# FINAL REPORT

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

## Contents



## 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 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.*

*Fig. 1 Dendrolimus sibiricus, adult*

*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).

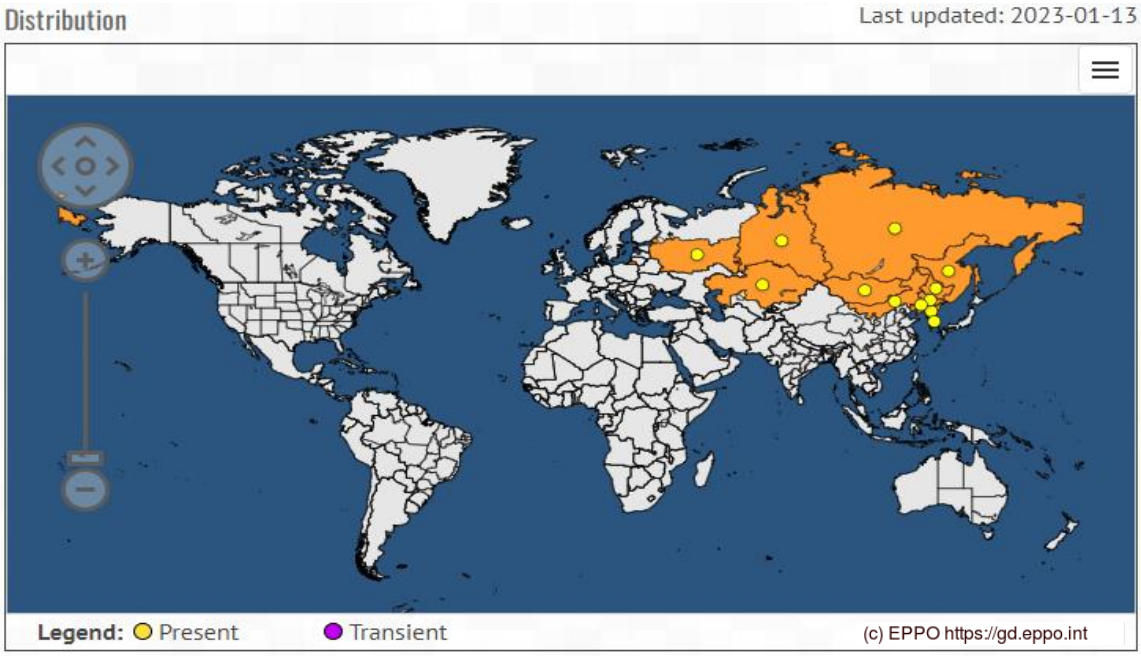


Fig. 2 Current distribution of *D. sibiricus*

## 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).

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

| 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) |

## 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.

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

| **Assigned value of the samples** | **EPPO 2021,** *COI* 5´ | **Kononov *et al.* 2016, *ITS2*** |
|---|---|---|
| 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* | - |

## 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*, *5.8S* rRNA) (query date October 2022).

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

| **Gene** | **GenBank** | **Q-Bank** | **Bold** |
|---|---|---|---|
| ***COI*** | 56 | 13 | 50 |
| ***COII*** | 16 | 0 | 0 |
| ***ITS2*** | 31 | 0 | 0 |

***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.

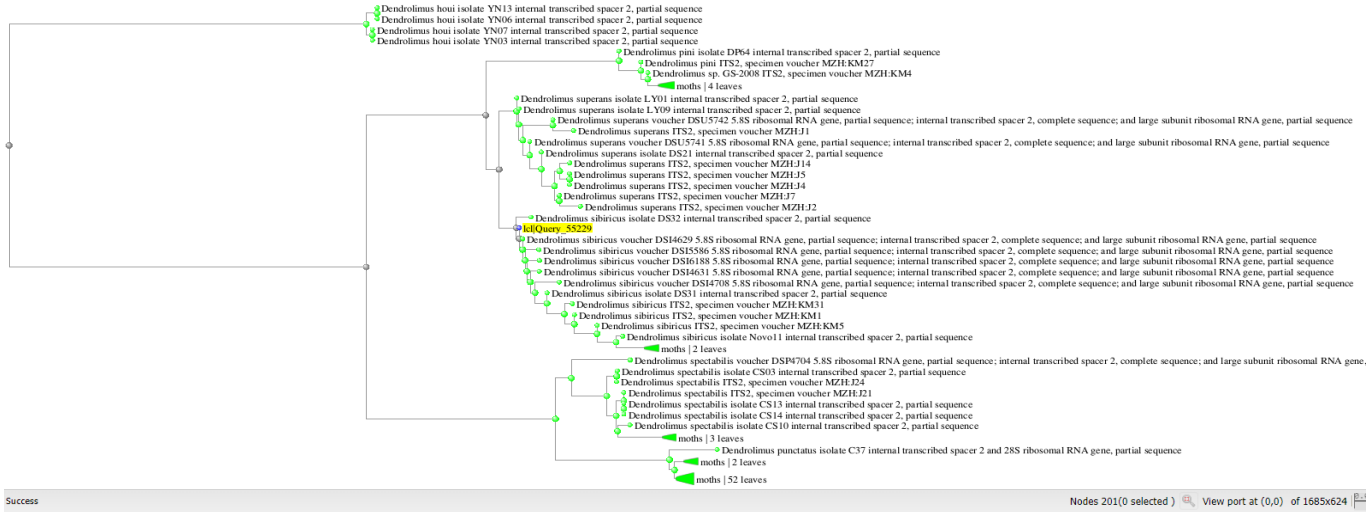



Figure 3: Neighbor joining tree on the *ITS2* locus shows clustering of different Dendrolimus species in a way that allows clear separation.

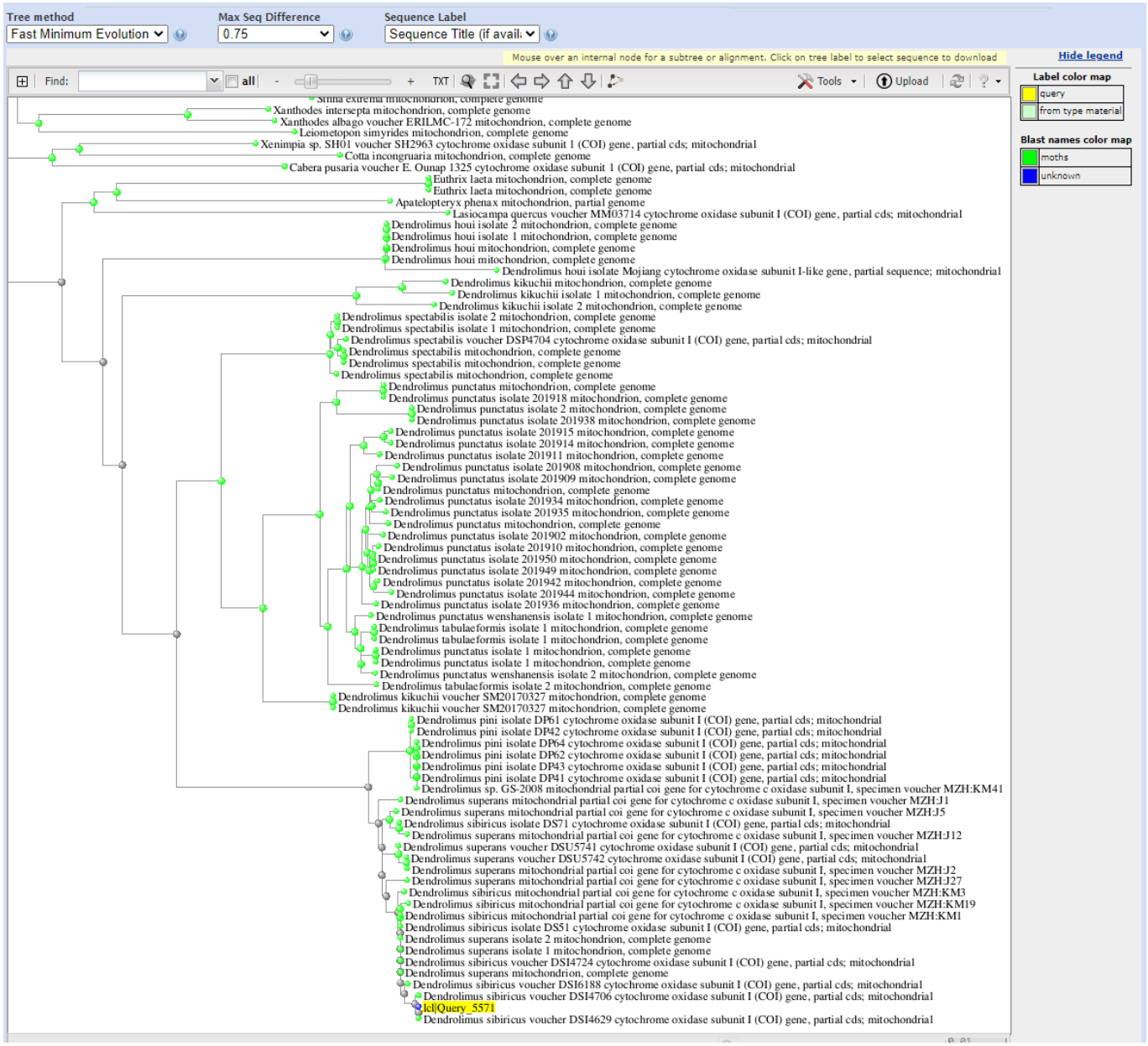



Figure 4: Neighbor joining tree on the 5' *COI* locus shows insufficient separation of *D. sibiricus* and *D. superans*

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
TTCATCATAATTTTTTTTATAGTAATACCAATTATAATTGGAGGATTTGGTAATTGATTA
GTACCTTTAATATTAGGGGCCCCTGACATAGCATTTCCACGAATAAATAATATAAGATTT
TGATTATTACCACCCTCTCTTACCTTATTAATTTCGAGAAGAATTGTAGAAAGTGGAGCC
GGAACTGGATGAACTGTTTATCCTCCTCTATCCTCTAATATTGCTCATAGAGGGAGATCA
GTTGATTTAGCTATTTTTTCCCTTCATCTTGCCGGAATTTCATCTATTTTAGGAGCAATT
AATTTTATTACAACAATTATTAACATAAAATTAAATAATATATCATTTGATCAAATACCT
TTATTTGTTTGAGCTGTAGGAATTACAGCATTTTTATTATTATTATCATTACCAGTTCTT
GCCGGAGCAATTACTATACTATTAACTGATCGAAATTTAAATACATCATTCTTTGACCCT
GCTGGAGGAGGAGATCCCATTTTATATCAACATTTATTTTGATTTTTTGGNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNATTATTTCTCAAGAAAGA
AATAAAAAAGAAACCTTTGGTTGTTTAGGAATAATTTATGCTATATTAGCAATTGGATTA
TTAGGATTTATTGTATGAGCTCATCACATATTTACAGTAGGTATAGATATTGATACTCGA
GCTTATTTTACCTCTGCAACTATAATTATTGCAGTACCAACAGGAATTAAAATTTTTAGA
TGATTAGCAACCCTTCATGGAACACAAATAAACTATAGCCCTTCCATACTTTGAAGATTA
GGATTTGTATTCTTATTTACTGTTGGAGGATTAACAGGAGTAATCCTTGCCAATTCTTCT
ATCGATATTACCCTTCATGATACTTACTATGTAGTAGCTCATTTTCATTATGTACTTTCA
ATAGGAGCAGTATTTGCTATTATAGGGGGATTTATTCATTGATACCCATTATTTACTGGC
TTATCTATAAATCCTTTTATATTAAAAATTCAATTTTTAATTATATTTATTGGAGTAAAT
TTAACATTTTTTCCTCAACACTTTTTAGGATTAGCTGGAATACCTCGACGATACTCTGAT
TACCCAGACTCATATATCTCATGAAATATTATTTCTTCATTAGGTTCTTATATTTCCTTA
TTAGGAGTTATAATAATATTAATTATTATTTGAGAATCAATAATTAACCAACGAATTAGA
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.

Participating staff:

Experts/ Supervisors: Richard Gottsberger, Helga Reisenzein
Technical staff/ Operators: Claudia Heiss, Chiara Pohn

Date: February 8, 2023

Helga Reisenzein
EURL Deputy Director

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.

## Appendix 2 – *In silico* data

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

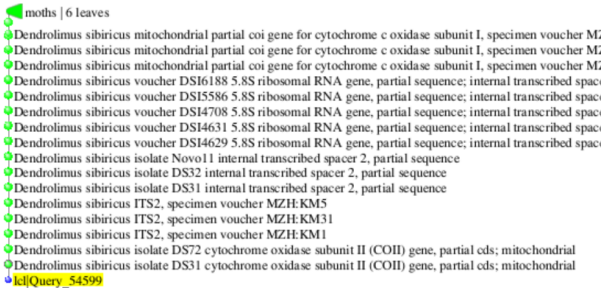
**5' *COI* sequencing:**

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.

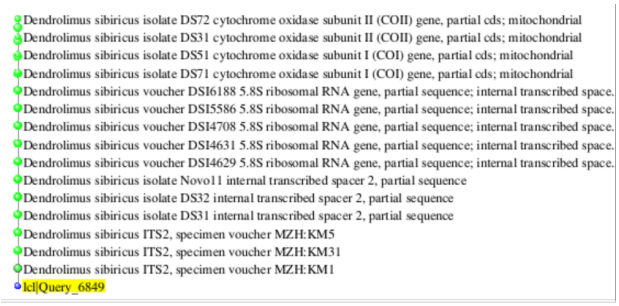
Fast Minimum Evolution tree for LepF:
Search set to Dendrolimus

Search set to *Dendrolimus sibiricus*




Fast Minimum Evolution tree for LepR:
Search set to Dendrolimus


Search set to *Dendrolimus sibiricus*



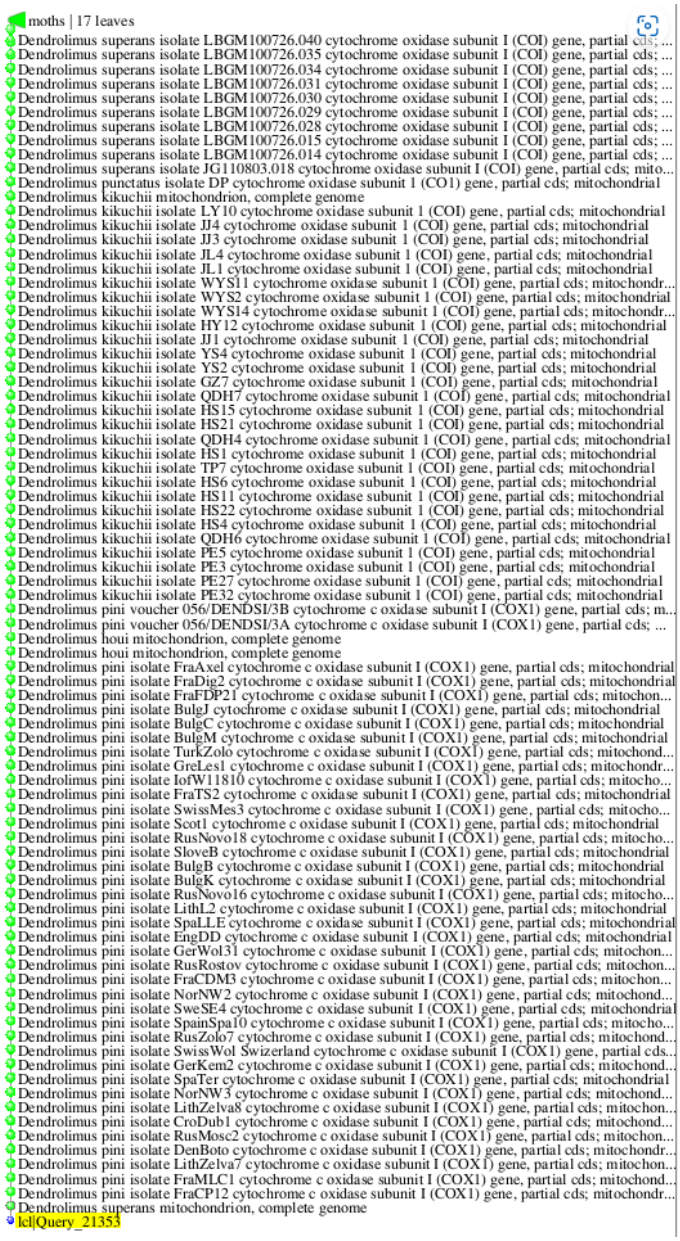
**3' *COI* sequencing:**

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.

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.

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

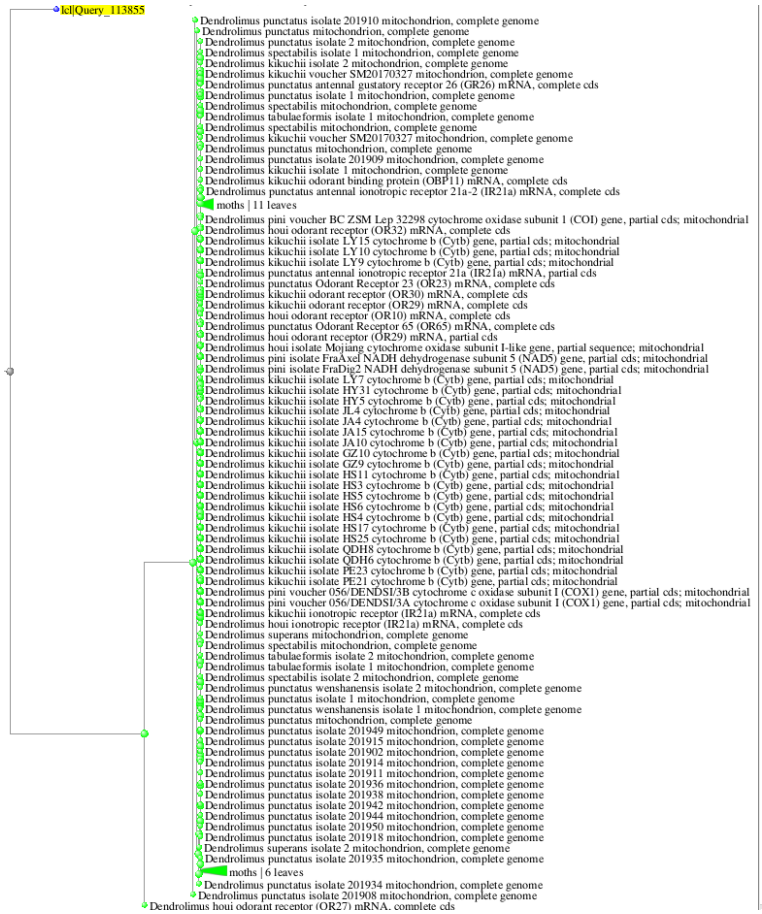
Search set to Dendrolimus




Search set to D. sibiricus

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:



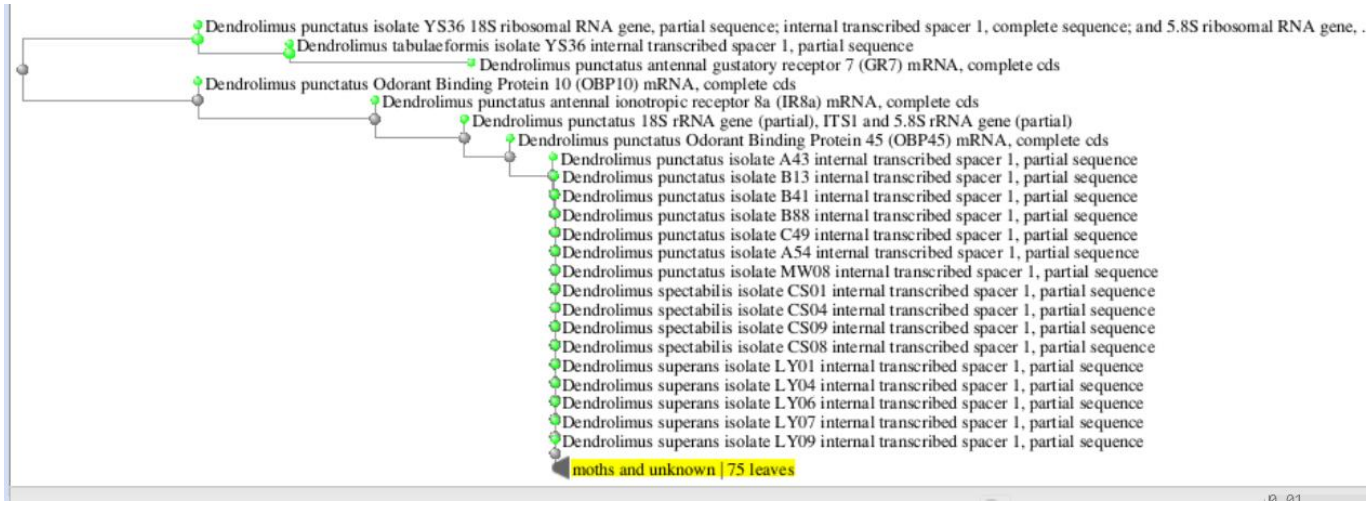

Seach set to *Dendrolimus sibiricus*


***ITS2* sequencing:**

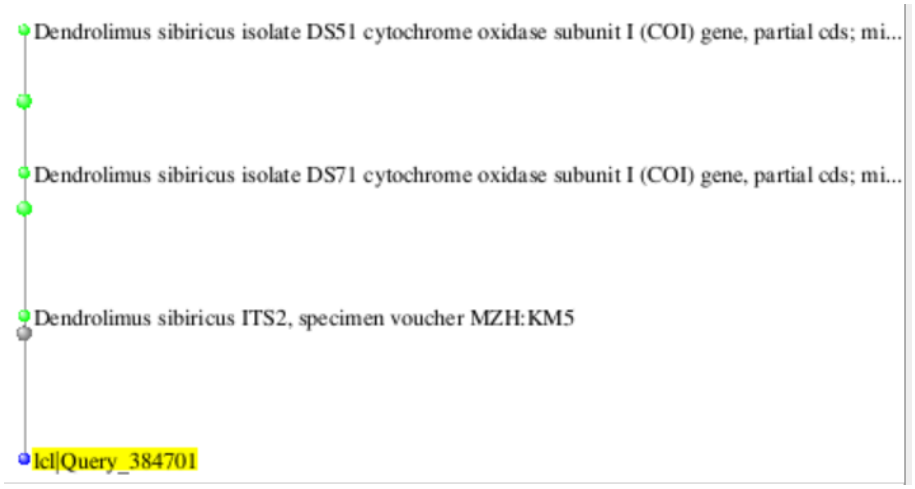
Kononov, A., Ustyantsev, K., Wang, B., Mastro, V. C., Fet, V., Blinov, A., & Baranchikov, Y. (2016). Genetic diversity among eight Dendrolimus species in Eurasia (Lepidoptera: Lasiocampidae) inferred from mitochondrial COI and COII, and nuclear ITS2 markers. BMC genetics, 17, 173-182.

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.

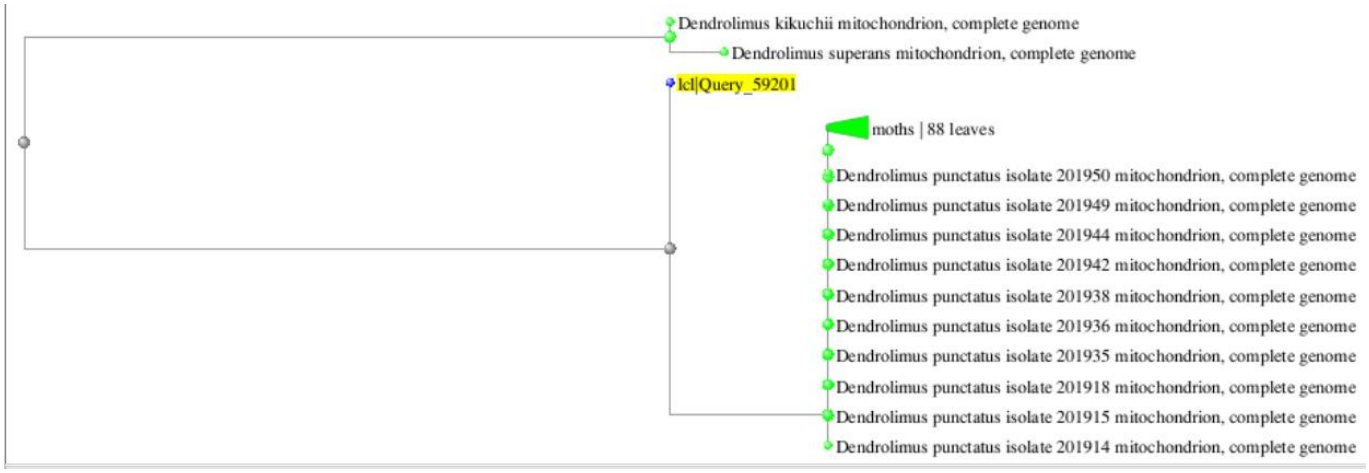
Fast Minimum Evolution trees for ITS2A:
Search set to Dendrolimus



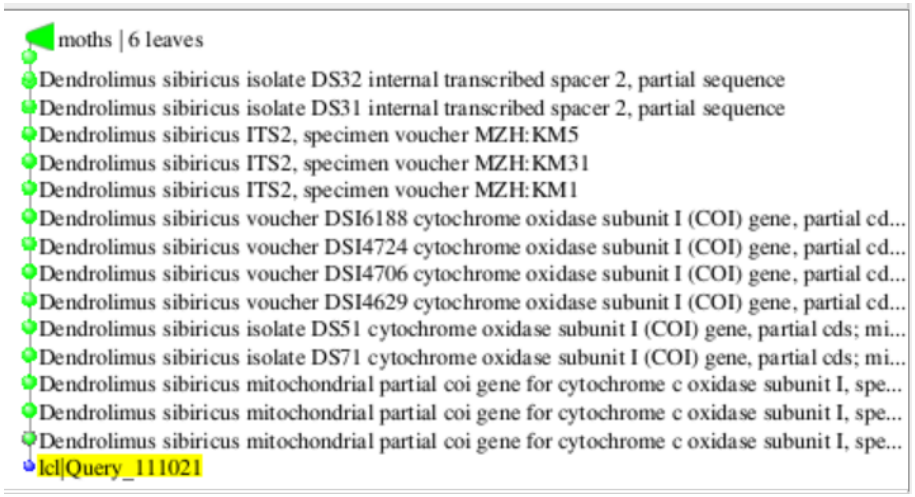
Search set to *Dendrolimus sibiricus*




Fast Minimum Evolution tree for ITS2B:
Search set to Dendrolimus




Search set to *Dendrolimus sibiricus*



## Appendix 3 – Specifications and parameters for the molecular tests

**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 µl | |
| Water | 6 | |
| Mastermix | 2 | 1x |
| Primer1: | 0,5 | 0,5µM |
| Primer2: | 0,5 | 0,5µM |
| ∑ | 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 µl | |
| Water | 6 | |
| Mastermix | 2 | 1x |
| Primer1: | 0,5 | 0,5µM |
| Primer2: | 0,5 | 0,5µM |
| ∑ | 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 | ∞ | |