



FINAL REPORT

Pretesting of molecular identification tests for *Dendrolimus sibiricus* (Chetverikov, 1908)

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1. Introduction

The European Reference Laboratory for Insects and Mites has to select, adapt or develop reliable identification tests for the phytosanitary insect and mite species that are relevant for the European Union (included in the Commission Delegated Regulation (EU) 2019/1702 and in the EURL for Insects and Mites working programmes). One of the tasks of the EURL is to validate available diagnostic protocols before recommending their use to the National Reference Laboratories of the European Union. Pretesting of available tests is necessary to select the most reliable ones for the validation study.

The Siberian conifer silk moth Dendrolimus sibiricus (Lepidoptera: Lasiocampidae) (Fig.1) is considered a fairly recently



Fig. 1 Dendrolimus sibiricus, adult

separated species from *Dendrolimus superans*, making their differentiation more difficult (this is also why the taxon is sometimes referred to as *Dendrolimus superans ssp. sibiricus*). Both are closely related to *D. pini*.

D. sibiricus has thus far spread to China, Mongolia, North Korea and Russia, unlike *D. superans*, which is as of yet considered to be absent in Europe (only in north-east China, Japan and far eastern parts of Russia). So far, both species haven't been recorded in the European Union.

D. sibiricus is considered an European Union regulated species, listed among the EU quarantine pests (Annex II of the Commission Implementing Regulation (EU) 2019/2072) and among the EU priority pests (Commission Delegated Regulation (EU) 2019/1702). More details are available in the EFSA Pest Survey Card on this pest (EFSA 2020).

Distribution



Fig. 2 Current distribution of D. sibiricus

2. Scope of pretesting

The scope of this preliminary study was to identify molecular tests which are appropriate for the identification of *D. sibiricus*. Additionally, a database inventory for sequence records should led light on the application possibilities of sequencing as identification methods.

3. Test selection

For this pest species no international standards are available yet, however they are currently under preparation.

The EPPO PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests (EPPO, 2021) (Appendix 1), which includes tests for the DNA barcoding of arthropods in general was included in this pretesting.

According to Kononov *et al.* (2016), *D. sibiricus* can not be differentiated from *D. superans* and some *D. pini* genotypes sequencing the 5' end of the *COI* only.

ITS2 barcoding is mentioned in scientific literature as possible genetic locus for identification of *D. sibiricus* [Mikkola and Ståhls (2008), Kononov *et al.* (2016)].

Additionally, Mikkola and Ståhls (2008) give sequencing of the *COI* locus using CI-J-2183/ tl2-n-2014 (alias Jerry/Pat, called M5/3 by Kononov *et al.* 2016) primers as suitable for the identification of *D. sibiricus*.

For this pretesting study, ITS2 barcoding according to Kononov *et al.* (2016) was included, *COI* 3' end primers (CI-J-2183/ tl2-n-2014) were evaluated *in silico* only.

4. Composition of the sample set

For pretesting of tests indicated above, DNA was extracted from parts of single adult specimens (part of legs) of *Dendrolimus* spp. (Tab. 1).

Sample	Species	Host plant	Origin	Source
2258/19	D. sibiricus	Pine	Russia	Kulinich, Lethmayer (AGES)
1590/20	D. pini	Pine	Estonia	Ermakovich (PHML)
1282/21	D. sibiricus	Pine	Russia (Omsk)	Filippov, Taddei (ANSES)
1283/21	D. sibiricus	Pine	Russia (Omsk)	Filippov, Taddei (ANSES)
1501/21	D. sibiricus	Pine	Russia (Omsk)	Filippov, Taddei (ANSES)
1502/21	D. pini	Pine	France	Vardon, Taddei (ANSES)
1503/21	D. pini	Pine	France	Vardon, Taddei (ANSES)
1504/21	D. pini	Pine	France	Vardon, Taddei (ANSES)

Table 1: Details on single adult specimens for pretesting Dendrolimus spp. identification (sample set)

5. Specification of pretesting procedures

DNA extraction

For DNA extraction the DNeasy Blood and Tissue (Qiagen) on part of *Dendrolimus* spp. legs was used. Samples indicated in table 1 were tested undiluted and with at least one additional dilution (usually 1:20).

Molecular tests

Following tests for the identification of *D. sibiricus* were evaluated:

• ITS2 sequencing according to Kononov et al. (2016)

• Included in EPPO PM 7/129 (2) DNA barcoding (EPPO, 2016): Barcoding for arthropods (Appendix 1): LepF/R primer set

In silico:

• 3' COI sequencing according to Mikkola and Ståhls (2008)

6. Results of pretesting

In silico data

Primer BLAST of sequencing primers included in the *in silico* pretesting of this study (LepF/R for the 5' *COI*, Pat/Jerry for the 3' *COI*, and ITS2A/B for the *ITS2* locus) indicated them as inclusive for a multitude of Dendrolimus species. Neighbor joining trees were constructed to evaluate the possibility of differentiating the closely related *D. superans*, *D. sibiricus* and *D. pini* species. For both the 3' and 5' ends of the *COI* locus, the separation seems was not possible. Only on the *ITS2* locus clustering on species level is visable (Appendix 2).

Testing of the sample set

With the sample set of this pretesting, the results of the *in silico* data as well as evaluations in literature could be confirmed. Whereas 5' *COI* barcoding was not able to distinguish between *D. sibiricus* and *D. superans*, correct identification of *the D. sibiricus* samples was possible on the *ITS2* locus (Tabl.2). Amplification of *D. pini* sequences on the *ITS2* locus proved difficult, however, but this species could in turn be correctly identified via 5' *COI* barcoding in most cases.

1 0		0
Assigned value of the samples	EPPO 2021, <i>COI</i> 5′	Kononov <i>et al.</i> 2016, <i>ITS2</i>
2258/19		
Dendrolimus sibiricus	D. superans	D. sibiricus
1590/20		
Dendrolimus pini	D. superans	D. pini
1282/21		
Dendrolimus sibiricus	D. superans	D. sibiricus
1283/21		
Dendrolimus sibiricus	D. superans	D. sibiricus
1501/21		
Dendrolimus sibiricus	-	D. sibiricus
1502/21		
Dendrolimus pini	D. pini	-
1503/21		
Dendrolimus pini	D. pini	-
1504/21		
Dendrolimus pini	D. pini	-

Table 2: Results of pretesting for Dendrolimus spp. according to assigned values

7. Database inventory for sequence records

DNA barcoding relies on PCR of predetermined marker genes (e.g. for the *COI* gene), DNA sequencing and comparison of those sequences to a database of reference sequences (Armstrong and Ball 2005). Applying barcoding for insect identification requires enough sequence records from the species within the genus for a reliable comparision. Not only the number, but also the genetic and geographic diversity of the records and the quality are potential issues that should be considered.

Three different databases (NCBI GenBank, Bold and EPPO Q-Bank) were consulted for the inventory. As search parameters the genus and species name and the gene locus (*COI* and synonyms) were used. In addition, the reliability of the records was checked and, if reasonable, the search was extended to other gene loci.

Results

In all three consulted databases sequence records for *D. sibiricus* on the *COI* locus are avaible, with both the 5' and 3' end being represented in GenBank and Bold. GenBank also offered sequences on the *ITS2* and other loci (e.g. *COII*, *5.85* rRNA) (query date October 2022).

Gene	GenBank	Q-Bank	Bold
СОІ	56	13	50
COII	16	0	0
ITS2	31	0	0

Table 3: Number of sequence records per relevant gene for each database (data accessed October 2022).

Detailed information

NCBI GenBank and Bold database hold sequence records on the *COI* locus for multiple species of the Dendrolimus genus (e.g. *D. punctatus, D. kikuchii, D. pini D. sibiricus, D. superans*). In the EPPO Q-Bank 13 *COI* barcodes for *D. sibiricus* are deposited. 31 *ITS2* sequences are available in GenBank, in addition to several *COII* and 5.8S *rRNA* (included in the *ITS2* region) sequences.

Out of 184 *D. sibiricus* specimen found in the Bold database, 143 hold barcodes, 50 of which are public. The total Dendrolimus genus is credited with 1.047 specimens with barcodes out of 1.137 specimen records, belonging to 14 public species and an additional 18 non-public ones.

The geographic variation of the sequence records for *D. sibiricus* in Bold represents its known distribution, with the vast majority stemming from Russia and occasional specimens having been sampled in Mongolia and Kazakhstan. All records found in EPPO Q-Bank were larvae sampled in Russia.

Tree-based identification

To evaluate the species divergences within the genus, Neighbor Joining (NJ) trees of distance were constructed for the *ITS2* and *COI* loci using NCBI GenBank (max. seq. diff. of 0.75). Figure 3 confirmed the suitability of the *ITS2* locus for distinguishing multiple Dendrolimus species, whereas Figure 4 shows insufficient seperation of *D. sibiricus* and *D. superans* on the *COI* locus.



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Figure 3: Neighbor joining tree on the *ITS2* locus shows clustering of different Dendrolimus species in a way that allows clear separation.



Figure 4: Neighbor joining tree on the 5' COI locus shows insufficient separation of D. sibiricus and D. superans

Interestingly, many of the sequences for the 5' end of the COI locus deposited in the NCBI database and subsequently mined from there for the Bold database, included a gap of about 50bp in the middle of their 1371bp sequence (Fig. 5).

ACTCCTGGGTCTTTAATTGGAGATGATCAAATTTATAATACTATTGTCACAGCACATGCT TTCATCATAATTTTTTTTTTTATAGTAATACCAATTATAATTGGAGGATTTGGTAATTGATTA TGATTATTACCACCCTCTTTACCTTATTAATTTCGAGAAGAATTGTAGAAAGTGGAGCC GGAACTGGATGAACTGTTTATCCTCCTCTATCCTCTAATATTGCTCATAGAGGGAGATCA GTTGATTTAGCTATTTTTTCCCTTCATCTTGCCGGAATTTCATCTATTTTAGGAGCAATT TTATTTGTTTGAGCTGTAGGAATTACAGCATTTTTATTATTATTATCATTACCAGTTCTT GCCGGAGCAATTACTATACTATTAACTGATCGAAATTTAAATACATCATTCTTTGACCCT GCTGGAGGAGGAGATCCCATTTATATCAACATTTATTTTGATTTTTGGNNNNNNN AATAAAAAAGAAACCTTTGGTTGTTTAGGAATAATTTATGCTATATTAGCAATTGGATTA TTAGGATTTATTGTATGAGCTCATCACATATTTACAGTAGGTATAGATATTGATACTCGA GCTTATTTTACCTCTGCAACTATAATTATTGCAGTACCAACAGGAATTAAAATTTTTAGA TGATTAGCAACCCTTCATGGAACACAAATAAACTATAGCCCTTCCATACTTTGAAGATTA GGATTTGTATTCTTATTTACTGTTGGAGGATTAACAGGAGTAATCCTTGCCAATTCTTCT ATCGATATTACCCTTCATGATACTTACTATGTAGTAGCTCATTTTCATTATGTACTTTCA ATAGGAGCAGTATTTGCTATTATAGGGGGGATTTATTCATTGATACCCATTATTTACTGGC TTAACATTTTTTCCTCAACACTTTTTAGGATTAGCTGGAATACCTCGACGATACTCTGAT TACCCAGACTCATATATCTCATGAAATATTATTTCTTCATTAGGTTCTTATATTTCCTTA TTAGGAGTTATAATAATAATAATTATTATTTGAGAATCAATAATTAACCAACGAATTAGA ATCTTTACTTTAAACATAAGATCTTCTATTGAATGATATCAAAATTTACCT

Figure 5: Peculiarity recognised in several COI sequences of D. sibiricus.

8. Discussion

Molecular identification of *Dendrolimus sibiricus* (3' and 5' *COI* barcoding, *ITS2* sequencing) was pretested in this study. *ITS2* sequencing is crucial for the differentiation of *D. sibiricus* and *D. superans*, which is not possible on the 5' *COI* locus (typically used for barcoding). 3' *COI* sequencing was only tested *in silico* during this pretesting, but seems promising, providing enough resolution for these closely related species.

Additionally, the identification of the non-target *D. pini* is difficult using *ITS2* sequencing alone, as amplification of the sequence isn't always successful.

Based on this pretesting study, the use of more than one test seems necessary for accurate identification of *D. sibiricus*. Therefore, 5' *COI* barcoding combined with *ITS2* sequencing (Kononov *et al.* 2016) will be validated, in addition to 3' *COI* sequencing. The sample set for the validation will be extended to more specimens of both target and closely related non target Lepidoptera specimens.

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Date: February 8, 2023

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Richard Gottsberger Scientific Project Leader – Molecular Unit

Appendix 1 - References

EFSA (European Food Safety Authority), Wilstermann, A, Schrader, G, Kinkar, M, Vos, S, 2020. Pest survey card on Dendrolimus sibiricus. EFSA supporting publication 2020:EN-1779. 23 pp. doi: 10.2903/sp.efsa.2020.EN-1779.

EPPO (2021). EPPO standards PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests. Bulletin OEPP/EPPO Bulletin, 51 (1): 100–143.

Hajibabaei, M., Janzen, D. H., Burns, J. M., Hallwachs, W., & Hebert, P. D. (2006). DNA barcodes distinguish species of tropical Lepidoptera. Proceedings of the National Academy of Sciences, 103(4), 968-971.

Kononov, A., Ustyantsev, K., Wang, B., Mastro, V. C., Fet, V., Blinov, A., & Baranchikov, Y. (2016). Genetic diversity among eight Dendrolimus species in Eurasia (Lepidoptera: Lasiocampidae) inferred from mitochondrial COI and COII, and nuclear ITS2 markers. BMC genetics, 17, 173-182.

Mikkola, K., & Ståhls, G. (2008). Morphological and molecular taxonomy of Dendrolimus sibiricus Chetverikov stat. rev. and allied lappet moths (Lepidoptera: Lasiocampidae), with description of a new species. Entomologica Fennica, 19(2), 65-85.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H. & Flook, P., 1994: Evolution, weighting and phylogenetic utility of mitochondrial gene-sequences and a compilation of conserved polymerase chain-reaction primers. — Annals of the Entomological Society of America 87: 651–701.

Search set to Dendrolimus or *Dendrolimus sibiricus,* respectively.

5' COI sequencing:

EPPO (2021). EPPO standards PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests. Bulletin OEPP/EPPO Bulletin, 51 (1): 100–143.

Hajibabaei, M., Janzen, D. H., Burns, J. M., Hallwachs, W., & Hebert, P. D. (2006). DNA barcodes distinguish species of tropical Lepidoptera. Proceedings of the National Academy of Sciences, 103(4), 968-971.

Fast Minimum Evolution tree for LepF: Search set to Dendrolimus

^a Dendrolinus punctatus Odorant Receptor 56 (OR56) mRNA, partial cds
 Dendrolimus punctatus isolate 201910 mitochondrion, complete genome Dendrolimus punctatus isolate 201910 mitochondrion, complete genome Dendrolimus punctatus isolate 2 mitochondrion, complete genome Dendrolimus spectabilis isolate 1 mitochondrion, complete genome Dendrolimus spectabilis mitochondrion, complete genome Dendrolimus punctatus isolate 201909 mitochondrion, complete genome Dendrolimus kituchii iootate 201909 mitochondrion, complete genome Dendrolimus kituchii isolate 1 mitochondrion, complete genome Dendrolimus kituchii donart treeptor (DR9) mRNA, complete cds Dendrolimus pini solate FraMLC1 cytochrome coxidase subunit 1 (COX) gene, partial cds; mitochondrial Dendrolimus pini isolate FraMLC1 cytochrome to cytakse subunit 1 (COX) gene, partial cds; mitochondrial Dendrolimus pini isolate FraMLC1 cytochrome b (Cytb) gene, partial cds; mitochondrial Dendrolimus punctatus antenal isolate JY 50 (chrome b (Cytb) gene, partial cds; mitochondrial Dendrolimus punctatus antenal isolate LY 52 (SCAL) mRNA, complete cds Dendrolimus punctatus antenal
Dendroimus pini isolaie FraDig2 NADH denytorgenake subunit3 (NADS) gene, partial cds; mitochondrial Dendroimus kikuchi isolate LY7 cytochrome b (Cytb) gene, partial cds; mitochondrial Dendroimus kikuchi isolate HY5 cytochrome b (Cytb) gene, partial cds; mitochondrial Dendroimus kikuchi isolate HY5 cytochrome b (Cytb) gene, partial cds; mitochondrial Dendroimus kikuchi isolate HS5 cytochrome b (Cytb) gene, partial cds; mitochondrial Dendroimus kikuchi isolate JA5 cytochrome b (Cytb) gene, partial cds; mitochondrial Dendroimus kikuchi isolate JA6 cytochrome b (Cytb) gene, partial cds; mitochondrial Dendroimus kikuchi isolate JA6 cytochrome b (Cytb) gene, partial cds; mitochondrial Dendroimus kikuchi isolate JA15 cytochrome b (Cytb) gene, partial cds; mitochondrial Dendroimus kikuchi isolate JA16 cytochrome b (Cytb) gene, partial cds; mitochondrial Dendroimus kikuchi isolate JA16 cytochrome b (Cytb) gene, partial cds; mitochondrial
 Dendrolimus kikuchi isolate (ZZ 10 cytochrome b (Cytb) gene, partial cds; mitochondrial Dendrolimus kikuchi isolate (ZZ 9 cytochrome b (Cytb) gene, partial cds; mitochondrial Dendrolimus kikuchi isolate HS1 cytochrome b (Cytb) gene, partial cds; mitochondrial Dendrolimus kikuchi isolate HS5 cytochrome b (Cytb) gene, partial cds; mitochondrial Dendrolimus kikuchi isolate HS5 cytochrome b (Cytb) gene, partial cds; mitochondrial Dendrolimus kikuchi isolate HS5 cytochrome b (Cytb) gene, partial cds; mitochondrial Dendrolimus kikuchi isolate HS5 cytochrome b (Cytb) gene, partial cds; mitochondrial Dendrolimus kikuchi isolate HS4 cytochrome b (Cytb) gene, partial cds; mitochondrial
Dendrolimus kikuchi isolate HS25 cytochrome b (Cyto) gene, partial cds; mitochondrial Dendrolimus kikuchi isolate HS25 cytochrome b (Cyto) gene, partial cds; mitochondrial Dendrolimus kikuchi isolate DDH6 cytochrome b (Cyto) gene, partial cds; mitochondrial Dendrolimus kikuchi isolate DDH6 cytochrome b (Cyto) gene, partial cds; mitochondrial Dendrolimus kikuchi isolate PE21 cytochrome b (Cyto) gene, partial cds; mitochondrial Dendrolimus kikuchi isolate PE21 cytochrome b (Cyto) gene, partial cds; mitochondrial Dendrolimus kikuchi isolate PE21 cytochrome b (Cyto) gene, partial cds; mitochondrial Dendrolimus pini voucher 056/DENDSI/3B cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Dendrolimus pini voucher 056/DENDSI/3B cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
Dendrolimus kikuchii ionotropic receptor (IR21a) mRNA, complete cds Dendrolimus houi ionotropic receptor (IR21a) mRNA, complete cds Dendrolimus superans mitochondrion, complete genome Dendrolimus superans mitochondrion, complete genome Dendrolimus tabulaeformis isolate 2 mitochondrion, complete genome Dendrolimus tabulaeformis isolate 1 mitochondrion, complete genome Dendrolimus spectabilis solate 2 mitochondrion, complete genome
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 Dendrolimus punctatus isolate 201908 mitochondrion, complete genome Dendrolimus houi odorant receptor (OR27) mRNA, complete cds

Search set to Dendrolimus sibiricus

moths | 6 leaves

Dendrolimus sibiricus mitochondrial partial coi gene for cytochrome c oxidase subunit I, specimen voucher M: Dendrolimus sibiricus mitochondrial partial coi gene for cytochrome c oxidase subunit I, specimen voucher M: Dendrolimus sibiricus mitochondrial partial coi gene for cytochrome c oxidase subunit Specimen voucher M: Dendrolimus sibiricus voucher DSI6188 5.85 ribosomal RNA gene, partial sequence; internal transcribed space Dendrolimus sibiricus voucher DSI586 5.85 ribosomal RNA gene, partial sequence; internal transcribed space Dendrolimus sibiricus voucher DSI4629 5.85 ribosomal RNA gene, partial sequence; internal transcribed space Dendrolimus sibiricus voucher DSI4629 5.85 ribosomal RNA gene, partial sequence; internal transcribed space Dendrolimus sibiricus voucher DSI4629 5.85 ribosomal RNA gene, partial sequence; internal transcribed space Dendrolimus sibiricus voucher DSI4629 5.85 ribosomal RNA gene, partial sequence; Dendrolimus sibiricus solate DS32 internal transcribed spacer 2, partial sequence Dendrolimus sibiricus solate DS32 internal transcribed spacer 2, partial sequence Dendrolimus sibiricus solate DS32 internal transcribed spacer 2, partial sequence Dendrolimus sibiricus solate DS32 internal transcribed spacer 2, partial sequence Dendrolimus sibiricus IS32, specimen voucher MZH-KM31 Dendrolimus sibiricus IS32, specimen voucher MZH-KM11 Dendrolimus sibiricus IS32, specimen voucher MZH-KM11 Dendrolimus sibiricus isolate DS32 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial Dendrolimus sibiricus isolate DS31 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial Dendrolimus sibiricus isolate DS31 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial Dendrolimus sibiricus isolate DS31 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial Dendrolimus sibiricus isolate DS31 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial Dendrolimus sibiricus mitochondrial partial coi gene for cytochrome c oxidase subunit I, specimen voucher M

Fast Minimum Evolution tree for LepR: Search set to Dendrolimus

<text> . hitochondrial ves 64 cytochrome oxidase subuma and a subu Dendrolimus pini isolate DP64 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial

Search set to Dendrolimus sibiricus

Dendrolimus sibiricus isolate DS72 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial Dendrolimus sibiricus isolate DS31 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial Dendrolimus sibiricus isolate DS51 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial Dendrolimus sibiricus isolate DS71 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial Dendrolimus sibiricus voucher DS16188 5.8S ribosomal RNA gene, partial sequence; internal transcribed space. Dendrolimus sibiricus voucher DS15586 5.8S ribosomal RNA gene, partial sequence; internal transcribed space. Dendrolimus sibiricus voucher DSI4708 5.8S ribosomal RNA gene, partial sequence; internal transcribed space.
 Dendrolimus sibiricus voucher DSI4631 5.8S ribosomal RNA gene, partial sequence; internal transcribed space. Dendrolimus sibiricus voucher DS14629 5.85 ribosomal RVA gene, partial sequence; internal transcribed space.
 Dendrolimus sibiricus isolate Novol1 internal transcribed spacer 2, partial sequence
 Dendrolimus sibiricus isolate DS32 internal transcribed spacer 2, partial sequence Dendrolimus sibiricus isolate DS31 internal transcribed spacer 2, partial sequence Dendrolimus sibiricus ISOate DS31 mierna transchee Spac Dendrolimus sibiricus ITS2, specimen voucher MZH:KM5 Dendrolimus sibiricus ITS2, specimen voucher MZH:KM31

Dendrolimus sibiricus ITS2, specimen voucher MZH:KM1
 Icl[Query_6849

3' COI sequencing:

Mikkola, K., & Ståhls, G. (2008). Morphological and molecular taxonomy of Dendrolimus sibiricus Chetverikov stat. rev. and allied lappet moths (Lepidoptera: Lasiocampidae), with description of a new species. Entomologica Fennica, 19(2), 65-85.

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Fast Minimum Evolution trees for Primer forward: C1-J-2183 (alias Jerry, called M5 by Kononov et al. 2016) 5'-CAACATTTATTTTGATTTTTTGG-3' Search set to Dendrolimus

•	moths 17 leaves	
2	Dendrolimus superans isolate LBGM100726.040 cytochrome oxidase subunit I (COI) gene, partial cost	
ą	Dendrolimus superans isolate LBGM100726.035 cytochrome oxidase subunit I (COI) gene, partial cds;	
9	Dendrolimus superans isolate LBGM100726.034 cytochrome oxidase subunit I (COI) gene, partial cds;	·
ž	Dendrolimus superans isolate LBGM100/26.031 cytochrome oxidase subunit 1 (COI) gene, partial cds;	1
ą	Dendrolimus superans isolate LBGM100726.029 cytochrome oxidase subunit 1 (COI) gene, partial cds:	1
9	Dendrolimus superans isolate LBGM100726.028 cytochrome oxidase subunit I (COI) gene, partial cds;	
X	Dendrolimus superans isolate LBGM100726.015 cytochrome oxidase subunit I (COI) gene, partial cds;	·
ð	Dendrolimus superans isolate LGUM100720.014 cytochrome oxidase subunit 1 (COI) gene, partial cus;	1
ģ	Dendrolimus punctatus isolate DP cytochrome oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	1
2	Dendrolimus kikuchii mitochondrion, complete genome	
ð	Dendrolimus kikuchii isolate JJ4 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	
ą	Dendrolimus kikuchii isolate JJ3 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	
9	Dendrolimus kikuchii isolate JL4 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	
J	Dendrolimus kikuchii isolate JL1 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	
ą	Dendrolimus kikuchii isolate WYS2 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	1
9	Dendrolimus kikuchii isolate WYS14 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondr	
ž	Dendrolimus kikuchii isolate HY12 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	
ą	Dendrolimus kikuchii isolate YS4 cytochrome oxidase subunit 1 (COI) gene, partial cds, initochondrial	
ģ	Dendrolimus kikuchii isolate YS2 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	
X	Dendrolimus kikuchii isolate GZ7 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial Dendrolimus kikuchii isolate GZ7 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	
ā	Dendrolinus kikuchii isolate HST cytochrome oxidase subunit 1 (COI) gene, partial cds, mitochondrial	
ģ	Dendrolimus kikuchii isolate HS21 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	
1	Dendrolimus kikuchii isolate QDH4 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	
J	Dendrolimus kikuchii isolate TP7 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	
¢	Dendrolimus kikuchii isolate HS6 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	
Y	Dendrolimus kikuchii isolate HS11 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	
ð	Dendrolimus kikuchii isolate HS22 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	
ģ	Dendrolimus kikuchii isolate QDH6 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	
9	Dendrolimus kikuchii isolate PE5 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	
J	Dendrolimus kikuchii isolate PE3 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	
ą	Dendrolimus kikuchii isolate PE32 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	
1	Dendrolimus pini voucher 056/DENDSI/3B cytochrome c oxidase subunit I (COX1) gene, partial cds; m.	ł
J	Dendrolimus pini voucher 056/DENDSI/3A cytochrome c oxidase subunit I (COX1) gene, partial cds;	
ą	Dendrolimus houi mitochondrion, complete genome	
9	Dendrolimus pini isolate FraAxel cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondria	l
ž	Dendrolimus pini isolate FraDig2 cytochrome c oxidase subunit I (COXI) gene, partial cds; mitochondria	4
ą	Dendrolimus pini isolate BulgJ crochrome c oxidase subunit I (COXI) gene, partial cds; mitochondrial	1
9	Dendrolimus pini isolate BulgC cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	
J	Dendrolimus pini isolate BulgM cytochrome c oxidase subunit I (COXI) gene, partial cds; mitochondrial Dendrolimus pini isolate TurkZolo cytochrome c oxidase subunit I (COXI) gene, partial cds; mitochond	
ą	Dendrolimus pini isolate GreLesl cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondr.	1
1	Dendrolimus pini isolate IofW11810 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitocho	
J	Dendrolimus pini isolate Prais2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Dendrolimus pini isolate SwissMes3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	
4	Dendrolimus pini isolate Soti cytochrome e oxidase subunit I (COX1) gene, partial eds; mitochondrial	T
9	Dendrolimus pini isolate RusNovo18 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitocho	·
3	Dendrolimus pini isolate SloveB cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	
ģ	Dendrolimus pini isolate BulgK cytochrome c oxidase subunit I (COXI) gene, partial cds; mitochondrial	
9	Dendrolimus pini isolate RusNovo16 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitocho	·
J	Dendroimus pini isolate LithL2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Dendrolimus pini isolate SnaLLE cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	a
ģ	Dendrolimus pini isolate EngDD cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	1
2	Dendrolimus pini isolate GerWol31 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochon	·I
J	Dendrolimus pini isolate Ruskostov cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochon.	1
đ	Dendrolimus pini isolate NorNW2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochond	1
9	Dendrolimus pini isolate SweSE4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondria	4
J	Dendroimus pini isolate SpainSpa10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochon.	·
ş	Dendrolimus pini isolate SwissWol Swizerland cytochrome c oxidase subunit I (COX1) gene, partial cds.	1
9	Dendrolimus pini isolate GerKem2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochond.	4
3	Dendroimus pini isolate SpaTer cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial Dendrolimus pini isolate NorNW3 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochond	
ą	Dendrolimus pini isolate LithZelva8 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochon	1
9	Dendrolimus pini isolate CroDub1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochond	·
3	Dendrolimus pini isolate RusMosc2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochon	·
ą	Dendrolimus pini isolate LithZelva7 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochon.	1
9	Dendrolimus pini isolate FraMLC1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochond.	-
J	Dendrotimus pini isolate FraCP12 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondr	·
1	IclQuery 21353	
		1

Search set to D. sibiricus
^a Dendrolimus sibiricus isolate DS51 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial
Dendrolimus sibiricus isolate DS71 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial
Bendrolimus sibiricus isolate DS72 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
Dendrolimus sibiricus isolate DS31 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
O Dendrolimus sibiricus voucher DSI6188 cytochrome oxidase subunit I (COI) gene, partial cds; mitochon
Pendrolimus sibiricus voucher DSI4724 cytochrome oxidase subunit I (COI) gene, partial cds; mitochon
Φ Dendrolimus sibiricus voucher DSI4706 cytochrome oxidase subunit I (COI) gene, partial cds; mitochon
Pendrolimus sibiricus voucher DSI4629 cytochrome oxidase subunit I (COI) gene, partial cds; mitochon
Dendrolimus sibiricus mitochondrial partial coi gene for cytochrome c oxidase subunit I, specimen vouc
Pendrolimus sibiricus mitochondrial partial coi gene for cytochrome c oxidase subunit I, specimen vouc
O Dendrolimus sibiricus mitochondrial partial coi gene for cytochrome c oxidase subunit I, specimen vouc
φDendrolimus sibiricus voucher DSI6188 5.8S ribosomal RNA gene, partial sequence; internal transcribe
ΦDendrolimus sibiricus voucher DSI5586 5.8S ribosomal RNA gene, partial sequence; internal transcribe
ΦDendrolimus sibiricus voucher DSI4708 5.8S ribosomal RNA gene, partial sequence; internal transcribe
φ Dendrolimus sibiricus voucher DSI4631 5.8S ribosomal RNA gene, partial sequence; internal transcribe
φ Dendrolimus sibiricus voucher DSI4629 5.8S ribosomal RNA gene, partial sequence; internal transcribe
ϕ Dendrolimus sibiricus isolate Novol 1 internal transcribed spacer 2, partial sequence
ϕ Dendrolimus sibiricus isolate DS32 internal transcribed spacer 2, partial sequence
ϕ Dendrolimus sibiricus isolate DS31 internal transcribed spacer 2, partial sequence
Dendrolimus sibiricus ITS2, specimen voucher MZH:KM5
Pendrolimus sibiricus ITS2, specimen voucher MZH:KM31
Dendrolimus sibiricus ITS2, specimen voucher MZH:KM1

Lcl|Query_34641

Fast Minimum Evolution trees for Primer reverse: tl2-n-3014 (alias Pat, called M3 by Kononov *et al.* 2016) 5'-TCCAATGCACTAATCTGCCATATTA-3' Search set to Dendrolimus: Seach set to *Dendrolimus sibiricus*



ITS2 sequencing:

Kononov, A., Ustyantsev, K., Wang, B., Mastro, V. C., Fet, V., Blinov, A., & Baranchikov, Y. (2016). Genetic diversity among eight Dendrolimus species in Eurasia (Lepidoptera: Lasiocampidae) inferred from mitochondrial COI and COII, and nuclear ITS2 markers. BMC genetics, 17, 173-182.

Mikkola, K., & Ståhls, G. (2008). Morphological and molecular taxonomy of Dendrolimus sibiricus Chetverikov stat. rev. and allied lappet moths (Lepidoptera: Lasiocampidae), with description of a new species. Entomologica Fennica, 19(2), 65-85.

Fast Minimum Evolution trees for ITS2A:

Search set to Dendrolimus



Search set to Dendrolimus sibiricus



Fast Minimum Evolution tree for ITS2B: Search set to Dendrolimus



Search set to Dendrolimus sibiricus

1	moths 6 leaves
1	Dendrolimus sibiricus isolate DS32 internal transcribed spacer 2, partial sequence
6	Dendrolimus sibiricus isolate DS31 internal transcribed spacer 2, partial sequence
4	Dendrolimus sibiricus ITS2, specimen voucher MZH: KM5
4	Dendrolimus sibiricus ITS2, specimen voucher MZH:KM31
4	Dendrolimus sibiricus ITS2, specimen voucher MZH:KM1
4	Dendrolimus sibiricus voucher DSI6188 cytochrome oxidase subunit I (COI) gene, partial cd
1	Dendrolimus sibiricus voucher DSI4724 cytochrome oxidase subunit I (COI) gene, partial cd
4	Dendrolimus sibiricus voucher DSI4706 cytochrome oxidase subunit I (COI) gene, partial cd
4	Dendrolimus sibiricus voucher DSI4629 cytochrome oxidase subunit I (COI) gene, partial cd
1	Dendrolimus sibiricus isolate DS51 cytochrome oxidase subunit I (COI) gene, partial cds; mi
4	Dendrolimus sibiricus isolate DS71 cytochrome oxidase subunit I (COI) gene, partial cds; mi
1	Dendrolimus sibiricus mitochondrial partial coi gene for cytochrome c oxidase subunit I, spe
1	Dendrolimus sibiricus mitochondrial partial coi gene for cytochrome c oxidase subunit I, spe
4	Dendrolimus sibiricus mitochondrial partial coi gene for cytochrome c oxidase subunit I, spe
1	cl[Query_111021

Specification of the PCR Assay 1 (COI Barcoding according to EPPO PM7/129(2))

Name of the primer incl. sequence, literature reference, fragment length in bp:

LepF: 5'- ATTCAACCAATCATAAAGATATTGG-3' LepR: 5'- TAAACTTCTGGATGTCCAAAAAAAATCA-3'

Literature: Hajibabaei, M., Janzen, D. H., Burns, J. M., Hallwachs, W., & Hebert, P. D. (2006). DNA barcodes distinguish species of tropical Lepidoptera. Proceedings of the National Academy of Sciences, 103(4), 968-971.

Fragment length: 709bp

PCR - Parameters:

Thermocyler used: Biometra T3000 Thermal cycler

Mastermix: 5x HOT FIREPol® Master Mix, Solis Biodyne:

Composition:		Final concentration:
	Volume per reaction μ l	
Water	6	
Mastermix	2	1x
Primer1:	0,5	0,5μM
Primer2:	0,5	0,5μM
Σ	9	
DNA	1	

PCR conditions:

	°C	Duration (min., sec.)	Nr. of Cycles
Start	95	15 min	1
Denaturation	95	45 sec	5
Annealing	44	45 sec	
Extension	72	45 sec	
Denaturation	95	45 sec	35
Annealing	49	45 sec	
Extension	72	45 sec	
Final extension	72	7 min	1
Cooling	15	∞	

Specification of the PCR Assay 2 [ITS sequencing according to Kononov et al. (2016)]

Name of the primer incl. sequence, literature reference, fragment length in bp:

ITS2A: 5'- TGTGAACTGCAGGACACAT-3' ITS2B: 5'- TATGCTTAAATTGAGGGGGT-3'

Kononov, A., Ustyantsev, K., Wang, B., Mastro, V. C., Fet, V., Blinov, A., & Baranchikov, Y. (2016). Genetic diversity among eight Dendrolimus species in Eurasia (Lepidoptera: Lasiocampidae) inferred from mitochondrial COI and COII, and nuclear ITS2 markers. BMC genetics, 17, 173-182.

Fragment length: 504bp

PCR - Parameters:

Thermocyler used: Biometra T3000 Thermal cycler

Mastermix: 5x HOT FIREPol® Master Mix, Solis Biodyne:

Composition:		Final concentration:
	Volume per reaction μ l	
Water	6	
Mastermix	2	1x
Primer1:	0,5	0,5μM
Primer2:	0,5	0,5μΜ
Σ	9	
DNA	1	

PCR conditions:

	°C	Duration (min., sec.)	Nr. of Cycles
Start	95	15 min	1
Denaturation	95	30 sec	40
Annealing	52	30 sec	
Extension	72	30 sec	
Final extension	72	7 min	1
Cooling	15	∞	