

# Identifying earthworms (Oligochaeta, Megadrili) of the Southern Kuril Islands using DNA barcodes

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## Abstract

*Identifying earthworms (Oligochaeta, Megadrili) of the Southern Kuril Islands using DNA barcodes.* The Kuril Islands are a volcanic archipelago located between Hokkaido and Kamchatka. In this study we investigated earthworm fauna of three of the Southern Kuril Islands, Kunashir, Shikotan, and Yuri, using both morphological analysis and DNA barcoding. Our results highlight the potential of DNA barcoding for studying earthworm fauna: while previous studies reported only six earthworm species and subspecies on the Southern Kurils, we detected 15 genetic clusters. Six of them correspond to European cosmopolites; six, to Asian species; and three, to unidentified species. While no European earthworms were found on Yuri that is uninhabited since WWII, they dominated on larger and inhabited Kunashir and Shikotan, suggesting that they are recent invaders. Of the six Asian species, five had *cox1* sequences identical or very closely related to published sequences from the mainland or the Japanese islands and thus are recent invaders.

Key words: Earthworms, Megadrili, *cox1*, Barcoding, Kuril islands

## Resumen

*Identificación de las lombrices (Oligochaeta, Megadrili) del sur de las Islas Kuriles utilizando códigos de barras de ADN.* Las Islas Kuriles forman un archipiélago volcánico situado entre Hokkaido y Kamchatka. En este estudio analizamos las lombrices de tres de las islas Kuriles meridionales: Kunashir, Shikotan y Yuri, utilizando el análisis morfológico y los códigos de barras de ADN. Nuestros resultados ponen de relieve el potencial de los códigos de barras de ADN para estudiar las lombrices: si bien en estudios anteriores solo se habían registrado seis especies y subespecies de lombriz en las islas Kuriles meridionales, nosotros detectamos 15 grupos genéticos. Seis de ellos son especies cosmopolitas europeas; seis, especies asiáticas; y tres, sin determinar. A pesar de que no se encontraron lombrices europeas en Yuri, que está deshabitada desde la Segunda Guerra Mundial, estas especies dominaron en las islas Kunashir y Shikotan, que son más grandes y están habitadas, lo que sugiere que se trata de especies invasoras recientes. De las seis especies asiáticas, cinco tenían secuencias *cox1* idénticas o muy emparentadas con las secuencias publicadas encontradas en el continente o en las islas del Japón y, por tanto, se trata de invasoras recientes.

Palabras clave: Lombrices, Megadrili, *cox1*, Código de barras de ADN, Islas Kuriles

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## Introduction

DNA barcoding is a powerful tool for identification of organisms (Hebert et al., 2003). It allows one to work with specimens that cannot be identified using their morphology, e.g., juvenile or degraded ones (Hajibabaei et al., 2006). In addition, DNA barcoding is promising for groups with problematic taxonomy, e.g. earthworms (Decaëns et al., 2013). Although earthworms are fairly well studied, recent molecular research demonstrated outstanding cryptic diversity within most earthworm species, which is exacerbated by the paucity of diagnostic morphological characters in this group (King et al., 2008; Rougerie et al., 2009; James et al., 2010; Marchán et al., 2017).

Earthworms are important components of soil biota, generally believed to increase general ecosystem productivity (Darwin, 1881; Lee, 1985). However, human-mediated earthworm invasions are becoming problems in some regions (Hendrix et al., 2008): they may alter community composition, promote colonization of ecosystems by other invasive species (Roth et al., 2015), and even decrease ecosystem productivity by depleting available nutrients (Resner et al., 2015).

The Southern Kurils is an archipelago located to the north of Hokkaido. There are several studies on the earthworm fauna of Southern Kurils (Perel, 1979; Marusik, 2002; Gongalsky et al., 2014). Six earthworm species and subspecies were reported: *Metaphire hilgendorfi* (Michaelsen, 1982) (= *Pheretima hilgendorfi* = *Amyntas hilgendorfi*), *Eisenia japonica* (Michaelsen, 1892), *Lumbricus rubellus* Hoffmeister, 1843, *Dendrobaena octaedra* (Savigny, 1826), *Dendrodrilus rubidus tenuis* (Eisen, 1874), and *Allolobophora parva* (Eisen, 1874). While the latter four are cosmopolites of European origin (Hendrix et al., 2008), the former two are local Asian species from the Japanese archipelago.

The aim of this study was to investigate earthworm samples from three Southern Kuril Islands, Kunashir, Shikotan and Yuri. These islands with areas of 1,500, 225, and 10 km<sup>2</sup>, respectively, are characterized by moderate marine climate (Krestov et al., 2009; Pietsch et al., 2003; Razzhigaeva et al., 2014). Shikotan is covered by meadows and dark coniferous, birch and alder forests. Kunashir contains diverse biotopes with a mixture of boreal and subtropical species. Yuri is completely devoid of trees and is covered by meadows and bogs. We used DNA barcoding in order to update species composition of these islands and attempt to detect species origins. We wanted to clarify the origin of its Asian earthworm fauna (ancient vs. recent) and to examine the patterns of their coexistence with European cosmopolites, in terms of genetic diversity and relative abundance.

## Material and methods

Specimens were collected in 2012–2016 (fig. 1, table 1). A part of the material from the south of Kunashir (13 individuals) was taken from the study

of Gongalsky et al., 2014). Taxonomic nomenclature follows Blakemore, 2003). In addition to morphological identification, a set of specimens, including juvenile individuals, was taken for DNA barcoding. DNA isolation, amplification, and sequencing were performed as described in Shekhovtsov et al., 2013). All obtained *cox1* sequences were deposited in GenBank under accession numbers KX400579–KX400731 and KY750693–KY750709 (see table 1).

Nucleotide and gene diversity values and mismatch distributions were calculated using Arlequin v.3.5.2.2 (Excoffier and Lischer, 2010). Divergence times for the *E. japonica* dataset were calculated by BEAST v.1.7.2 (Drummond et al., 2012) and Tracer v.1.6.0 (Rambaut et al., 2014). It is problematic to calibrate molecular clock for earthworms (Fernández et al., 2016), as earthworm fossils are almost absent and cannot be attributed to any particular taxon. There are calibration methods based on outgroup clitellate fossils (Marchán et al., 2017), however, they seem to be more applicable to deeper time spans and high-level taxa. Here we used the molecular clock rate of 2.5 % sequence divergence per million years obtained using vicariance dating (Pérez-Losada et al., 2011) as a rough estimate. Phylogenetic trees were built using MrBayes v.3.2.0 (Ronquist and Huelsenbeck, 2003) and MEGA v.5.0 (Tamura et al., 2011). For Bayesian analysis, the HKY+I+G model was suggested by MrModeltest (Nylander, 2004). Two independent analyses were performed using ‘metropolis coupled Monte Carlo’ simulations for 10 million generations, sampling a tree every 10,000 generations. For minimum evolution and maximum likelihood algorithms, bootstrapping was performed with 1,000 replications.

## Results

A total of 170 individuals from Kunashir, Shikotan, and Yuri were barcoded using the *cox1* gene. We detected 15 clusters corresponding to distinct Operational Taxonomic Units that may represent different earthworm species or mitochondrial genetic lineages (fig. 2). Six of these OTUs corresponded to well-known European cosmopolites: *L. rubellus*, *A. caliginosa* (Savigny, 1826), *Aporrectodea rosea* (Savigny, 1826), *Octolasion tyrtaeum* (Savigny, 1826) (also incorrectly referred to as *Octolasion lacteum* (Örley, 1885)), *D. octaedra*, and *D. r. tenuis*. Three of those, *O. tyrtaeum*, *A. caliginosa*, and *A. rosea* were reported from Southern Kurils for the first time. *Lumbricus rubellus* was prevalent on Kunashir, making up 47 % of the total sample (and all European cosmopolites accounted for about 60%). We should also note that *D. octaedra* and *D. r. tenuis* were relatively under-represented in this study due to sampling bias as we took few litter samples.

All mtDNA haplotypes of European cosmopolites were identical to the representatives of these species found in European populations (Fernández et al., 2012, 2016; Porco et al., 2013; Shekhovtsov et al., 2014a, 2014b, 2016). For the two most frequent

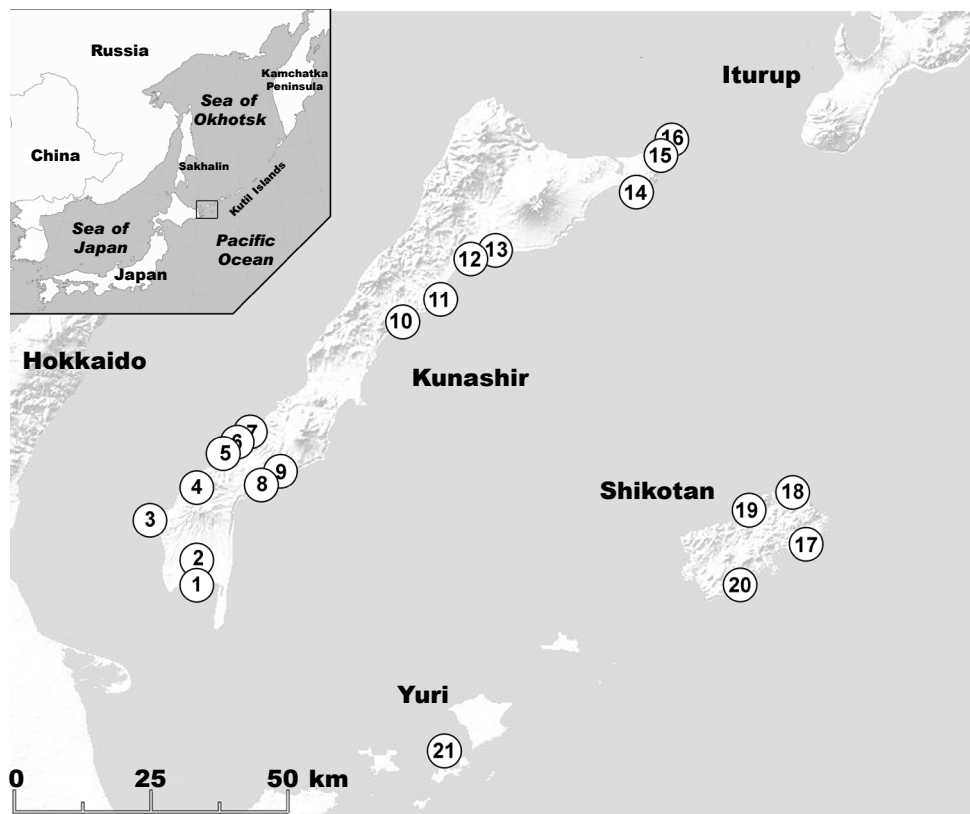


Fig. 1. Locations sampled in this study. Upper left, small scale map of the region; location of the studied islands is shown by a square. (For abbreviations of locations see table 1).

Fig. 1. Lugares muestreados en este estudio. En la esquina superior izquierda, mapa de la región en pequeña escala; la ubicación de las islas estudiadas se indica con un cuadrado. (Para las abreviaturas de los lugares muestreados, véase tabla 1).

European species found in this study, *L. rubellus* and *A. caliginosa*, we detected high genetic diversity (table 2), similar to that found in native and introduced populations by Porco et al. (2013) and Martinsson and Erséus (2017). Other European cosmopolites were represented only by a few individuals and lacked genetic diversity.

Another five OTUs could be identified as species of the family Megascolecidae: *M. hilgendorfi* (Michaelsen, 1892), *Amyntas agrestis* (Goto and Hatai, 1899), *Amyntas phaselus* (Hatai, 1930), *A. vittatus* (Goto and Hatai, 1898), and *A. tokioensis* (Beddard, 1892). For the latter four this is the first report for the Kuril islands, as well as for Russia as a whole.

The Asian *M. hilgendorfi* was represented by two haplotypes, one of which was identical to AB542629 GenBank from Hokkaido, and the second one differed from those by 2 %. All sequences of *A. phaselus* were identical to a specimen collected on the Ulleung-do island (South Korea) by Blakemore (2013a) and 99 % similar to an individual from northern Honshu (AB542519). *Amyntas vittatus* from Kunashir was

identical to the AB542566 accession from Hokkaido. *Amyntas agrestis* haplotypes were closely (99 %) related to those from Shikoku and Honshu (AB542597, AB542599–AB542602). Two individuals of *A. tokioensis* differed by one nucleotide substitution, and by two substitutions from AB542557 GenBank entry from Sendai (Honshu).

Our specimens of *E. japonica* from the Kuril Islands were unrelated to ones from Honshu (Blakemore, 2012b), South Korea (Blakemore, 2013b), or Shikoku (GenBank accessions AB542698, AB543188, AB543237) (fig. 3A). In contrast to other Asian species, we detected several closely related haplotypes of *E. japonica* that could have diverged on the Kuril islands. The estimate of divergence time for our *E. japonica* sample was 7,580 years (95 % CI, 3,750–11,800 years). Mismatch distribution data (fig. 3B) suggests a recent demographic expansion event for *E. japonica*.

There were another three clusters on the tree (fig. 2). Two of those (shown as E2716 and E2727 on figure 2) were small unpigmented juvenile worms, and E2727

Table 1. Locations sampled in this study: N, number of individuals; r, river; v, village; p, peninsula; l, lake; t, town; GenBank, GenBank accession numbers.

Tabla 1. Lugares muestreados en este estudio: N, número de individuos; r, río; v, aldea; p, península; l, lago; t, ciudad; GenBank, números de accesión en la base de datos GenBank.

Location	N	Species	GenBank
<b>Kunashir</b>			
1 Mouth of r. Golovnina	2	<i>M. hilgendorfi</i> , E2954	KX400703–KX400704
2 Watershed of r. Golovnina and r. Khlebnikova	12	<i>M. hilgendorfi</i> , <i>L. rubellus</i>	KX400720–KX400731
3 Cape Ivanovskii	20	<i>A. phaselus</i> , <i>L. rubellus</i> , <i>A. caliginosa</i> , <i>E. japonica</i>	KX400646–KX400649, KX400659–KX400672
4 Caldera of Golovnina volcano	30	<i>M. hilgendorfi</i> , <i>M. agrestis</i> , <i>E. japonica</i> , <i>A. vittatus</i> , <i>L. rubellus</i>	KX400652–KX400658, KX400680–KX400690, KX400701–KX400702, KX400705–KX400714
5 V. Alekhino	4	<i>M. hilgendorfi</i> , <i>E. japonica</i>	KX400613–KX400614, KX400631–KX400632
6 Cape Znamenka	9	<i>M. hilgendorfi</i> , <i>L. rubellus</i> , <i>E. japonica</i>	KX400615–KX400623
7 West bank of l. Pechianoye	4	<i>M. hilgendorfi</i> , <i>M. agrestis</i>	KX400697–KX400700
8 r. Andreevka	4	<i>L. rubellus</i>	KX400609–KX400612
9 Mouth of r. Belkina	5	<i>A. caliginosa</i> , <i>L. rubellus</i>	KX400624–KX400628
10 Mouth of r. Ilyushina	2	<i>A. caliginosa</i> , <i>A. phaselus</i>	KX400650–KX400651
11 Lower reaches of r. Philatova	2	<i>E. japonica</i>	KX400629–KX400630
12 Lower reaches of r. Saratovskaya	6	<i>A. phaselus</i> , <i>E. japonica</i> , <i>D. rubidus</i> , <i>D. octaedra</i>	KX400640–KX400645
13 Mouth of r. Tyatina	11	<i>L. rubellus</i>	KX400633–KX400639
14 Lovtsova p., shore of Spokoistvia bay	7	<i>M. hilgendorfi</i> , <i>L. rubellus</i> , <i>E. japonica</i>	KX400673–KX400679
15 Lovtsova p., near l. Nadya	8	<i>L. rubellus</i> , <i>D. octaedra</i>	KX400715–KX400719
16 Lovtsova p., lighthouse	5	<i>L. rubellus</i>	KX400691–KX400696
<b>Shikotan</b>			
17 N43.8100 E146.7941	6	<i>A. caliginosa</i> , <i>L. rubellus</i>	KX400579–KX400584
18 N43.8828 E146.8447	8	<i>L. rubellus</i> , E2716, E2717	KX400585–KX400592
19 Krabozavodskoy t., courtyard	9	<i>E. japonica</i> , <i>A. caliginosa</i> , <i>L. rubellus</i> , <i>A. rosea</i> , <i>O. tyrtaeum</i>	KX400593–KX400600, KX400608
20 Northwest of Tserkovnaya bay, 100 m from the sea	7	<i>E. japonica</i> , <i>A. caliginosa</i> , <i>A. rosea</i> , E2727	KX400601–KX400607
<b>Yuri</b>			
21	17	<i>E. japonica</i> , <i>M. hilgendorfi</i> , <i>M. agrestis</i> , <i>A. phaselus</i> , <i>A. tokioensis</i> , E2954	KY750693–KY750709
<b>Total</b>	<b>170</b>		

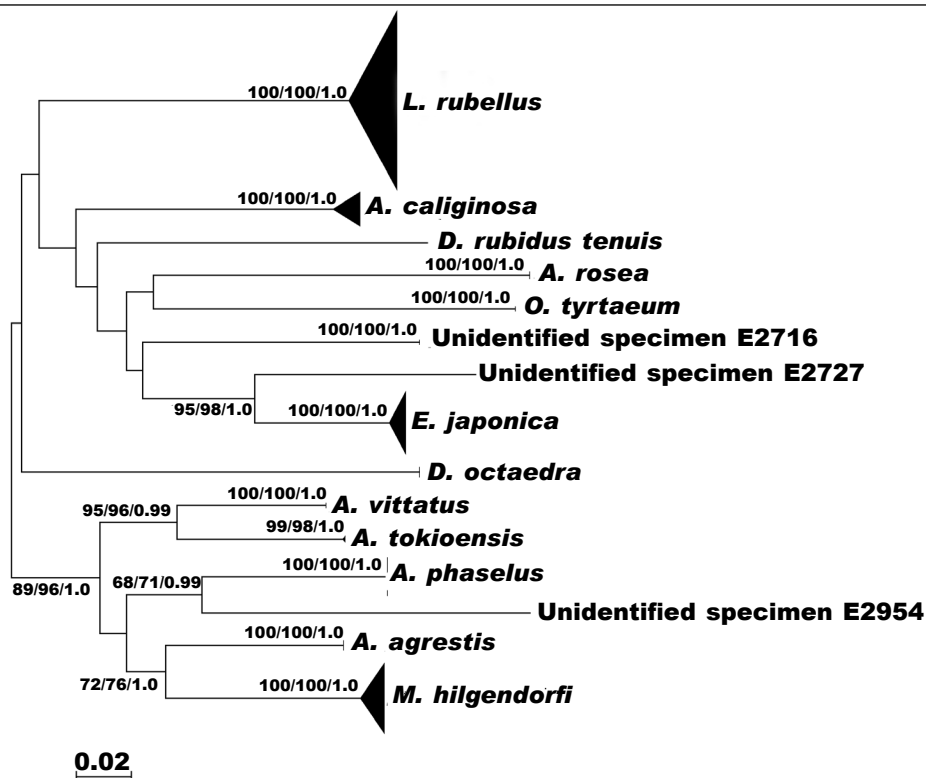


Fig. 2. Minimum evolution phylogenetic tree of the obtained *cox1* sequences. Numbers near branches indicate bootstrap support for minimum evolution/maximum likelihood/Bayesian posterior probabilities. Species are collapsed into triangles; the base of the triangle is proportional to the number of sequences and its height, to nucleotide diversity within the sample.

Fig. 2. Árbol filogenético de las secuencias *cox1* obtenidas con el método de la mínima evolución. Los números cercanos a las ramas indican la valoración de bootstrap para los algoritmos de mínima evolución, máxima probabilidad y probabilidad a posteriori de Bayes. Las especies convergen en triángulos cuya base es proporcional al número de secuencias y su altura, a la diversidad nucleotídica de la muestra.

was found alongside *A. caliginosa* and *A. rosea* and was provisionally identified as one of those species. E2954 was found together with *M. hilgendorfi* and generally resembled its morphology. All these three clusters undoubtedly represent distinct species, as their *cox1* sequences had approximately 10 % nucleotide differences from any GenBank or BOLD entries.

## Discussion

Our results indicate that DNA barcoding is a promising tool for studying earthworm biodiversity: all previous studies reported six species and subspecies from the Kuril islands, while our rather limited sample yielded fifteen. There are two explanations for this. First, barcoding allows one to identify juvenile or poorly preserved specimens. Second, many earthworm species look similar, and so the species representing small minority of the sample tend to be grouped with more numerous species and overlooked. An example

to this was discovered by Chang et al., 2016): in the USA, three Megascolecidae species, *M. hilgendorfi*, *A. agrestis*, and *A. tokioensis* often occur together, but are often identified as one of the two former species. Co-occurrence of these species was also detected in this study.

Multiple studies demonstrated very high genetic differences between even closely located populations in endemic earthworm species (e.g., Novo et al., 2009, 2015; Shekhovtsov et al., 2013, 2015). On the other hand, in the case of cosmopolitan earthworms a single *cox1* haplotype may be spread on multiple continents (e.g., Fernández et al., 2011, 2016; Porco et al., 2013; Martinsson et al., 2015). Based on these contrasting patterns one can hypothesize on the origin of the studied populations.

There is almost 400 species of European Lumbricidae endemic to Southern Europe (Hendrix et al., 2008), while only 20–30 of them managed to colonize other regions, and about a half of those are cosmopolites widespread throughout the world. Six of them

Table 2. Earthworm species detected in this study: N, number of individuals; n, number of unique haplotypes; GD, gene diversity; pi, nucleotide diversity; SD, standard deviation.

Tabla 2. Especies de lombriz detectadas en este estudio: N, número de individuos; n, número de haplotipos únicos; GD, diversidad génica; pi, diversidad nucleotídica; SD, desviación estándar.

	N	n	GD (SD)	pi (SD)
European cosmopolites				
<i>Lumbricus rubellus</i>	69	12	0.776 (0.035)	0.0223 (0.0112)
<i>Aporrectodea caliginosa caliginosa</i>	13	5	0.833 (0.060)	0.0126 (0.0070)
<i>A. rosea</i>	3	1	0	0
<i>Octolasion tyrtaeum</i>	2	1	0	0
<i>Dendrobaena octaedra</i>	4	1	0	0
<i>Dendrodrilus rubidus tenuis</i>	1	1	–	–
Asian species				
<i>Eisenia japonica</i>	24	16	0.949 (0.028)	0.0093 (0.0051)
<i>Metaphire hilgendorfi</i>	27	2	0.519 (0.028)	0.0087 (0.0048)
<i>M. agrestis</i>	4	1	0	0
<i>Amyntas phaselus</i>	15	1	0	0
<i>A. vittatus</i>	2	1	0	0
<i>A. tokioensis</i>	2	2	1.000 (0.500)	0.0015 (0.0021)
Unidentified specimens				
E2727	1	1	–	–
E2954	1	1	–	–
E2716	2	1	0	0
Total	170			

were found by us on Kunashir and Shikotan, and all had haplotypes identical to those found in European populations, which confirms recent introduction. The same was true for five species of the family Megascolecidae, which also indicates that these species were introduced from the Japanese Archipelago.

Our results indicate that the earthworms of the Southern Kurils are mostly of recent invasive origin, and the native earthworm fauna of the Southern Kurils failed to survive the Last Glacial Period (with the only possible exception of *E. japonica*). A similar pattern was observed for terrestrial isopods, which find suitable habitats across both islands but do not host any indigenous species (Gongalsky et al., 2014). All terrestrial isopods were either the cosmopolitan *Porcellio scaber* Latreille, 1804 or amphibiotic species occurring on the littoral zone of the islands. The latter undoubtedly have much better dispersal ability and may have arrived more recently.

It should be noted that in many Asian regions, invasive earthworms of European origin often seem to be more vigorous than local species, either replacing them in many habitats (Tiunov et al., 2006; Hendrix et al.,

2008) or co-habiting (Blakemore, 2009, 2012). Asian species are also important as invasive earthworms, e.g., in North America (Hendrix and Bohlen, 2002; Callahan et al., 2003; Blakemore, 2009; Snyder et al., 2011; Greiner et al., 2012). The Southern Kurils is a region where both groups meet. Judging by our collection sample, European and Asian earthworms successfully coexist on Kunashir and Shikotan.

It is also interesting that only Asian earthworms were found on Yuri (we managed to collect only a small sample, but the island is also small and ecologically homogeneous). Yuri hosted a Japanese village before the WWII but was uninhabited since that time (apart from military units). On the contrast, Kunashir and Shikotan have functioning Russian settlements. These facts may suggest that Asian earthworms were introduced by Japanese settlers prior to WWII, while European cosmopolites are the results of post-WWII introduction.

The only possibly native species on the studied Southern Kuril islands is *E. japonica*. The obtained *cox1* haplotypes were unrelated to those from the Japanese Archipelago or Southern Korea, which

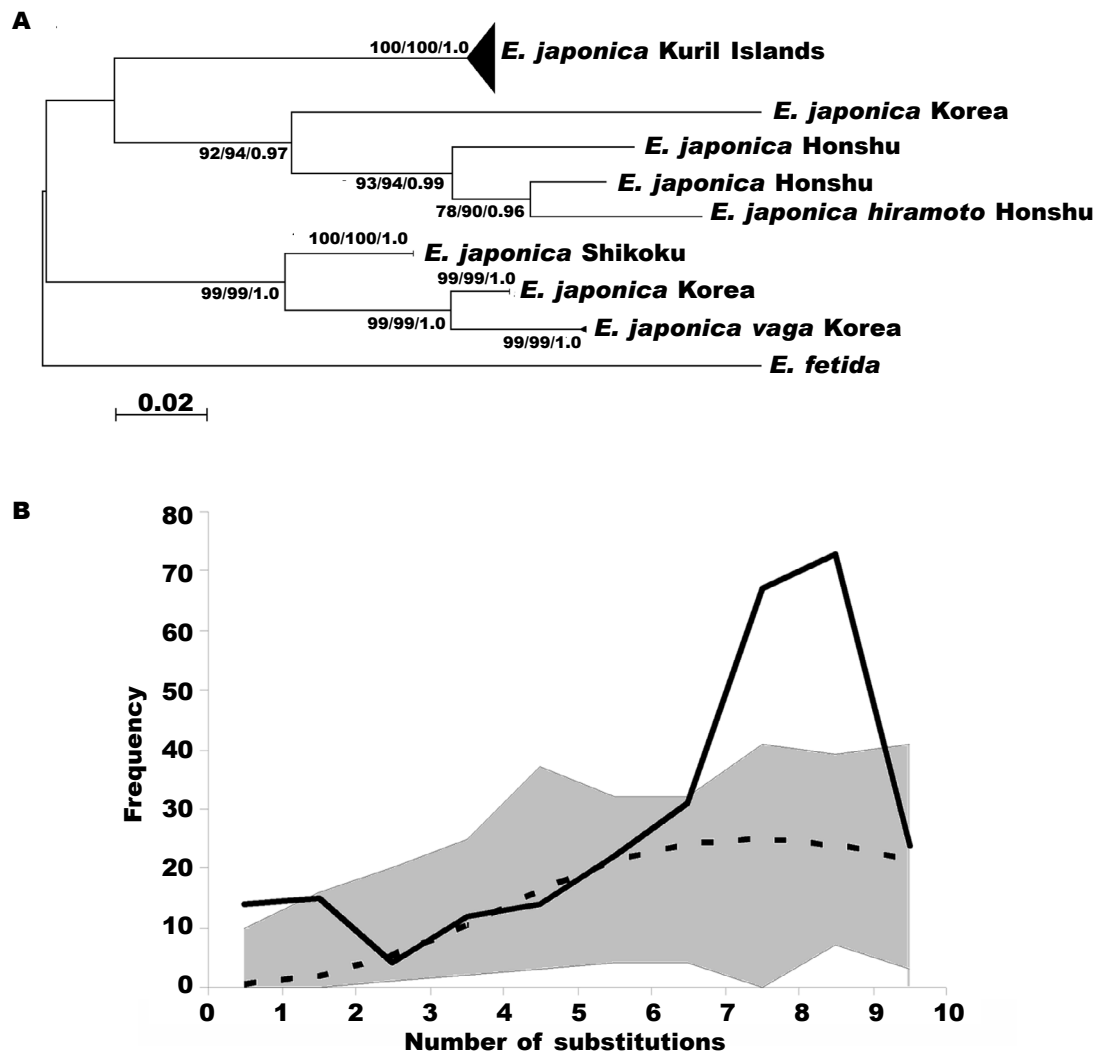


Fig. 3. A, minimum evolution phylogenetic tree constructed for *E. japonica* sequences; branches are collapsed into triangles; the base of the triangle is proportional to the number of sequences, and its height to nucleotide diversity within the sample; B, mismatch distribution for *E. japonica* sample from the Kuril Islands: horizontal axis, number of pairwise differences; vertical axis, total number of substitutions; solid line, observed distribution; dotted lined, modeled distribution; gray shading, 95% confidence interval for the modeled distribution.

Fig. 3. A, árbol filogenético construido con el método de la mínima evolución para las secuencias de *E. japonica*; las ramas convergen en triángulos cuya base es proporcional al número de secuencias y su altura, a la diversidad nucleotídica de la muestra; B, distribución de las diferencias entre pares de secuencias para la muestra de *E. japonica* de las islas Kuriles: eje horizontal, número de diferencias por pares; eje vertical, número total de sustituciones; línea continua, distribución observada; línea punteada, distribución obtenida por el modelo; sombreado gris, intervalo de confianza del 95% para la distribución obtenida por el modelo.

indicates that this species may actually represent a species complex like the congeneric *E. nordenskioldi* (Eisen, 1879) (Blakemore, 2013c; Shekhovtsov et al., 2013). Currently available data imply that *E. japonica* populations from the Kurils underwent a recent demographic expansion after the end of the Last Glacial Period, although further studies of other islands of the archipelago are required to make strong conclusions.

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