



Title	Reconstruction of mitochondrial genomes from raw sequencing data provides insights on the phylogeny of Ixodes ticks and cautions for species misidentification
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Citation	Ticks and Tick-Borne Diseases, 13(1), 101832 <a href="https://doi.org/10.1016/j.ttbdis.2021.101832">https://doi.org/10.1016/j.ttbdis.2021.101832</a>
Issue Date	2022-01-01
Doc URL	<a href="http://hdl.handle.net/2115/87841">http://hdl.handle.net/2115/87841</a>
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2 **Reconstruction of mitochondrial genomes from raw**  
3 **sequencing data provides insights on the phylogeny of**  
4 ***Ixodes* ticks but suggests the caution for species**  
5 **misidentification**

6  
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34

35 **Abstract**

36 High-throughput sequencing (HTS) technology has profoundly been involved in sequencing whole genomes  
37 of several organisms in a fast and cost-effective manner. Although HTS provides an alternative  
38 biomonitoring method to the time-consuming and taxonomy-expertise dependent morphological approach,

39 still we cannot rule out the possibility of the impediment and misidentification biases. In this article we aim  
40 to retrieve whole mitochondrial genome (mitogenome) sequences from publicly available raw sequencing  
41 data for phylogenetic comparison of *Ixodes persulcatus*. For this comparison, we sequenced whole  
42 mitogenomes of four *I. persulcatus* ticks from Japan and constructed mitogenomes from raw sequencing  
43 data of 74 *I. persulcatus* ticks from China. Bayesian phylogenetic trees were inferred by the concatenated  
44 fifteen mitochondrial genes. We further tested our results by the phylogenetic analysis of cytochrome c  
45 oxidase subunit 1 (*cox1*) gene and internal transcribed spacer 2 (ITS2) sequences. Our findings showed that  
46 70 constructed mitogenomes from China were clustered with the sequenced four mitogenomes of *I.*  
47 *persulcatus* from Japan. We also revealed that mitogenome sequences retrieved from two data sets  
48 CRR142297 and CRR142298 were clustered with *Ixodes nipponensis*. Moreover, other two mitogenome  
49 sequences from CRR142310 and CRR142311 formed a clade with *Ixodes pavlovskyi*. The phylogenetic  
50 analysis of *cox1* gene and ITS2 sequences confirmed the identification errors of these four samples. The  
51 overall phylogenetics in our study concluded that accurate morphological identification is necessary before  
52 implementing HTS to avoid any misidentification biases.

53 **Keywords:** High-throughput sequencing; *Ixodes persulcatus*; mitogenome; phylogenetic analysis.

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## 56 1. Introduction

57 Ticks are the second most important vectors of human and animal pathogens among arthropods after  
58 mosquitoes. For instance, *Ixodes persulcatus* in Asia and Russia is a vector for tick-borne encephalitis virus  
59 (TBEV) (Mansfield et al., 2009) and Lyme borreliosis (Murase et al., 2013). Climate change and the expansion  
60 of the human-animal interface have contributed to the increase of emerging tick-borne diseases all over the  
61 globe (Cavicchioli et al., 2019). Therefore, correct identification of several tick species is crucial for  
62 studying ticks and tick-borne pathogens (TBPs).

63 To date, the rapid evolution of high-throughput sequencing (HTS) platforms have provided opportunities to  
64 have insights in many fields related to genomics (Lee et al., 2013). For example, it has allowed the  
65 description of microbial communities of ticks from different ecosystems (Estrada-Peña et al., 2018; Tokarz  
66 et al., 2019), the characterization of TBEV (Paulsen et al., 2021) and the detection of several viral populations  
67 in ticks (Qiu et al., 2019). In addition, these platforms have contributed to sequencing mitochondrial

68 genomes (mitogenomes) from various species of invertebrates (McKenna et al., 2010) including ticks  
69 (Burnard and Shao, 2019). However, phylogenetic relationships of ticks based on mitochondrial and nuclear  
70 genomes remain uncertain due to the limited number of genomes available (Liu et al., 2018). Nevertheless,  
71 it is challenging to sequence and assemble the nuclear genomes of ticks if compared to the mitochondrial  
72 genomes (Nene, 2009). Thus, mitogenomes are used to infer phylogenetic relationships of ticks as well as  
73 insects at different taxonomic levels (Burger et al., 2013; Burger et al., 2014; Carpi et al., 2016; Kelava et  
74 al., 2021; Mans et al., 2019; Mans et al., 2015; Mans et al., 2021; McCooke et al., 2015; Nakao et al., 2021).  
75 Recently, a hybrid assembly approach that utilizes Illumina short-read and Pacific Biosciences long-read  
76 data was applied for the genome studies of several tick species including *I. persulcatus*, *Haemaphysalis*  
77 *longicornis*, *Dermacentor silvarum*, *Hyalomma asiaticum*, *Rhipicephalus sanguineus*, and *Rhipicephalus*  
78 *annulatus* (Guerrero et al., 2019, 2021; Jia et al., 2020). Although the output of these studies can provide  
79 key features of the tick metabolism, population structure and genetic diversity of ticks and the associated  
80 pathogen composition and ecology, bias is possible in sequence data due to the under-sampled regions that  
81 may lead to loss of important regions during assembly (Ross et al., 2013). Proper data processing is also  
82 prerequisite as exemplified by the misidentification of TBPs due to poor knowledge on tick microbiome  
83 (Buysse and Duron, 2021). Nevertheless, making HTS data available for the public can significantly improve  
84 the scientific progress (Resnik, 2010). That is, if the data and supporting information are accurate and  
85 available to the scientific community. A good example for utilizing publicly available data resources is the  
86 recent phylogenomic analysis of the inward rectifier potassium (Kir) channels in ticks (Saelao et al., 2021).  
87 The aim of our study was to reconstruct mitogenome sequences of 74 *I. persulcatus* genomic sequences  
88 publicly available at the Genomic Sequence Archive (GSA) and to make a comparison with those from four  
89 *I. persulcatus* ticks sampled from different regions of Japan. The results provided an insight of the  
90 phylogenetic relationships of *I. persulcatus* with other *Ixodes* species but suggests the caution for the biases  
91 possibly caused by species misidentification in some samples.

## 92 **2. Materials and methods**

### 93 *2.1 Specimen collection and DNA extraction*

94 A total of four adult ticks were collected from Hokkaido prefecture (n = 2), Nagano prefecture (n = 1),  
95 Fukushima prefecture (n = 1), Japan between 2013 and 2014. The collected ticks were morphologically  
96 identified as two adult males and two adult females of *I. persulcatus* based on a standard key under a

97 stereomicroscope (Yamaguti et al., 1971). More specifically, in order to distinguish *I. persulcatus* from  
98 *Ixodes pavlovskyi* and *Ixodes nipponensis*, the following morphological characteristics were investigated:  
99 the length of the internal spur of coxa I, the shape of the spiracular plate, the length and color of the legs,  
100 and the apex of the hypostome as reported elsewhere (Nakao et al., 1992). Individual tick specimens were  
101 cut into half with a sterile blade. A half was crushed with stainless beads using a Micro Smash MS-100R  
102 (TOMY, Tokyo, Japan) at 2,500 rpm for 30 s. The DNA was extracted using a blackPREP Tick DNA/RNA  
103 Kit (Analytikjena, Germany) according to the manufacturer's instructions.

#### 104 2.2 Construction of NGS libraries and whole mitogenome sequencing

105 The entire mitogenome sequence of *I. persulcatus* was amplified in two overlapping PCRs (long-range and  
106 short). Long-range PCR primers: mtG\_K23: 5'-TCCTACATGATCTGAGTTYAGACCG-3' and mtG\_K26:  
107 (5'- ACGGGCGATATGTRCATATTTTAGAGC-3') and short PCR primers (I\_gap\_F3: 5'-  
108 TTTYWAATTAAGATAGAAACCAACCTG-3' and I\_gap\_R3 5'-AAATGTAAGGAGCATCACTCADA-  
109 3') were designed by aligning complete mitogenomes of genus *Ixodes* deposited in the database. The long-  
110 range and short PCRs were performed as previously described (Kelava et al., 2021) with modifications.  
111 Briefly, a 50 µl-reaction mixture of long-range PCR was performed containing 10 µl of 5 × PrimeSTAR  
112 GXL Buffer (Mg<sup>2+</sup> Plus) (TaKaRa Bio Inc., Shiga, Japan), 4.0 µl of dNTP Mixture (2.5 mM each), 200 nM  
113 of each primer, 1.0 µl of PrimeSTAR® GXL DNA Polymerase (TaKaRa Bio Inc.), and 2.0 µl of template  
114 DNA. The reaction conditions were 45 cycles of 98 °C for 10 s, 60 °C for 15 s, and 68 °C for 10 min. Short  
115 PCR was performed in a 25 µl-reaction mixture containing 12.5 µl of 2 × Gflex PCR Buffer (Mg<sup>2+</sup>, dNTP  
116 plus) (TaKaRa Bio Inc.), 0.5 µl of Tks Gflex DNA Polymerase (1.25 units/µl) (TaKaRa Bio Inc.), 200 nM  
117 of each primer, and 1.0 µl of template DNA. The reaction conditions were 94 °C for 60 s, 45 cycles of 98 °C  
118 for 10 s, 55 °C for 15 s, 68 °C for 60s, and a final extension of 68 °C for 5 min. Product of the PCR were  
119 analyzed by electrophoresis in a 1.5% agarose gel stained with Gel-Red™ (Biotium, Hayward, CA). PCR  
120 products were purified with a NucleoSpin Gel and PCR Clean-Up Kit (TaKaRa Bio Inc.).

121 Illumina sequencing libraries were constructed from the purified amplicons of two universal tick  
122 mitogenome PCRs (Kelava et al., 2021) using the Nextera DNA Library Prep Kit (Illumina, Hayward, CA)  
123 and were sequenced with the MiSeq reagent kit v3 for 600 cycles on an Illumina MiSeq platform. Geneious  
124 v10.2.6 (Biomatters Ltd., Auckland, New Zealand) was used to map the reads against a reference  
125 mitochondrial genome (accession number: NC\_004370). The complete mitogenome sequences obtained

126 were submitted to the DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp>) with the accession numbers  
127 LC595234-LC595237.

### 128 2.3 Retrieving mitogenome sequences from the database

129 We downloaded the forward and reverse raw sequence reads of the 74 *I. persulcatus* ticks sampled from  
130 China available at GSA (<https://bigd.big.ac.cn/gsa/browse/CRA002715>). Pair-end raw sequence reads were  
131 paired, merged and mapped to *I. persulcatus* reference mitogenome sequence (accession number:  
132 NC\_004370) using Geneious v10.2.6.

### 133 2.4 Phylogenetic analysis of the mitogenome sequences

134 Consensus sequences were aligned with the four assembled *I. persulcatus* mitogenomes from Japan, and  
135 reference mitochondrial genomes of *I. persulcatus* (accession numbers: NC\_004370 and KU935457), *I.*  
136 *nipponensis* (accession number: MT371808), *I. pavlovskyi* (accession numbers: NC\_023831 and LC595233),  
137 *Ixodes simplex* (accession number: KY457531), *Ixodes hexagonus* (accession number: AF081828), *Ixodes*  
138 *rubicundus* (accession number: KY457530), *Ixodes holocyclus* (accession number: MH043266), *Ixodes*  
139 *uriae* (accession number: AB087746), *Ixodes ricinus* (accession number: KF197132) and *Ixodes tasmani*  
140 (accession number: MH043271). Fifteen mitochondrial genes were used to infer phylogenetic trees. We  
141 concatenated the sequences of 13 protein-coding (*cox1*, *cox2*, *cox3*, *nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*,  
142 *nad6*, *cytb*, *atp6*, and *atp8*) and two ribosomal RNA (12S rRNA and 16S rRNA) genes and used the  
143 concatenated sequence to construct phylogenetic trees. We used BEAST version 1.4, a program for Bayesian  
144 analysis of molecular sequences using Markov Chain Monte Carlo (MCMC) to create the Bayesian  
145 phylogeographic trees (Drummond and Rambaut, 2007).

### 146 2.5 Analysis of *cox1* gene and internal transcribed spacer 2 (ITS2) sequences

147 We inferred a phylogeny from the *cox1* gene, which is considered a reliable marker for identifying tick  
148 species using the maximum clade credibility (MCC) phylogenetic analysis. A total of 74 *cox1* gene  
149 sequences were obtained from the raw reads of the *I. persulcatus* ticks reported in Jia et al (2020), after being  
150 merged and mapped to *I. persulcatus* reference *cox1* gene sequence (accession number: NC\_004370) using  
151 Geneious v10.2.6. The *cox1* gene sequences were aligned and the MCC phylogenetic trees were inferred  
152 using a generalized skyline plot model, that was embedded in a Bayesian MCMC analysis in BEAST version  
153 1.4.

154 To exclude the possibility that mitochondrial introgression has recently occurred in these ticks, we also

155 studied the internal transcribed spacer 2 (ITS2) sequences of CRR142297, CRR142298, CRR142310,  
156 CRR142311, CRR142260, and CRR142300. Briefly, the merged raw reads were mapped to *I. persulcatus*  
157 reference ITS2 sequence (accession number: JQ737128) using Geneious v10.2.6. Consensus sequences were  
158 aligned with the reference ITS2 sequences of *I. persulcatus* (accession numbers: JQ625713, JF703107,  
159 JQ737128, AB032834, D88868, and D88874), *I. nipponensis* (accession numbers: D88851, D88846, and  
160 D88850), *I. pavlovskyi* (accession numbers: D88859, and D88860), *Ixodes laguri* (accession number:  
161 MF979542). The 18 ITS2 sequences were aligned with the MAFFT analytical tool (Katoh and Standley,  
162 2013). Neighbor-joining consensus phylogenetic tree was constructed by the Tamura-Nei 93 as a substitution  
163 model, with 1,000 bootstrap replications.

### 164 **3. Results and discussion**

165 The mean number of total pair-end reads obtained from MiSeq was 282,721 per sample. After filtration, an  
166 average of 65.4% of reads were successfully mapped against the reference mitogenome. The length of  
167 complete mitogenomes of this study ranged between 14,542 bp and 14,549 bp with a mean sequencing depth  
168 of  $\times 2,709$  (min =  $\times 947$ , max =  $\times 4,407$ ). Each mitogenome encoded 13 protein-coding, two ribosomal  
169 RNA (rRNA) (12S and 16S), and 22 transfer RNA genes with one non-coding control region in the same  
170 arrangement with that of the *I. persulcatus* reference mitogenome sequence (accession number: NC\_004370).  
171 Mapping of 74 published sample sequences from China was successful in detecting the mitogenomes with  
172 the length between 14,543 bp and 14,571 bp (Table 1). In average, 0.001095% (min = 0.000039%, max =  
173 0.004567%) of the reads were mapped on each mitogenome and the sequencing depth ranged between  $\times 11$   
174 and  $\times 2345$  (mean =  $\times 469$ ). The detailed results of the mapping are provided in Supplementary Table S1.  
175 All genes were encoded in accordance with *I. persulcatus* reference mitogenome sequence.  
176 MCC phylogenetic tree of the consensus mitogenomes revealed that sequences CRR142297 and  
177 CRR142298 (identified as *I. persulcatus* (Jia et al., 2020)) clustered with *I. nipponensis*. Moreover, two other  
178 sequences, CRR142310 and CRR142311 (also identified as *I. persulcatus* (Jia et al., 2020)) clustered with *I.*  
179 *pavlovskyi* (Figure 1). In addition, MCC phylogenetic analysis of 90 *cox1* gene sequences (1,534 nt)  
180 confirmed the incorrect identification of the above-mentioned four samples (Figure 2). Moreover, neighbor-  
181 joining consensus phylogenetic analysis revealed that ITS2 sequences from CRR142297 and CRR142298  
182 clustered with *I. nipponensis*. Similarly, ITS2 sequences from CRR142310 and CRR142311 clustered with  
183 *I. pavlovskyi* reference sequences separately from *I. persulcatus* reference sequences (Figure 3).

184 Although *I. nipponensis* is more common in Korea and Japan, it has been detected from Hunan province in  
185 China (Cheng et al., 2018). In addition, *I. pavlovskyi* was recorded among the *Ixodes* ticks of China for the  
186 first time in 2016 from samples deposited in the medical entomology gallery of China (Guo et al., 2016).  
187 Unfortunately, the authors of Jia et al (2020) have not provided clear supplementary data that will accurately  
188 identify the geographic localities of the specimens used for genome sequencing. However, we noticed that  
189 the sample IDs of each misidentified *I. nipponensis* (JLip#) and *I. pavlovskyi* (NXip#) were similar (Figure  
190 1), which may suggest that these samples were probably collected from the same geographical locations.  
191 However, natural hybridization between *I. persulcatus* and *I. pavlovskyi* has been reported from Siberia (Rar  
192 et al., 2019) where putative hybrids (i.e. identified as one species based on the morphological appearance  
193 and to another species based on *cox1* gene and ITS2) were detected. This phenomenon could explain the  
194 misidentification of CRR142310 and CRR142311, but to the best of our knowledge, no natural hybridization  
195 between *I. persulcatus* and *I. nipponensis* has ever been reported. Thus, the misidentification of CRR142297  
196 and CRR142298 is still difficult to explain, especially since mitochondrial introgression could be excluded  
197 based on co-segregation of the *cox1* and ITS2 genes.

198 Although *I. persulcatus*, *I. pavlovskyi*, and *I. nipponensis* can carry similar communities of TBPs (Masuzawa  
199 et al., 1999; Seo et al., 2021; St. John et al., 2021), the prevalence of these pathogens are highly variable  
200 between these tick species (Rar et al., 2017) as well between the vertebrate hosts of ticks (Swei and Kwan,  
201 2017). Hence, accurate identification of ticks is essential for providing correct epidemiology of the  
202 associated TBPs.

203 We have not scrutinised the identification of other species studied before, but we urge caution when using  
204 the publicly available data from this study. It may also be noted that while the quality of the genomes  
205 published in in Jia et al. (2020) are reported as above 90% complete using BUSCO analysis as criteria, the  
206 deposited protein coding datasets show completeness from 60-80% as determined with BUSCO, which put  
207 these genomes on par with that of other sequenced tick genomes (Mans, 2020). This would imply that while  
208 the genes are present in the assemblies, the identification and extraction of the protein coding sequences, and  
209 the annotation is currently incomplete.

## 210 **Funding**

211 This research was supported by KAKENHI (16H06431, 19H03118, 19F19097, 20K21358 and 20KK0151)  
212 and the Japan Program for Infectious Diseases Research and Infrastructure (20wm0225016j0001) from the



213 Japan Agency for Medical Research and Development (AMED).

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348

#### 349 **Figure Legends**

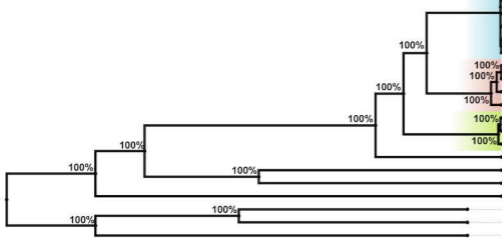
350 **Figure 1. Bayesian Maximum Credibility (MCC) tree of 15 concatenated mitochondrial gene**  
351 **sequences of 10 *Ixodes* tick species.** Cyan, light red and green colors highlight the *Ixodes persulcatus*, *I.*  
352 *pavlovskyi*, and *I. nipponensis* clades, respectively. The other 7 *Ixodes* species; *I. simplex*, *I. hexagonus*, *I.*  
353 *rubicundus*, *I. holocyclus*, *I. uriae*, *I. ricinus* and *I. tasmani* are not highlighted. Brown and cyan colored text  
354 (sequence names) indicates sequences obtained from China and Japan, respectively.

355 **Figure 2. Bayesian Maximum Credibility (MCC) tree of *cox1* gene sequences (1,534 bp) of 10 *Ixodes***  
356 **species.** Cyan, light red and green colors highlight the *Ixodes persulcatus*, *I. pavlovskyi*, and *I. nipponensis*

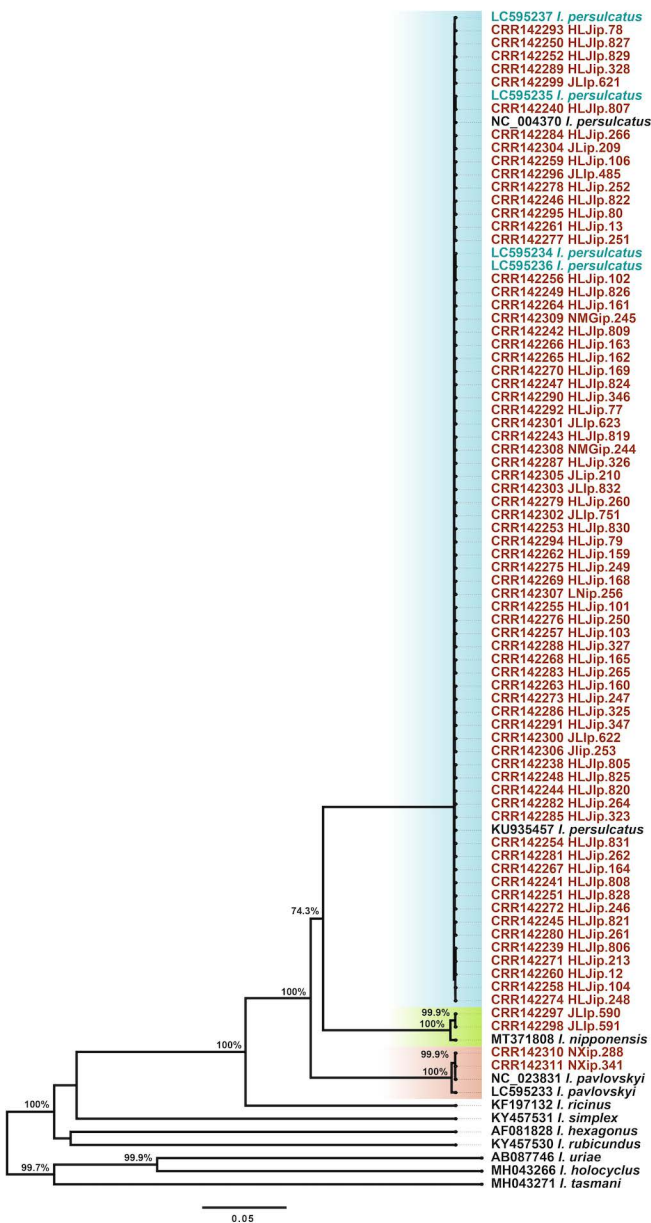
357 clades, respectively. The other 7 *Ixodes* species; *I. simplex*, *I. hexagonus*, *I. rubicundus*, *I. holocyclus*, *I.*  
358 *uriae*, *I. ricinus* and *I. tasmani* are not highlighted. Brown and cyan colored text (sequence names) indicates  
359 sequences obtained from China and Japan, respectively.

360 **Figure 3. Neighbor-joining consensus phylogenetic tree of ITS2 sequences of four *Ixodes* species.**  
361 Bootstrap values are shown on each node. Cyan, light red and green colors highlight the *Ixodes persulcatus*,  
362 *I. pavlovskyi*, and *I. nipponensis* clades, respectively. Text (sequence names) in brown indicates sequences  
363 obtained from China.

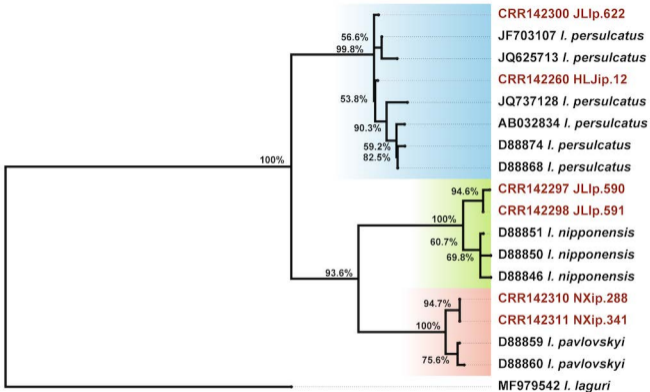
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 CRR142296 JLip.485  
 CRR142301 JLip.623  
 CRR142272 HLJip.246  
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 LC595236 *I. persulcatus*  
 CRR142256 HLJip.102  
 LC595234 *I. persulcatus*  
 KU935457 *I. persulcatus*  
 NC\_004370 *I. persulcatus*  
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 LC595235 *I. persulcatus*  
 CRR142240 HLJip.807  
 CRR142310 NXip.288  
 CRR142311 NXip.341  
 LC595233 *I. pavlovskyi*  
 NC\_023831 *I. pavlovskyi*  
 CRR142297 JLip.590  
 CRR142298 JLip.591  
 MT371808 *I. nipponensis*  
 KF197132 *I. ricinus*  
 AF081828 *I. hexagonus*  
 KY457531 *I. simplex*  
 KY457530 *I. rubicundus*  
 AB087746 *I. uriae*  
 MH043266 *I. holocyclus*  
 MH043271 *I. tasmani*



0.05







0.05

**Table 1: Summary of mapping of high-throughput sequencing reads to *I. persulcatus* reference mitogenome sequence.**

	Mean	Min.	Max.
Mitogenome length (bp)	14,547	14,543	14,571
Total number of reads	28,027,633	860,432	160,707,036
Number of reads mapped to mitogenome	30,153	623	194,603
Percent of mapped reads	0.001095	0.000039	0.004567
Mapped nucleotides	6,820,950	155,999	34,097,385
Sequencing depth	469	11	2,345