI* haplotypes, the outgroup was removed and then an unrooted ML tree (Fig. 1d) was reconstructed. The resultant tree

showed two distinctive phylogenetic groups, namely A-A-A-A and the other groups (including at least one G or more in the four positions), regardless of collection localities. The A-A-A-A group included 35 of the 58 *COI* haplotypes. The others could be divided into the A-A-G-A group ($N=12$: ECH11, 12, 15, 16, 18, 23, 27, 28, 32, 33, 36, and 49), the G-A-G-A group ($N=1$: ECH35), and the G-G-G-G group ($N=8$: ECH01, 07, 19, 29, 41, 45, 48, and 54).

As shown in Table S2 and Fig. S3, ECH01 was a dominant member of the G-G-G-G group with the most individuals (27), which appeared across all the South Korean populations examined here. As shown in Fig. 1d, the A-A-A-A group is likely to be an ancestral type because it was most frequently found in the other species within Ellobiidae (data not shown) and its haplotype diversity was the highest among the four genetic groups. Given that the G-G-G-G group exhibited much lower haplotype diversity than the A-A-A-A group, and was not observed in any other ellobiid species (data not shown), it is reasonable to suggest that the G-G-G-G group is a derived rather than an ancestral type. Thus, as shown in Fig. 1d, it is conceivable that unidirectional and stepwise A→G transition events from A-A-A-A to G-G-G-G may have been occurred in *E. chinense*. Within the A-A-A-A and A-A-G-A groups, parsimoniously informative A→G transition event was found at the sites 120 (ECH12, 15, 16, 18, 23, 38, 40, 44, and 49) and 183 (ECH3, 9, 10, 20, 43, 50, and 55), with a few exceptional cases, for example, G→A at the sites 216 (ECH12, 15, 16, 18, and 23) and 372 (ECH12, 15, and 23), and 429 (ECH37, 38, 46, and 47; ECH19 found in the G-G-G-G group).

As indicated in Table 2, the nucleotide diversity (π) is relatively low among the nine populations of *E. chinense*, ranging from 0.00749 (population BG) to 0.01042 (SC) with an average of 0.00865, whereas the haplotype diversity was very high across these populations, ranging from 0.924 (YG) to 1.000 (SC and JB) with an average of 0.939). All values of Tajima's D and Fu's F_S were congruently negative, with averages of -1.87100 ($P < 0.05$) and -2.75886 (not statistically significant), respectively, indicating that the signature of demographic expansions recently occurred in *E. chinense*.

Population genetic structure of E. chinense based on COI

As shown in Table S3, the pairwise F_{ST} values between nine *E. chinense* populations were highly low and statistically non-significant, ranging from -0.21177 (JB and GY) to 0.09091 (JB and JK), indicating the lack of genetic structure. Analysis of molecular variance (AMOVA) for the nine populations of *E. chinense* in South Korea and Japan (Table S4) revealed that 101.05% of the variance was allocated into the level of individuals within each population, while differentiation between populations (-1.05%) did not appear to contribute to the overall variance.

Upon the results of TCS network analysis, all the *COI* haplotypes were connected by forming a single, large network, conforming that no distinct genetic structure existed among populations (Fig. S4a). The G-G-G-G group, with its representative member ECH01, was located rather at a distal position on the TCS network, which implies that these haplotypes may have recently emerged during the expansion of northwestern Pacific *E. chinense* populations. The phylogenetic network analysis (Fig. S4b) also

indicated that a distinct genetic structure representing the geographical distributions of *E. chinense* did not exist. Indeed, all haplotypes were grouped into a single broad cluster with overlaps between populations, which is in agreement with the results of the TCS network analysis (Fig. S4a). In addition, the mismatch distribution analysis showed a clearly unimodal curve (Fig. S4c), evidently implying the metapopulation of *E. chinense* that may have experienced a recent demographic expansion^{12,13} or through a range expansion with high levels of migration between neighboring demes^{14,15}.

The PCoA shown in Fig. 2a provided additional evidence that, regardless of the geographical distribution of Northwestern Pacific *E. chinense*, there was consistent support for the existence of four distinct phylogenetic groups, i.e., A-A-A-A, A-A-G-A, G-A-G-A, and G-G-G-G (as also shown in Fig. 1). In this analysis, the plausible ancestral A-A-A-A group (with the highest degree of haplotype diversity) was placed mainly around the bottom of the second quadrant, whereas the A-A-G-A group was in the left of the fourth quadrant, the G-A-G-A group (ECH35 only) in the right middle of the fourth quadrant, and the G-G-G-G group (represented by ECH01) was in the first quadrant. The three variation types within the A-A-A-A group, namely A-A-A-G, A-G-A-A, and A-G-A-G, appeared in the ML tree (Fig. 1d; Fig. S3) and were spatially separated from the remaining orthodox members of A-A-A-A, which were located along the Y axis between the first and second quadrants. Based on the results of PCoA (Fig. 2a) and the ML tree (Fig. 1a), we confidently suggest that the four phylogenetic groups of *E. chinense* arose as a result of serial, stepwise A→G transition events in the following order: A-A-A-A (ancestral) → A-A-Ĝ-A → Ĝ-A-Ĝ-A → G-Ĝ-G-Ĝ (derived). The other local A→G transition events occurred within the A-A-A-A group (A-A-A-Ĝ, A-Ĝ-A-A, and A-Ĝ-A-Ĝ) (Fig. 1d; Fig. S3); these could be considered the fifth spatially separated phylogenetic group of *E. chinense*, based only on PCoA (Fig. 2a), being located between the phylogenetic groups G-G-G-G and A-A-A-A but apart from A-A-A-A.

Covariance of four COI hotspots linked to the conformation of four G-quadruplexes

Similar sequence interval lengths existed between the four adenine/guanine hotspots of *COI* characterized by unidirectional A→G transitional changes: G-(75 bp)-G-(72 bp)-G-(66 bp)-G. Additionally, the covariance analysis indicated that there was a high degree of covariance among these (see the four thick and bold linkage lines in Fig. 2b): out of 58 haplotypes, 44 were covariate between 207 and 282 sites, 44 between 207 and 420 sites, 41 between 282 and 420 sites, 37 between 207 and 354 sites, 32 between 282 and 354 sites, and 32 between 282 and 420 sites (Data S3 in detail). Given the similar intervals and covariance characteristics, we used QGRS-Conserve to search for sequence motifs that could possibly indicate the formation of G-quadruplex structures in relation to the four hotspots on the CDS of the *COI* barcoding region. Our results revealed possible sequences associated with the conformation of four different G-quadruplexes represented by each of the four hotspots (Fig. 2c). The first hotspot located at site 207 may be involved in forming a G-quadruplex structure such as G₂-N₆-G₂-N₁₅-G₂-N₁₁-ĜA; the second hotspot at site 282 may be involved in forming G₂-N₂-G₂-N₅-G₂-N₅-ĜT or G₂-N₂-G₂-N₄-Ĝ₂-N₁-GA; the third hotspot at site 354 may be involved in forming G₂-N₁-G₂-N₄-G₂-N₁₈-CĜ; and the fourth hotspot, found at site 420, may be involved in the formation of TĜ-N₈-G₂-N₁₀-G₂-N₂₁-CG.

G-quadruplex structures generally have 1-7 nucleotides between the four guanine-tracks each consisting of two or three guanines, e.g., G₂₋₃-N₁₋₇-G₂₋₃-N₁₋₇-G₂₋₃-N₁₋₇-G₂₋₃; two (for G₂) or three (for G₃) G-quadruplexes can form a stacked structure. Considering the typical features of G-quadruplex structures, each of the putative motifs searched for G-quadruplex structure formation is likely to exhibit two G-quadruplexes stacked together (Figs 2c and d). However, the number of nucleotides between the G-tracks is much larger than usual for some sites, and some G tracks consist of only one G (not two or three). Thus, the G-quadruplex structures formed from the putative motifs are expected to be relatively weak. Assuming that all four putative motifs found in our analyses are capable of forming G-quadruplex structures, the four consecutive G-quadruplexes with intervals of 57/60, 39/41, and 65 bp in order might play a role in reducing or inhibiting transcription or translation of *COI* expression, i.e., they may be an exemplar of downregulation of gene expression; the ribosome stalling process (Fig. 2d).

Genetic diversity of E. chinense inferred from microsatellite markers

The 10 polymorphic microsatellite loci were amplified from 54 individuals collected from the four natural populations (BG, YG, HK, and HS) of *E. chinense* in South Korea (Table 1), and the PCR products were then sequenced and analyzed to elucidate the level of genetic diversity and the pattern of genetic structure of *E. chinense*. Fig. 3a shows the microsatellite diversity indices related to genetic diversity for the four *E. chinense* populations. The number of observed alleles per locus (N_A) varied greatly among loci, from 14 (ECHm41) to 29 (ECHm36). The number of effective alleles per locus (N_E) ranged from 5.377 (ECHm24) to 11.858 (ECHm40), with the mean of 9.495. Overall, the effective number of alleles ($N_E = 9.495$) was smaller than the mean number of alleles ($N_A = 13.4$). The expected mean heterozygosity (H_E) per locus ranged from 0.793 (ECHm24) to 0.913 (ECHm40), while the average value was 0.883. The observed heterozygosity per locus (H_O) was from 0.533 (ECHm24) to 0.969 (ECHm40), with a mean value of 0.806. Thus, the expected heterozygosity ($H_E = 0.883$) was larger than the observed heterozygosity ($H_O = 0.806$). The average of the genetic differentiation coefficient F_{ST} was estimated relatively low to be 0.038; it ranged from 0.020 (ECHm40) to 0.051 (ECHm27). Table S5 provides more detail on the genetic diversity estimates within each of the four populations (BG, YG, HK, and HS) of *E. chinense*.

Genetic structure of E. chinense based on microsatellite markers

According to Table S6, the estimated F_{ST} values, showing the degree of genetic differentiation among pairs of populations, were statistically significant ($P < 0.05$) only between the populations BG and HS ($F_{ST} = 0.120$) and HK and HS ($F_{ST} = 0.0184$). In contrast, pairwise R_{ST} was not significant among populations. Generally, pairwise genetic differentiation among populations was low, indicating that the *E. chinense* populations were not genetically differentiated from each other.

The distributions of molecular genetic variation among and within populations, as estimated by AMOVA, are shown in Table S7; only 0.85% of the total molecular variation was attributed to interpopulation differentiation, whereas 86.40% of the variation was within populations. This supports the conclusion that significant genetic differences did not exist among the four examined *E. chinense* populations.

Based on microsatellite markers, the 54 *E. chinense* individuals were further examined to determine population stratification. The optimal number of clusters, K , according to the method of Evanno et al. (2005)¹⁶, was 2 (Fig. 3b). However, with this method, it is only possible to infer with confidence about clusters ≥ 2 . Indeed, the LnP (D) plot shows a strong drop off in model fit only after $K=2$ (data not shown); thus, the ancestry proportions were plotted for each individual given two clusters. All individuals were equally admixed (Fig. 3b) across the range, supporting a single genetic cluster. IBD analysis indicated that the pairwise observed genetic differences were not correlated with the pairwise geographical distances among the four examined *E. chinense* populations (Fig. 3c). Consistently, the PCoA results (Fig. 3d) showed that a distinct genetic structure did not exist and that all populations were clustered into a single genetic group. Taken together, the Bayesian clustering approach, IBD analysis, and PCoA provided consistent results that indicated a lack of spatial genetic clustering among the four *E. chinense* populations from South Korea and Japan. These results are coincident with the metapopulation of *E. chinense* through recent expansion, as inferred from *COI* haplotypes (Figs 1 and 2).

Discussion

The land snail *E. chinense* inhabits on the shoreline adjacent to areas of brackish water and prefers to dwell in salt grass colonies, which are distributed widely across northwestern Pacific coastal areas including those in South Korea, southern Japan, Hong Kong, and southern China. Over recent decades, population numbers have decreased sharply due to coastal development, tidal flat reclamation, and salt marsh destruction. *E. chinense* is also known to be a group of organisms that are particularly more susceptible to human activities than other counterparts of marine ecosystems¹⁷. Consequently, *E. chinense* is currently designated as an endangered species II class by the Ministry of Environment in the South Korean government, and a vulnerable species by the Japanese government and IUCN; thus, it has been under continuous management at both national international levels¹⁷.

The phylogenetic and population genetic analyses of *E. chinense* conducted here based on *COI* and microsatellite markers consistently showed the absence of any distinct genetic structure with which to distinguish populations; this may be attributable to their larvae being able to easily cross oceanic barriers via Kuroshio warm currents¹⁸. It is most likely that *E. chinense* exhibits typical metapopulation characteristics, which are commonly observed in other marine organisms¹⁹: 1) high haplotype diversity but low nucleotide diversity, 2) lack of genetic structure along populations, and 3) low genetic differentiation (frequent genetic flows between populations). Given the general metapopulation characteristics, it is reasonable to conclude that *E. chinense* populations inhabiting South Korea and Japan are a single broad metapopulation that has arisen due to repeating extinctions and migrations caused by continuous and rapid changes in environmental and habitat conditions during the Late-Middle and Late Pleistocene (Figs 4b and S5).

As shown in Fig. 4a, a part of the Kuroshio warm current passing through Japan diverges near Haenam to the south and west coasts of the Korean Peninsula. Thus, it is assumed that *E. chinense* larvae spread

east along the southern coast and north along the western coast according to the influence of the Kuroshio warm currents. In summary, our results strongly suggest that South Korean and Japanese populations of *E. chinense* possess metapopulation characteristics associated with Late-Middle and Late Pleistocene expansions (Fig. 4b), and that all northwestern Pacific *E. chinense* affected by passes of the Kuroshio warm current are likely to be a single, large metapopulation (Fig. 4a). However, our study is limited because we included only one *E. chinense* sample from Japan and did not analyze samples from southern China and Hong Kong given the difficulties in international collection of endangered species. Therefore, we cannot conclusively state that northwestern Pacific *E. chinense* from the Korean Peninsula, southern Japan, southern China, and Hong Kong exhibit overall metapopulation dynamics. Indeed, further research is required in which additional *E. chinense* samples from southern Japan, southern China, and Hong Kong are collected and comprehensive analyzed.

According to the results of our molecular clock analysis based on 58 *COI* haplotypes, the first appearance of the subfamily Ellobiinae including *E. chinense* was estimated to be at ca. 58.0 mya (Figs 4 and S5) around the Paleocene-Eocene Thermal Maximum (PETM; 55 mya) when a massive release of CO₂ caused by ocean volcanic activities that abruptly raised earth's temperature²⁰. Due to the PETM, the struggle for life resulted in evolutionary adaptations that had led to the birth of a number of new animal groups. The PETM was immediately succeeded by the Eocene Optimum, verdant and productive period covered with luxuriant vegetation²¹. Considering the speculation, it is convinced that the origin of Ellobiinae is most likely to have first appeared at the Eocene Optimum (called 'Dawn of the New') immediately after the PETM, given both divergence time estimation error ranges and well-known rapid mutation rate of mitochondrial genes. As shown in Fig. 4, the A-A-Ĝ-A group diverged from the ancestral type A-A-A-A 0.85 mya, Ĝ-A-Ĝ-A from A-A-Ĝ-A 0.61 mya, and then G-Ĝ-G-Ĝ from Ĝ-A-Ĝ-A 0.55 mya. Considering global temperature related to sea level changes over the past 0.85 mya, it is likely that augmentation of *COI* haplotype diversity, i.e., the number of branch bifurcations, is congruent with interglacial periods exhibiting relatively higher global temperatures and sea levels. The divergence time between the ancestral A-A-A-A group and the other derived groups was estimated at 0.85 mya, which corresponds to the end of the global early Pleistocene cold episode (1.7–0.85 mya), which was succeeded by the appearance of warm sea currents in East Asia, the South China Sea, North Pacific Ocean, and Japanese islands²². After the Mid-Pleistocene Transition (1.25-0.77 mya), which is also known as the Mid-Pleistocene Revolution, divergences of Ĝ-A-Ĝ-A (0.61 mya) and G-Ĝ-G-Ĝ (0.55 mya) also occurred during interglacial periods (EPICA community members, 2004), as shown in Fig. 4B. In particular, explosive augmentation of *COI* haplotype numbers and demographic population expansion, as expected from the negative values of Tajima's *D* and Fu's *F_S* neutrality tests in the present study, may have occurred largely during the Late-Middle Pleistocene (i.e., the Late Chibanian Age; 0.350–0.126 mya) and Late Pleistocene (i.e., the Tarantian Age; 0.126–0.012 mya), and might have continued to the Last Glacial Maximum (0.027–0.002 mya) (Fig. 4b).

Intriguingly, we found that four adenine-to-guanine transition hotspots at the 207, 282, 354, and 420 sites of the *COI* haplotype alignment of *E. chinense* could determine the production of genetic groups in the

species, regardless of geographical distribution. Based on our PCoA, it seems possible to divide the *COI* haplotypes into five different genetic groups within a single metapopulation of *E. chinense*. The largest group, A-A-A-A, which is plausibly ancestral, includes 35 of the 58 haplotypes; the most derived group, G-G-G-G, consists of only 8 haplotypes. Despite a few G→A transitions, such as at the 216, 372, and 429 sites, purine base transitions seem to be biased to unidirectional A→G transitions, including the four hotspot sites and two other sites, 120 and 183. In particular, the unidirectional A→G transition events are shown in the serial and stepwise transitions of the four hotspots in *COI*. Such a strong unidirectional tendency can produce guanine richness under plausible positive selection pressure. For example, a positive selective bias from adenine-to-guanine transition has recently been characterized in soft-bodied cephalopods by Moldovan et al. (2020)²³. These authors suggested that edited adenines tend to be substituted to guanines, and this tendency is supported by positive selection at highly edited sites. Furthermore, they suggest that such A→G transition bias can yield beneficial effects of increased phenotypic diversity in a low-polymorphic population, which can enhance adaptation, and facilitate the evolutionary process. Similar observations were recently reported in *Drosophila* and human mRNA editing sites²⁴. Therefore, guanine-rich DNA sequences, resulting from the serial and stepwise A→G transitions reported here, could increase phenotypic diversity in a low-polymorphic metapopulation such as that of *E. chinense*, which might have enhanced the possibility of adaptation in terrestrial and brackish water areas as the species passed through repeated long glacial and relatively short interglacial periods during the Late-Middle Pleistocene and Late Pleistocene (Fig. 4b).

We found strong covariance among the four guanine hotspot sequences in *COI*, each of which may link respectively to a putative G-quadruplex motif. Guanine-rich sequences are known to be capable of producing noncanonical conformations of G-quadruplexes (also known as G-tetrads or G4) in DNA or RNA sequences²⁵. In the nuclear genome, G-quadruplex motifs have been associated with genome instability and gene expression defects; however, they are mainly recognized as regulatory structures that control gene expression or positively affect telomere capping²⁶. Recent studies²⁷ have reported that G-quadruplex structures can form in the mitochondrial genome and even on the CDS in protein-coding genes²⁸. Although G-quadruplexes typically have harmful effects, those formed in mitochondrial genomes can help in the response to certain environmental stresses such as oxidative stress²⁹. Deficiency in *COI* within mitochondria caused by G-quadruplexes could result in positive selection bias that reduces the production of reactive oxygen leading to less oxidative damage and a selective advantage during competition with other mitochondria within the same cell, which could generate homoplasmy for *COI* deficiency. Hershman et al. (2008)³⁰ suggested that cells with cytochrome c oxidase deficiency are apoptosis resistant and therefore more likely to survive. Active *COI* oxidizes cytochrome c, which activates pro-caspase 9 leading to apoptosis. Assuming that the four guanine hotspots link to create four G-quadruplex structures, they may function in the regulation (mainly inhibition) of *COI* expression at the transcription or translation levels, and in modulating (mainly reducing) the concentration of activated *COI* within the mitochondria. This would reduce oxidative damage and increase resistance to apoptosis with lower reactive oxygen production. Such G-quadruplex conformation-related oxidative stress and apoptosis resistance could provide a selective advantage in

competition with other mitochondria in a cell. According to a previous study on the energy metabolism of the garden snail *Helix aspersa*, natural selection can reduce energy metabolism in a land snail³¹. Intriguingly, the slow behaviors of land snails that have led to reduced energy consumption might be associated with downregulation of *COI* expression modulated by G-quadruplex motifs on the CDS of *COI* (as we hypothesized; see Fig. 2).

Determining the genetic diversity and population genetic structure of isolated and threatened species is of great importance when planning a suitable conservation strategy³², especially for the selection of appropriate populations and management methods aimed at maintaining genetic variation. Highly diverse populations can be targeted for protection while small and depleted populations can be subjected to management actions that restore their diversity. For example, enhancement or reinforcement plans aim to add individuals to existing populations while reintroduction plans aim to re-establishment a species within its natural habitat where it has become extinct³³. From the present study, the genetic resources of the endangered *E. chinense* could be essential for successfully designing conservation strategies aimed at preserving its population and restoring its habitat. In research into endangered species, such as *E. chinense*, permission from local and national governments must be acquired, via strict evaluation processes, prior to sample collection, which makes it difficult to conduct further research and collect samples. Moreover, direct collection of endangered species from foreign countries is largely prohibited by law, which explains the rareness, to date, of international-scale sample collections, and related research. Considering the difficulty in performing population genetic, phylogenetic, and demographical research on endangered species, the present study, which includes a well-organized research model, has great value for the global protection of *E. chinense*.

Declarations

Acknowledgements

This research was financially supported by National Institute of Biological Resources, Ministry of Environment, Republic of Korea (granted to U.W.H.; no. NIBR202005202).

Author Contributions

U.W.H. conceived the study. C.R.S., E.H.C., S.Y.B., J.J., and U.W.H. designed and set up the experiment. C.R.S., B.P., H.J.K., H.K.O., S.H.K., J.R.L., S.J.S., and D.M.P. performed the sample collection, C.R.S., E.H.C., G.K., J.H., H.Y.S., Y.S.L., Y.S.L., and U.W.H. analyzed the data. C.R.S., E.H.C., G.K., S.Y. and U.W.H. wrote the paper.

Additional information

The sequence data obtained here are publicly available from NCBI GenBank under the accession numbers mentioned in the Materials and Methods. The sequence alignment data sets of *COI* haplotypes, the result of covariate calculation, and the raw data of plausible G-quadruplex sequence motifs are available at Dryad Digital Repository, <https://doi.org/10.5061/dryad.fbg79cnt4>.

References

1. Pfeiffer, L. Descriptions of seven new species of land shells from the collection of H. Cuming, Esq. *Zool. Soc. Lond.* **32**, 603–605 (1864).
2. Yoo, J. S. Korean shells in colour. 1-196 (IlJiSa Publishing Co., 1976).
3. Ministry of Oceans and Fisheries. Conservation and management of marine ecosystems act. Korea Ministry of Government Legislation. Retrieved from <https://www.law.go.kr/LSW/eng/engLsSc.do?menuId=2&query=CONSERVATION+AND+MANAGEMENT+OF+MARINE+ECOSYS-TEMS+ACT#liBgcolor1> (2017).
4. Mable, B. K. Conservation of adaptive potential and functional diversity: integrating old and new approaches. *Genet.* **20**, 89–100 (2019).
5. Lande, R. Genetics and demography in biological conservation. *Science* **241**, 1455–1460; 1126/science.3420403 (1988).
6. Wang, Y. *et al.* The genetic diversity and population structure of *Sophora alopecuroides* (Faboideae) as determined by microsatellite markers developed from transcriptome. *PLoS one* **14**, e0226100; 10.1371/journal.pone.0226100 (2019).
7. Baek, S. Y. *et al.* *COI*-based genetic structure of an exotic snapping turtle *Chelydra serpentina* imported to South Korea. *Syst. Evol. Diversity* **36**, 354-362; 10.5635/ASED.2020.36.4.043 (2020).
8. Kim, G. *et al.* The complete mitochondrial genome of an Asian crested ibis *Nipponia nippon* (Pelecaniformes, Threskiornithidae) from South Korea. *Mitochondrial DNA* **4**, 3707-3708 (2019).
9. Choi, E. H. *et al.* Development and characterization, and application of ten polymorphic microsatellite markers in the crested ibis *Nipponia nippon* from South Korea. *Syst. Evol. Diversity* **36**, 154-158; 10.5635/ASED.2020.36.2.029 (2020).
10. Spiegel, J., Adhikari, S. & Balasubramanian, S. The structure and function of DNA G-quadruplexes. *Trends Chem.* **2**, 123–136; 10.1016/j.trechm.2019.07.002 (2019).
11. Endoh, T. & Sugimoto, N. Mechanical insights into ribosomal progression overcoming RNA G-quadruplex from periodical translation suppression in cells. *Rep.* **6**, 22719; 10.1038/srep22719 (2016).
12. Rogers, A. R. & Harpending, H. Population growth makes waves in the distribution of pairwise genetic differences. *Biol. Evol.* **9**, 552–569 (1992).
13. Slatkin, M. & Hudson, R. R. Pairwise comparisons of mitochondrial DNA sequences in stable and exponentially growing populations. *Genetics* **129**, 555–562.

14. Excoffier, L. Patterns of DNA sequence diversity and genetic structure after a range expansion: lessons from the infinite-island model. *Ecol.* **13**: 853–864; 10.1046/j.1365-294x.2003.02004.x (2004).
15. Ray, N., Currat, M. & Excoffier, L. Intra-deme molecular diversity in spatially expanding populations. *Biol. Evol.* **20**, 76–86; 10.1093/molbev/msg009 (2003).
16. Evanno, G., Regnaut, S. & Goudet, J. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Ecol.* **14**, 2611–2620; 10.1111/j.1365-294x.2005.02553.x (2005).
17. Lim, S. Y, Lee, C. S., Kim, M. S. & Yoo, S. H. The conservation value of endangered marine species: the case of the *Ellobium chinense*. *Korean Soc. Mar. Environ.* **21**, 645–654; 10.7837/kosomes.2015.21.6.645 (2015).
18. Kim, M. C. State of legally protected species from freshwater or estuary in Korea. *Conserv.* **175**, 23–29 (2016).
19. Peng, X. *et al.* Genetic diversity and metapopulation structures of two intertidal species along the coast of Zhejiang, China: implications for conservation. *Mitochondrial DNA* **30**, 674–681; 10.1080/24701394.2019.1616702 (2019).
20. Wieczorek, R., Fantle, M. S., Kump, L. R. & Ravizza, G. Geochemical evidence for volcanic activity prior to and enhanced terrestrial weathering during the Paleocene Eocene Thermal Maximum. *Cosmochim. Acta* **119**, 391–410; 10.1016/j.gca.2013.06.005 (2013).
21. Hyland, E. G. & Sheldon, N. D. Coupled CO₂-climate response during the Early Eocene Climatic Optimum. *Palaeoclimatol. Palaeoecol.* **369**, 125–135; 10.1016/j.palaeo.2012.10.011 (2013).
22. Kang, S., Lim, D. & Kim, S. Benthic foraminiferal assemblage of Seogwipo Formation in Jeju Island, South Sea of Korea: Implication for late Pliocene to early Pleistocene cold episode in the northwestern Pacific margin. *Int.* **225**, 138-146; 10.1016/j.quaint.2010.04.009. (2010).
23. Moldovan, M. A., Chervontseva, Z. S., Bazykin, G. A. & Gelfand, M. S. Adaptive evolution at mRNA editing sites in soft-bodied cephalopods. **8**, e10456. 10.7717/peerj.10456 (2020).
24. Popitsch, N. *et al.* A-to-I RNA editing uncovers hidden signals of adaptive genome evolution in animals. *Genome Biol. Evol.* **12**, 345–357; 10.1093/gbe/evaa046A (2020).
25. Falabella, M., Fernandez, R. J., Johnson, B. & Kaufman, B. A. Potential roles for G- quadruplexes in mitochondria. *Med. Chem.* **26(16)**, 2918–2932; 10.2174/0929867325666180228165527 (2018).
26. Smith, J. S. *et al.* Rudimentary G-quadruplex-based telomere capping in *Saccharomyces cerevisiae*. *Struct. Mol. Biol.* **18**, 478–485; 10.1038/nsmb.2033 (2011).
27. Byrd, A. K. *et al.* Evidence that G-quadruplex DNA accumulates in the cytoplasm and participates in stress granule assembly in response to oxidative stress. *Biol. Chem.* **291**, 18041–57; 10.1074/jbc.M116.718478 (2016).
28. Dong, D. W. *et al.* Association of G-quadruplex forming sequences with human mtDNA deletion breakpoints. *BMC Genomics* **15**, 677 (2014).

29. Falabella, M. *et al.* G-quadruplex dynamics contribute to regulation of mitochondrial gene expression. *Rep.* **9**, 5605; [10.1038/s41598-019-41464-y](https://doi.org/10.1038/s41598-019-41464-y) 1 (2019).
30. Hershman, S. G. *et al.* Genomic distribution and functional analyses of potential G- quadruplex-forming sequences in *Saccharomyces cerevisiae*. *Nucleic Acids Res.***36**, 144–156; [1093/nar/gkm986](https://doi.org/10.1093/nar/gkm986) (2008).
31. Artacho, P. & Nespolo, R. F. Natural selection reduces energy metabolism in the garden snail, *Helix aspersa* (*Cornu aspersum*). *Evolution*, **63**, 1044-1050; [10.1111/j.1558-5646.2008.00603.x](https://doi.org/10.1111/j.1558-5646.2008.00603.x). (2009).
32. Perina, A. *et al.* Assessment of genetic diversity and population structure of the common littoral shrimp *Palaemon serratus* (Pennant, 1777) by microsatellites: towards a sustainable management. *Aquatic Conserv. Mar. Freshw. Ecosyst.* **29**, 528–536; [1002/aqc.3011](https://doi.org/10.1002/aqc.3011) (2019).
33. IUCN/SSC. *Guidelines for re-introductions*. IUCN species survival commission. Gland and Cambridge: IUCN. (1998).
34. Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mar. Biol. Biotechnol.* **3**, 294–299 (1994).
35. Hall, T. A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **41**, 95–98; [14601/Phytopathol_Mediterr-14998u1.29](https://doi.org/10.14601/Phytopathol_Mediterr-14998u1.29) (1999).
36. Hyun, Y. S. *et al.* Isolation and characterization of 20 polynucleotide microsatellite markers in a vulnerable Korean snail, *Ellobium chinense*, using 454 pyrosequencing. *Genes & Genomics***39**, 155-160. [10.1007/s13258-016-0482-7](https://doi.org/10.1007/s13258-016-0482-7) (2017).
37. Schuelke, M. An economic method for the fluorescent labeling of PCR fragments. *Biotechnol.***18**, 233–234; [10.1038/72708](https://doi.org/10.1038/72708) (2000).
38. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.***24**, 4876– 4882 (1997).
39. Librado, P. & Rozas, J. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *J.* **25**, 1451–1452 (2009)
40. Excoffier, L. & Lischer, H. E. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Ecol. Resour.* **10**, 564–567; [10.1111/j.1755-0998.2010.02847.x](https://doi.org/10.1111/j.1755-0998.2010.02847.x) (2010).
41. Tajima, F. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123**, 585–595 (1989).
42. Fu, Y. X. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* **147**, 915–925 (1997).
43. Bryant, D. & Moulton, V. Neighbor-net: an agglomerative method for the construction of phylogenetic networks. *Biol. Evol.* **21**, 255–265; [10.1093/molbev/msh018](https://doi.org/10.1093/molbev/msh018) (2004).

44. Clement, M., Posada, D. & Crandall, K. A. TCS: a computer program to estimate gene genealogies. *Ecol.* **9**, 1657–1659; 10.1046/j.1365-294x.2000.01020.x (2000).
45. Perrier, X. & Jacquemoud-Collet, J. P. DARwin software, available at, <http://darwin.cirad.fr/Darwin> (2006).
46. Peakall, R. & Smouse, P. E. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Ecol. Notes* **6**, 288–295; 10.1111/j.1471-8286.2005.01155.x (2006).
47. Wright, S. The genetical structure of populations. *Eugen.* **15**, 323–354; 10.1111/j.1469-1809.1949.tb02451.x (1951).
48. Pritchard, J. K. et al. Inference of population structure using multilocus genotype data. *Genetics* **155**, 945–959 (2000).
49. Earl, D. A. & vonHoldt, B. M. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv. Genet. Resour.* **4**, 359–361; 1007/s12686-011-9548-7 (2012).
50. Bird, C. E., Karl, S. A., Mouse, P. E. & Toonen, R. J. Detecting and measuring genetic differentiation (eds. Held, C., Koenemann, S. & Schubart, C. D.) *Crustacean Issues: Phylogeography and Population Genetics in Crustacea*. CRC: Boca Raton: Florida. 26 (2011).
51. Frees, S., Menendez, C., Crum, M. & Bagga, P. S. QGRS-Conserve: a computational method for discovering evolutionarily conserved G-quadruplex motifs. *Genom.* **8**, 8; 10.1186/1479-7364-8-8 (2014).
52. Bouckaert, R. et al. BEAST2: a software platform for Bayesian evolutionary analysis. *PLoS Comput. Biol.* **10(4)**, e1003537; 1371/journal.pcbi.1003537 (2014).
53. Weigand, A. M. et al. Evolution of microgastropods (Ellobioidea, Carychiidae): integrating taxonomic, phylogenetic and evolutionary hypotheses. *BMC Evol. Biol.* **13**, 18; 1186/1471-2148-13-18 (2013).
54. Romero, P. E., Pfenninger, M., Kano, Y. & Klussmann-Kolb, A. Molecular phylogeny of the Ellobiidae (Gastropoda: Panpulmonata) supports independent terrestrial invasions. *Phylogenet. Evol.* **97**, 43–54; 10.1016/j.ympcv.2015.12.014 (2016).
55. Darriba, D., Taboada, G. L., Doallo, R. & Posada, D. jModelTest 2: more models, new heuristics and parallel computing. *Methods* **9**, 772 (2012).
56. Rambaut, A., Drummond, A. J., Xie, D., Baele, G. & Suchard, M. A. Posterior summarisation in Bayesian phylogenetics using Tracer 1.7. *Biol.* **67**, 901-904; 10.1093/sysbio/syy032 (2018).
57. Rambaut, A. & Drummond, A. J. TreeAnnotator v.2.3.0., available at, <http://beast2.org> (2014).
58. Rambaut, A. FigTree v.1.4., available at, <http://tree.bio.ed.ac.uk/software/figtree/> (2012).

Tables

Table 1. List of collection sites and the number of individuals of *Ellobium chinense* with genetic markers applied to each of the nine populations in South Korea and Japan.

| Populations | Collection sites | N | Genetic markers | | References |
|-------------|---|----|-----------------|-----------------|---------------------------|
| | | | COI | Microsatellites | |
| SC | Seo-myeon, Seocheon-gun, Chungnam, South Korea | 5 | ✓ | | Yi <i>et al.</i> (2019) |
| BG | Byeonsan-myeon, Buan-gun, Jeonbuk, South Korea | 16 | ✓ | | This study |
| | | | | ✓ | |
| | Simwon-myeon, Gochang-gun, Jeonbuk, South Korea | 5 | ✓ | | Yi <i>et al.</i> (2019) |
| YG | Nagwol-myeon, Yeonggwang-gun, Jeonnam, South Korea | 10 | | | This study |
| | | | ✓ | | |
| | Hongnong-eup, Yeonggwang-gun, Jeonnam, South Korea | 19 | ✓ | | This study |
| HK | Chillyang-myeon, Gangjin-gun, Jeonnam, South Korea | 11 | ✓ | | This study |
| | | | | ✓ (10)* | |
| | Bukpyeong-myeon, Haenam-gun, Jeonnam, South Korea | 5 | ✓ | | Yi <i>et al.</i> (2019) |
| JB | Anyang-myeon, Jangheung-gun, Jeonnam, South Korea | 1 | | | Kang <i>et al.</i> (2018) |
| | | | ✓ | | |
| | Hoecheon-myeon, Boseong-gun, Jeonnam, South Korea | 1 | ✓ | | This study |
| BY | Beolgyo-eup, Boseong-gun, Jeonnam, South Korea | 10 | | | This study |
| | | | ✓ | | |
| | Hwayang-myeon, Yeosu-si, Jeonnam, South Korea | 5 | ✓ | | Yi <i>et al.</i> (2019) |
| GY | Gwangyang-eup, Gwangyang-si, Jeonnam, South Korea | 9 | | | This study |
| | | | ✓ | | |
| HS | Seopo-myeon, Sacheon-si, Gyeongnam, South Korea | 10 | ✓ | | This study |
| | | | | | |
| | Chukdong-myeon, Sacheon-si, Gyeongnam, South Korea | 9 | ✓ | | This study |
| | Yonghyeon-myeon, Sacheon-si, Gyeongnam, South Korea | 5 | ✓ | | Yi <i>et al.</i> (2019) |

| | | | | | |
|--------------|---|------------|---|---|-----------------------------|
| | Jingyo-myeon, Hadong-gun, Gyeongnam, South Korea | 10 | ✓ | ✓ | This study |
| | Idong-myeon, Namhae-gun, Gyeongnam, South Korea | 8 | ✓ | ✓ | This study |
| JK | Oita Prefecture, Kyushu Island, Japan | 1 | ✓ | | Romero <i>et al.</i> (2015) |
| Total | | 140 | | | |

*Only 10 samples out of 11 were employed. 'N' indicates the number of individuals.

Table 2. The result summary of genetic diversity analyses and neutrality tests performed with 140 *COI* sequences from *Ellobium chinense* along the nine populations in South Korea and Japan.

| Populations | N | N _h | N _p | <i>h</i> | π | S | k | Tajima's <i>D</i> | Fu's <i>F_s</i> |
|-------------|-----|----------------|----------------|----------|---------|----|-------|-------------------|---------------------------|
| SC | 5 | 5 | 3 | 1.000 | 0.01042 | 13 | 6.200 | -0.04655 | -1.01140 |
| BG | 21 | 15 | 4 | 0.938 | 0.00749 | 22 | 4.457 | -1.02706 | -6.82701** |
| YG | 29 | 16 | 10 | 0.924 | 0.00912 | 31 | 5.429 | -1.13813 | -4.06280* |
| HK | 17 | 14 | 7 | 0.971 | 0.00882 | 25 | 5.250 | -1.16767 | -6.54343** |
| JB | 2 | 2 | 0 | 1.000 | 0.00840 | 5 | 5.000 | nd | nd |
| BY | 15 | 9 | 3 | 0.933 | 0.00791 | 17 | 4.705 | -0.29535 | -1.29300 |
| GY | 9 | 7 | 4 | 0.944 | 0.00952 | 17 | 5.667 | -0.45784 | -0.94084 |
| HS | 42 | 21 | 11 | 0.926 | 0.00890 | 37 | 5.296 | -1.33327 | -6.84025** |
| JK(Japan) | 1 | 1 | nd | nd | nd | nd | nd | nd | nd |
| Total/Mean | 140 | 58 | 42 | 0.939 | 0.00865 | 70 | 5.137 | -1.88691* | -2.75886 |

Diversity parameters are given for each locality: N = the number of *COI* sequences (individuals), N_h = the number of haplotypes, N_p = the number of private haplotypes, *h* = haplotype diversity, π = Jukes-Cantor corrected estimates of nucleotide diversity, S = the number of segregation sites, K = the average number of pairwise nucleotide differences, and 'nd' = not determined. Statistically significant values are written in bold: *P < 0.05 and **P < 0.01. The localities of the populations refer to Table 1 and Figure 1.

Figures

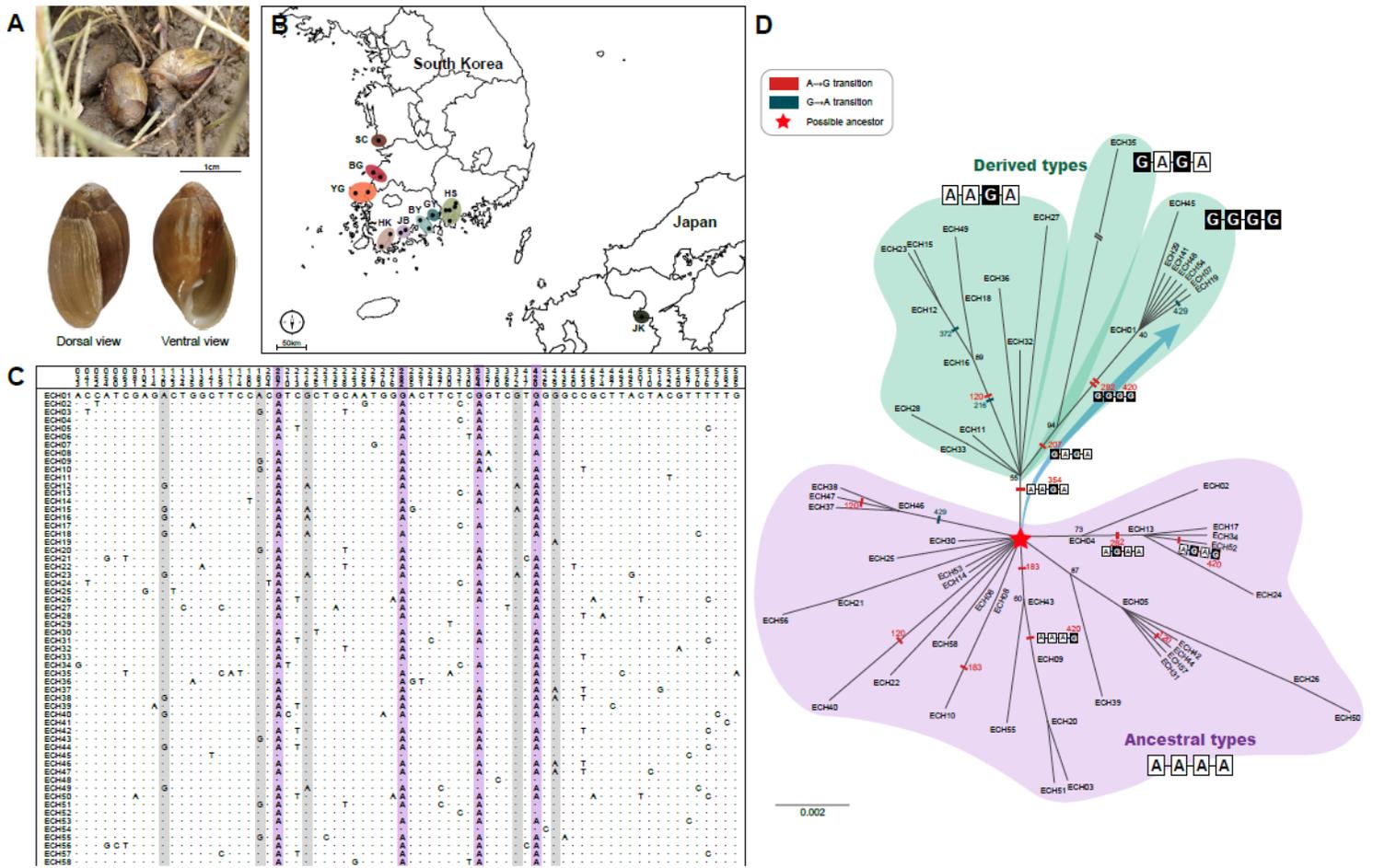


Figure 1

Photographs, sampling localities, polymorphic sites inferred from COI haplotypes, and an unrooted maximum likelihood tree for *Ellobium chinense* inhabiting the northwestern Pacific coast (South Korea and Japan). (a) A salt grass habitat of *E. chinense* (Hadong, Gyeongnam, South Korea; taken in July 2020), and dorsal and views of the snail's shell. Refer to Fig. S1 for the full landscape of the habitats. The scale bar indicates 1 cm. (b) The collection sites of *E. chinense* (marked with black dots) grouped into nine possible populations (colored circles). Full details are given in Table 1. (c) Alignment of 71 polymorphic sites inferred from the alignment of 58 COI haplotype sequences (Fig. S2) from 140 *E. chinense* individuals; 31 sites are parsimoniously informative. The four light pink columns indicate the four unidirectional stepwise A→G transition hotspots, which could be key sequences for haplotype classification. The five sites partially colored with light aquamarine are additional A→G transitions, with a few exceptional G→A transitions. Overall, guanine-rich COI sequences were evident, likely were caused by unidirectional, biased, A→G transitions during the evolution of *E. chinense*. (d) An unrooted maximum likelihood tree reconstructed with 58 COI haplotypes. Four different genetic groups are represented by four guanine hotspots: A-A-A-A, A-A-G-A, G-A-G-A, and G-G-G-G. These are depicted with white boxes for A and black boxes for G. The tendency for unidirectional, stepwise, A→G transitions from a plausible ancestor, A-A-A-A (pink star), to the most derived type, G-G-G-G, are shown with a large blue arrow. The additional A→G transitions and exceptional G→A transitions are marked with red and blue boxes,

respectively. Node confidence values near nodes are depicted with bootstrapping values in percent. Refer to Fig. S3 for a rooted maximum likelihood tree.

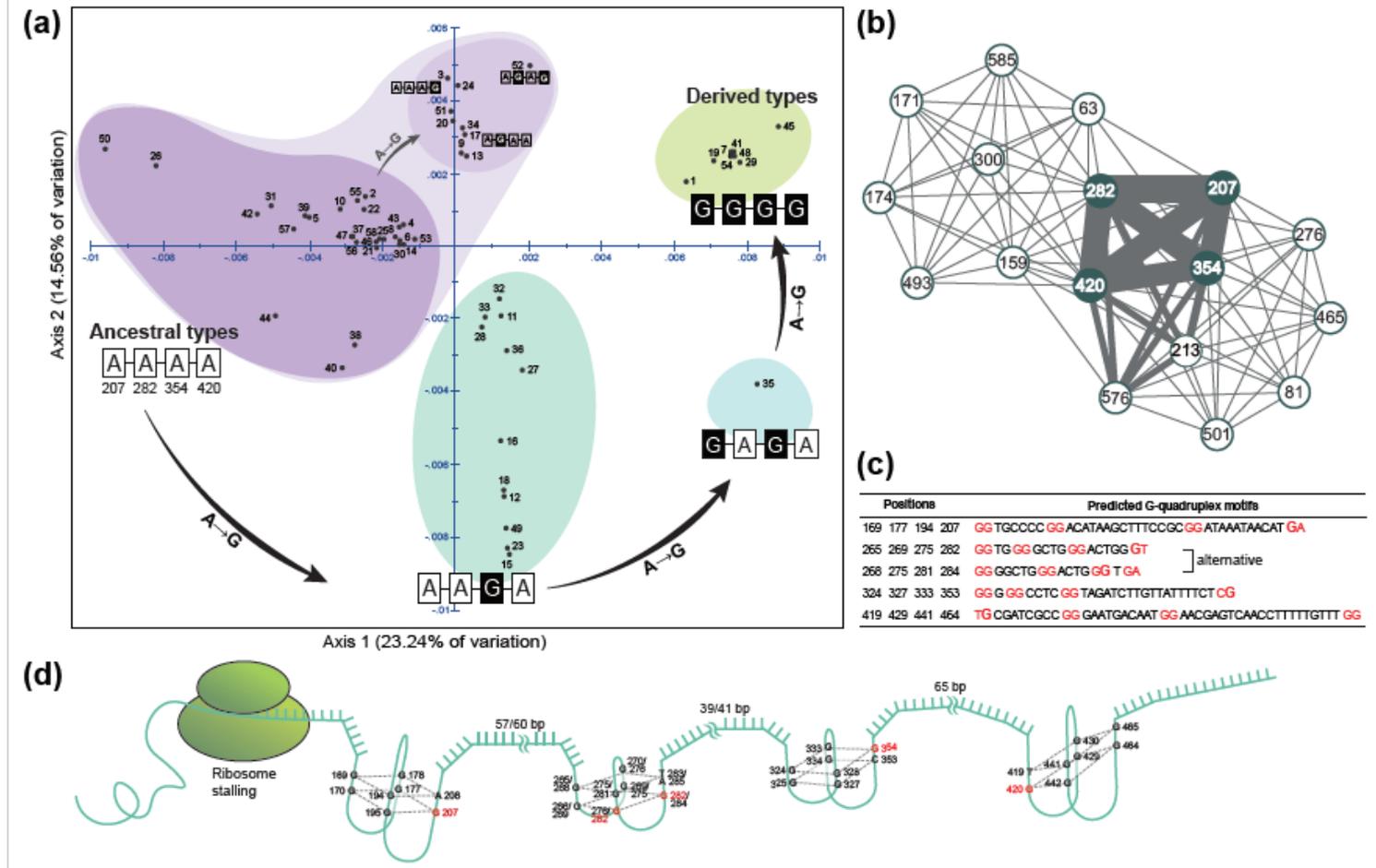


Figure 2

Results of principal coordinate analysis (PCoA), covariance of the four guanine hotspots, and plausible G-quadruplex motifs inferred from 58 COI haplotypes found in northwestern Pacific *Ellobium chinense*; hypothetical ribosome stalling caused by four plausible consecutive G-quadruplex structures in the mRNA of *E. chinense* COI. (a) Results of PCoA. Representation of the scores on the first two axes (Axis 1 = 23.33%; Axis 2 = 14.68%) from the matrix of genetic distances estimated with the 58 COI haplotypes. Refer to Table 1 for the number of individuals along with the collection site references of the haplotypes; refer to Figs 1C and S2 for sequence alignment of the 58 COI haplotypes. The colored circles indicate four or five genetic groups regardless of geographical distributions. The black arrows indicate unidirectional, stepwise, A→G transitions (b) Covariance test results for the four adenine/guanine hotspots that may be linked to the four guanine hotspots inferred from 58 COI haplotypes shown in Figs 1c and S2. In addition, a node in the network represented the position of each sequence, a link represented a covariance position, and the link thickness represented covariance frequency. The detailed data are listed in Data S3. (c) Plausible selected G-quadruplex motifs linked to the four adenine/guanine hotspots on COI. For the 282-site-related motif, the two alternatives are suggested. Refer to Data S3 for the raw data from the G-quadruplex sequence motif search in COI. (d) Hypothetical ribosome stalling in the COI translation

process caused by four consecutive G-quadruplex structures with intervals from 39 to 65 bp. Each structure consists of two stacked G-quadruplexes. For the 282-site-linked G-quadruplex structure, two alternative structures are depicted based on the two alternative motif sequences shown in (c).

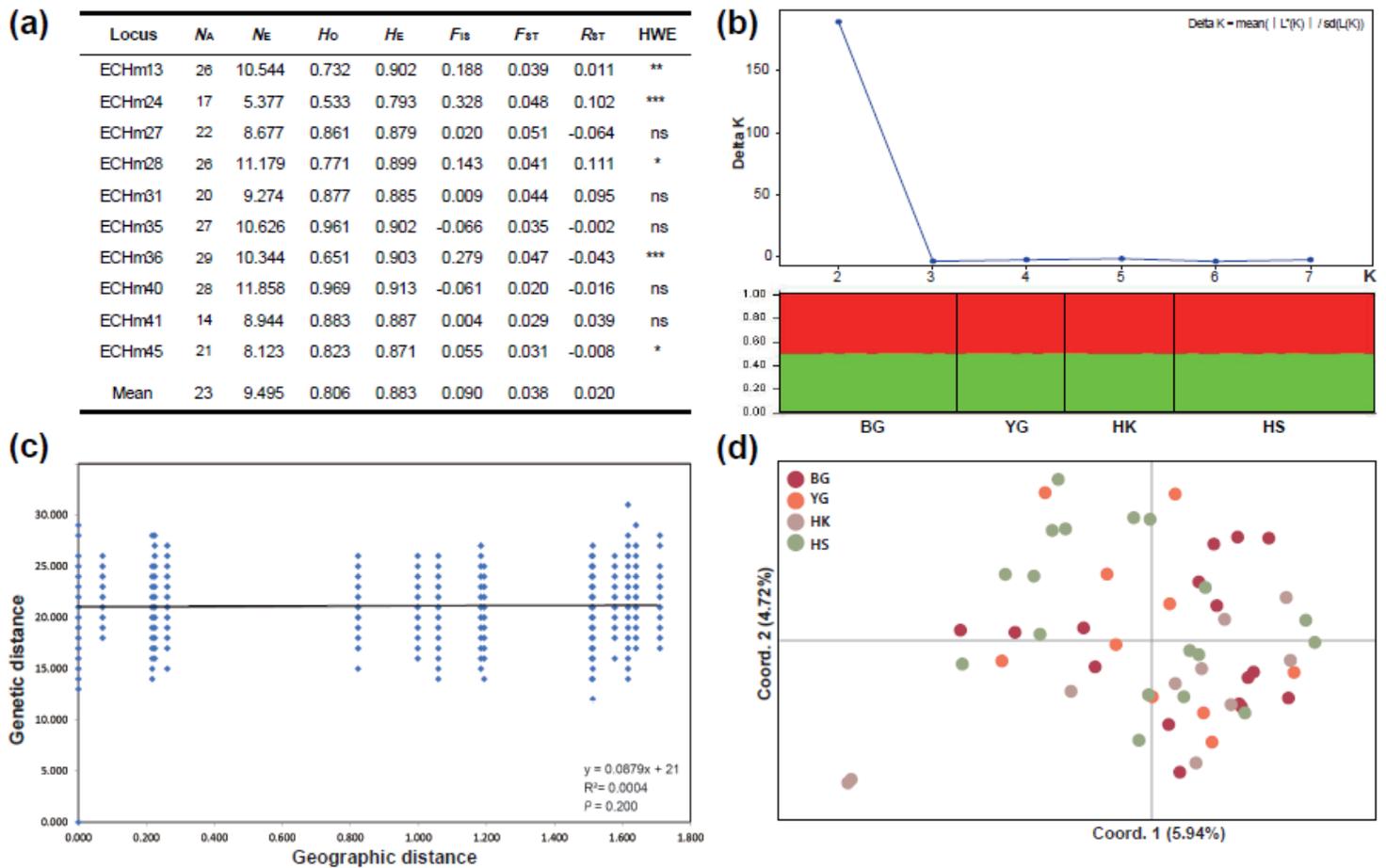


Figure 3

Genetic diversity, population genetic structure, isolation by distance (IBD), and principal component analysis (PCoA) based on 10 selected microsatellite markers and 54 *Ellobium chinense* individuals collected from South Korea. (a) Summary statistics for the genetic diversity of *E. chinense*. NA: mean number of alleles, NE: number of effective alleles, HO: observed heterozygosity, HE: expected heterozygosity, FIS: inbreeding coefficient (indicates populations with heterozygote deficit); HWE: loci showing a significant departure from Hardy-Weinberg equilibrium with a global test at the 5% level after a sequential Bonferroni correction (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns: not significant). (b) Determination of the true number of genetic clusters ($K=2$) by Bayesian clustering using the method of Evanno et al. (2005)16. K statistics are based on the rate of change in the log probability of data between successive K values. Bayesian clustering results were obtained using the STRUCTURE program48. Refer to Table 1 for information on the four populations: BG, YG, HK, and HS. (c) IBD test results showing that genetic distances are not correlated with geographical distances among the sample collection sites. (d) A two-dimensional plot from the PCoA results showing that a genetic structure did not exist among the four populations of *E. chinense* in South Korea. The percentage variation attributable to the three principle coordinate axes was 10.66% (Axis 1 = 5.94%; Axis 2 = 4.72%).

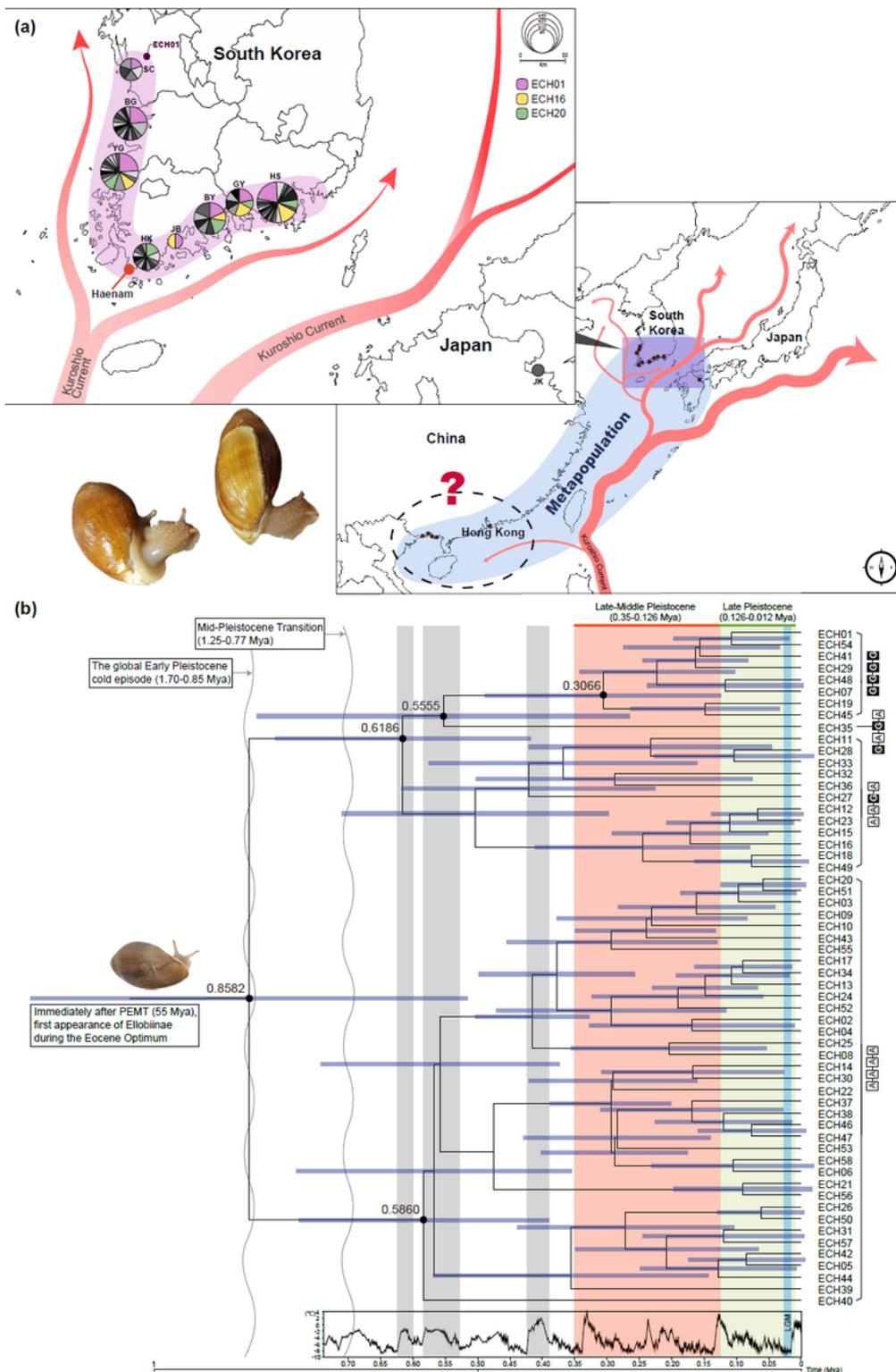


Figure 4

Geographical distribution of 58 COI haplotypes from *Ellobium chinense* in South Korea and Japan, and the results of molecular clock analysis using the haplotypes and BEAST 2.6.0. (a) Geographical distribution of 58 COI haplotypes from *E. chinense* shown on a map of South Korea and Japan. Each haplotype is represented by a pie chart sized in proportion to its frequency in each population. The haplotype ECH01 was observed in all populations. Results show that *E. chinense* from the northwestern

Pacific, including South Korea and Japan, exhibit metapopulation dynamics. Given the effects of the Kuroshio warm current, the southern China and Hong Kong populations could belong to the metapopulation (but this requires confirmation by further research). (b) Molecular clock analysis showing geological times, including the first appearance of the subfamily Ellobiinae (at the Eocene Optimum immediately after the Paleocene–Eocene Thermal Maximum; refer to Fig. S5), the divergence times of the four phylogenetic groups of *E. chinense*, and the periods of explosive population expansion with an increase in COI haplotype diversity [Late-Middle and Late Pleistocene prior to the Last Glacial Maximum (LGM)]. The gray columns mark interglacial periods related to haplotype divergences of *E. chinense* before Late-Middle Pleistocene. Light red and light-yellow boxes mark the periods of rapid population expansions and the number of bifurcations (=increased haplotype diversity), respectively. The four genetic groups, depicted by A-A-A-A, A-A-G-A, G-A-G-A, and G-G-G-G, are explained in detail in Figs 1 and 2. The full BEAST analysis results are presented in Fig. S5. Outgroups and the calibration point are described in detail in the Materials and Methods.

Supplementary Files

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- [Supplementaryinformation1.docx](#)