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A phylogeographic study of the Japanese earthworm, *Metaphire sieboldi* (Horst, 1883) (Oligochaeta: Megascolecidae): Inferences from mitochondrial DNA sequences

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ABSTRACT

To clarify the historical phylogeography of a Japanese earthworm, nucleotide sequence variations of *Metaphaire sieboldi* were investigated with respect to its geographic distribution. Sequencing of mitochondrial DNA (mtDNA), including the cytochrome oxidase subunit I (COI) region and the 16S rDNA region, of 72 samples including 4 outgroup taxa, and phylogenetic analyses using neighbour-joining (NJ) and Bayesian inference (BI) methods indicated that *M. sieboldi* forms a monophyletic group. This monophyletic group was divided into seven subgroups; most subgroups comprised individuals from several areas, except for southern Shikoku and southern Kyushu. The phylogenetic tree demonstrated that the Shikoku and Kinki populations were paraphyletic at the basal node, and individuals from Kyushu and western Shikoku were positioned at the advanced group. Estimated phylogeographic events based on mtDNA analyses include (1) *M. sieboldi* originated in Shikoku and/or Kinki, (2) individuals from Chugoku and Kyushu have a common ancestor from Shikoku, and (3) individuals from western Shikoku originated from Kyushu progenitors (about 0.4–0.5 mya) after separation by rising sea levels.

1. Introduction

Phylogeography, the combined analysis of genealogical and geographic data, has become a powerful tool for inferring historical biogeographic events. Patterns of variation in DNA markers have allowed inferences of past biogeographic events on every geographic scale from continental to local [1,2]. In addition, recent development of molecular techniques has allowed phylogenies to be reconstructed and systematic relationships to be evaluated among species or populations within a given species.

Modern fauna and flora species assemblages have been affected by eradications by glaciers and migration associated with connections and disjunctions of islands resulting from climate changes. After the Japanese archipelago was formed during the early Miocene (approx. 15–20 mya), glaciers did not form during the Quaternary except in alpine areas of northern Japan, thus, across most of Japan, glaciers have not affected the biota [20]. However, climatic changes did dramatically affect Japanese biota by changing the flora; evergreen broad-leaved forests present in warm periods were replaced with deciduous broad-leaved forests or coniferous forests during cold periods [36]. Moreover, land bridges, formed by lowering of the sea level after global cooling, affected the fauna of Japan, as animal populations that were initially divided by the sea could then cross the land bridges. Honshu, Shikoku, and Kyushu islands of Japan were repeatedly connected and divided by the raising and lowering of the sea level during the glacial period; therefore, the fauna and flora of Shikoku were affected by those of neighbouring areas, including the Kinki and Chugoku regions of Honshu, and Kyushu. Studies of fauna in western Japan such as Asian black bear *Ursus thibetanus* [38], sika deer *Cervus nippon* [37] and Japanese salamander *Hynobius boulengeri* [23], have shown that various taxa of Shikoku are partially composed of elements from neighbouring areas.

Earthworms are one of the most important components of the soil biota, and make up the largest contribution to the biomass in the temperate zone. They cannot readily cross seas, rivers, or mountains; therefore, the modes by which they expand their distribution are restricted, and it is uncertain how their present distribution pattern has been formed. The phylogeographic history of earthworms can be inferred from a phylogenetic analysis. Molecular phylogenetic studies of some earthworm groups have

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greatly increased in number (review in Dupont [11]); most of these studies are concerned with closely related species or variations within species [7–10,13,14,18,26] but none yet based on primary type specimens nor considering species' synonyms [5]. Recently, some studies have been conducted on the phylogeography of certain earthworm species in relation to palaeogeography [9,18].

Metaphire sieboldi (Horst, 1883) was the first earthworms described from Japan (its types are in Leiden Museum), it is a striking metallic blue only when adult and is one of the largest of Japanese species at ca. 30 cm [3,4]. This species occurs in extreme southwestern Chubu, Kinki, and the Chugoku (Honshu), as well as on the islands of Shikoku and Kyushu [19,21]. Given this distribution, it is intriguing to consider how such a large earthworm colonized Shikoku despite the apparent barrier to expansion presented by the sea. It is probably that *M. sieboldi* colonized from Honshu into Shikoku though the connected islands because earthworms could not survive in seawater. However, phylogeographic studies of Japanese earthworms have not been conducted to date.

It is clear that both demographic and phylogeographic forces shape the depth and distribution of lineages in a phylogenetic tree. In addition, selecting the correct genes in reconstructing a phylogeny is of importance due to differing evolutionary rates between different DNA regions. The cytochrome oxidase subunit I (COI) gene of mitochondrial DNA (mtDNA) has been extensively used in phylogenetic studies due to ease of primer design and the range of its phylogenetic signal [12]. The rate of evolution of this gene is also sufficiently rapid to allow discrimination at the species level [35], and have been shown to be informative in earthworms [7–9,13,14,18,25,26,29]. However, only COI gene is not enough to discriminate between individuals of *M. sieboldi*. Additionally, sequences of 16S ribosomal DNA (rDNA) of mtDNA are among the most frequently employed markers for phylogenetic analyses and have also been shown to be informative in earthworms [9,16,26]. Substitution rate of 16S rDNA gene was lower than those of COI gene [9,18]. However, some studies indicated that phylogeographic studies of earthworms were conducted using both COI and 16S genes because of accumulating informative characters. Therefore, the goal of this study was to provide the first broad-scale screening of mtDNA variation in *M. sieboldi*, to infer phylogeographic relationships within this species.

2. Materials and methods

2.1. Sampling of M. sieboldi and allied species

To obtain a comprehensive understanding of the underlying historical population structure of *M. sieboldi*, 71 individuals representing 64 populations were sampled from across the geographic range of the species (Table 1). When identical sequences were present in a single locality, one sequence was included and the others were omitted. For use as outgroup taxa, *M. megascolidioides* (Goto and Hatai, 1899), *M.* (now *Duplodicodrilus*) *schmardae* (Horst, 1883), *M. tosaensis* (Ohfuchi, 1938), and *Amynthas vittatus* (Goto and Hatai, 1898) were collected.

2.2. DNA extraction, amplification, and sequencing

The adult, clitellate earthworms were anesthetized in a 30–50% ethanol solution. Muscle tissue of anterior body wall was isolated and either stored in a freezer at −20 °C. All DNA extractions were performed using QIAGEN DNeasy™ kits, following the manufacturer's protocol for animal tissue samples. The isolated DNA was resuspended in TE buffer and stored at −20 °C until use. For all specimens, a 690-base fragment in the coding region of the COI and a 377-base fragment of the 16S rDNA were amplified using the following primers which were designed in this study: Meta-2F (5′ – ATR CCA GTA TTY ATT GGD GG -3′) and Meta-1R (5′- CTR AAT ACT TTR ATT CCT GT – 3′) for the COI region, 16S-Meta-F (5′ – AAC GGC CGC GGT AYM YTA AC -3′) AND 16S-Meta-R (5′ – CYW AAG CCA ACA TSG AGG TG -3′) for 16S rDNA. Double-stranded DNA was amplified by incubating at 94 °C for 10 s, followed by 45 cycles of incubation at 94 °C for 1.5 min, 48 °C for 2 min, and 72 °C for 3 min, with a final extension at 72 °C for 15 min. DNA was amplified using polymerase chain reaction (PCR) in a 50-μl reaction volume containing approximately 50 ng total DNA, 10 mM Tris–HCl buffer (pH 8.3) with 50 mM KCl and 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1.25 units *Taq* DNA polymerase (TAKARA), and 0.5 μM of each primer. After amplification, reaction mixtures were subjected to electrophoresis in 1% low-melting-temperature agarose gels and purified using QIAGEN QuickSpin™ kits following the manufacturer's specifications. The purified PCR products were sequenced using a Big DYE-terminator Cycle Sequencing Kit (ABI PRISM DNA Sequencing Kit; Perkin–Elmer Applied Biosystems) and an ABI PRISM 3100-*Avant* Genetic Analyzer according to the manufacturers' instructions. For sequencing, the same primers used for amplification were used.

2.3. Data analysis

To construct phylogenetic trees, sequences were aligned using Clustal W [34], and were improved manually using MEGA 4 [32]. The positions of deletions or insertions were determined manually. Alignment began at position 240 for COI gene and 11,689 for 16S rDNA of *Lumbricus terrestris* (Linnaeus, 1758), which was retrieved from GenBank (accession no. U24570 [6]).

In the phylogenetic analyses, the most appropriate model of DNA substitution was chosen using hierarchical likelihood ratio tests with PAUP 4.0b10 [30] and Modeltest v3.06 [27]. For the combined dataset of COI and 16S rDNA, Tamura-Nei's model [31] with invariable sites of 0.6385 and a gamma shape parameter of 0.7595 (TrN + I + G) was chosen (Base frequencies: A, 0.3764; C, 0.2241; G, 0.1174 and T, 0.2821. Substitution rates: A–C, 1.0000; A–G, 12.0519; A–T, 1.0000; C–G, 1.0000; C–T, 9.1328; and G–T, 1.0000). For the COI region, the general time-reversible model [33] with invariable sites of 0.4707 and a gamma shape parameter of 0.5544 (GTR + I + G) was chosen (Base frequencies: A, 0.4029; C, 0.1645; G, 0.1672; and T, 0.2654. Substitution rates: A–C, 3.1784; A–G, 6.4862; A–T, 4.1422; C–G, 0.5389; C–T, 19.5915; and G–T, 1.0000).

Neighbour-joining (NJ) analyses were performed using PAUP 4.0b10 on model of TrN + I + G with 1000 bootstrap replicates. Bayesian analysis was applied to generate a posterior probability distribution using the Metropolis-coupled Markov Chain Monte Carlo (MCMC) with MrBayes 3.0b4 [15,28]. The search was run for 4.5×10^6 generations, and every 100th tree was sampled. Posterior probabilities for each branch were calculated from the sampled trees.

3. Results

3.1. Sequence characteristics

MtDNA sequences from the COI region were determined from a total of 75 samples, including 71 samples of *M. sieboldi* and four outgroup species. The length of the COI region in all samples, including outgroups, was 690 bp, without insertions or deletions. Of the 690-nucleotide positions, 252 (36.5%) were polymorphic and 208 (30.1%) were parsimony informative. Among the *M. sieboldi* samples, the infraspecific sequence divergences of the COI gene ranged from 0.1% to 18.1%, and the mean infraspecific sequence divergences between phylogenetic groups of our study ranged 5.7%