



FINAL REPORT

Pretesting of molecular identification tests for *Aromia bungii* (Faldermann, 1835)

Contents

1. Introduction.....	2
2. Scope of pretesting	2
3. Test selection.....	3
4. Composition of samples	3
5. Specification of pretesting procedures	3
5.1 DNA extraction.....	3
5.2 Real-time PCR.....	3
5.3 Other molecular tests	4
6. Database inventory for sequence records	4
6.1 Results.....	4
6.2 Detailed information.....	4
6.3 Tree-based identification	5
7. Results of pretesting.....	5
8. Conclusions and discussion	6
9. Appendices	7
Appendix 1 - References.....	7
Appendix 2 – <i>In silico</i> data	8
Appendix 3 – Specifications and parameters for the molecular tests.....	21
Appendix 4 – Results of the real-time PCRs according to Rizzo <i>et al.</i> (2020).....	27
Appendix 5 – Results of the conventional PCRs according to Hajibabaei <i>et al.</i> (2006) Lee <i>et al.</i> (2021), Tamura <i>et al.</i> (2022), and Russo <i>et al.</i> (2020).....	27
Appendix 6 – Barcoding results according to Lee <i>et al.</i> (2021), Tamura <i>et al.</i> (2022), and Russo <i>et al.</i> (2020).....	28

1. Introduction



Fig. 1 Adult *Aromia bungii* specimen

(<https://www.boldsystems.org/pics/FBCOH/GBOL00002%2B1352481226.jpg>)

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.

Aromia bungii (Fig. 1), also called the peach musk beetle, is an oligophagous wood-borer of the family Cerambycidae from the order Coleoptera. The adult beetles measure between 22-38 mm in length and have a black body with a red pronotum.

Native to China and Vietnam, the beetle also occurs in Korea, Mongolia and Japan. In 2011 and 2016, isolated outbreaks were reported in the Bavaria region of Germany. Furthermore, in 2012 and 2016 the Campania region of Italy and in 2013 the Lombardy region of Italy were affected by this beetle (current spread see Fig. 2)

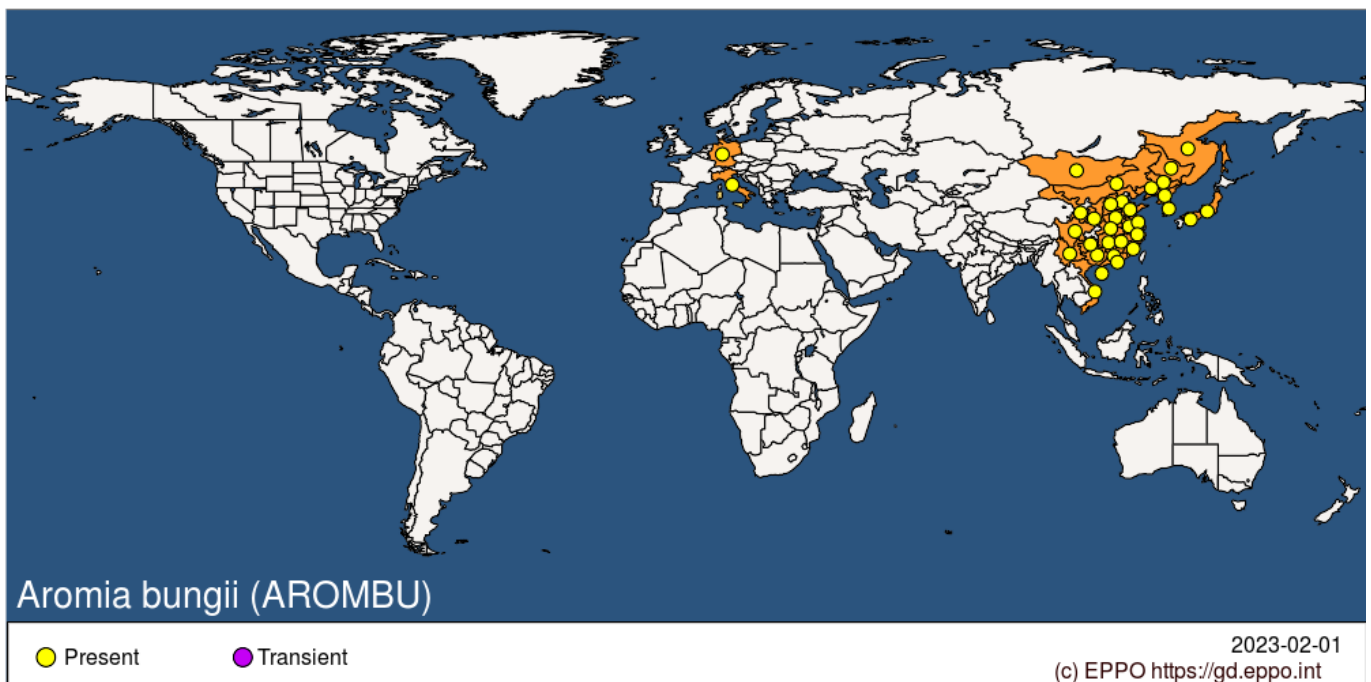


Fig. 2 Current distribution of *A. bungii*. according to EPPO global database (query 01.02.2023)

A. bungii typically infests healthy trees from the genus *Prunus*. In Germany the beetle has been found on *Prunus domestica* subsp. *insititia* and in Italy *Prunus armeniaca*, *Prunus domestica*, *Prunus persica*, *Prunus serotina*, *Prunus cerasifera* and *Prunus avium* trees have been infested.

A. bungii is a 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).

2. Scope of pretesting

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

3. Test selection

No published specific diagnostic protocols in international standards are available yet. An EPPO PM7 diagnostic protocol is under preparation. Molecular identification is possible by PCR amplification of the ribosomal *28S* and the mitochondrial *COI* genes (EFSA, 2019). Beside the barcoding primer sets listed in EPPO PM7/129(2), new primer sets for *A. bungii* were developed by Lee *et al.* 2021. Seven different haplotypes detected in Japanese population, designated A–G, based on the mitochondrial *COI* sequences are described by Tamura *et al.* (2022), whereas Korean samples were divided into three haplogroups, namely HG1–HG3. The identification of haplotypes can help to discriminate multiple introductions from human-mediated long-distance dispersal.

Rizzo *et al.* (2021) published two *A. bungii*-specific real-time PCRs, one TaqMan-based and one SYBR Green-based. Pest-specific real-time PCRs are a valuable tool in diagnostics, as they offer a quick and reliable detection method for specific pests. Additionally, a visual LAMP assay is included in this publication, however, this test was not considered as it only focuses on frass samples and on-site detection.

For pretesting the primer sets covered in the EPPO PM 7/129(2) DNA barcoding as an identification tool for a number of regulated pests (EPPO, 2021a, Appendix 1) were chosen as gold standard.

Additionally:

Specific identification tests in scientific literature for the identification of *A. bungii*.

- TaqMan real-time PCR test based on Rizzo *et al.* (2020); an inclusion in the EPPO diagnostic standard is under discussion
- SybrGreen real-time PCR based on Rizzo *et al.* (2020); an inclusion in the EPPO diagnostic standard is under discussion

Sequencing primers in scientific literature for the identification of *A. bungii*.

- 3 *COI* sequencing primer pairs according to Lee *et al.* (2021): ABC_F1/R2; ABC_F1/R3; ABC_F4/R2; expected amplicon sizes 1452bp, 808bp, and 736bp, respectively.
- A *COI* sequencing primer pair according to Tamura *et al.* (2022): CO1-Croz/tRNALeu-R; expected amplicon size 762bp
- A *28S* sequencing primer pair according to Russo *et al.* (2020): D2f/r; expected amplicon size 445bp

4. Composition of samples

For pretesting of tests indicated above, DNA of part of an adult specimen (part of leg) as well as frass of *A. bungii* was extracted destructively, whereas fragments of one larva were extracted non-destructively, using the DNeasy Blood and Tissue Kit (Qiagen) in all cases (see Tab. 1). The adult specimen included in this study was morphologically confirmed to the species level (pers. comm. C. Lethmayer, AGES). The larval sample was received as a non-target sample for the joined validation on *Anoplophora glabripennis/chinensis* with ANSES in 2022.

Table 1: Sample set for pretesting.

Sample	Species	Life stage	Host plant	Origin	Source
AnoL2100123	<i>A. bungii</i>	Larval fragments	Prunus sp.	Italy (Lombardia), 2019	ANSES (Taddei)
1771/18	<i>A. bungii</i>	Leg	na	Italy (Campania), 2017	AGES (Egartner)
1772/18	<i>A. bungii</i>	Frass	na	Italy (Campania), 2017	AGES (Egartner)

5. Specification of pretesting procedures

5.1 DNA extraction

For DNA extraction the DNeasy Blood and Tissue Kit (Qiagen) was used, either destructively for 1771/18 (leg) and 1772/18 (frass), or non-destructively for AnoL2100123 (larval fragments). Samples indicated in table 1 were tested undiluted and with at least one additional dilution (usually 1:20) for barcoding, and a dilution series was analysed with the two real-time PCRs (details see App. 3-6).

5.2 Real-time PCR

Following real-time PCR tests for the identification of *A. bungii* according to Rizzo *et al.* (2020) were evaluated:

- TaqMan based real-time PCR on the *COI* locus
- SYBR Green based real-time PCR on the *COII* locus

5.3 Other molecular tests

Included in EPPO PM 7/129(2) DNA barcoding (EPPO, 2021a):

- Barcoding for arthropods (Appendix 1); was tested for the identification of *A. bungii*.

Lee *et al.* (2021)

- 3 *COI* sequencing primer pairs: ABC_F1/R2; ABC_F1/R3; ABC_F4/R2

Tamura *et al.* (2022)

- *COI* sequencing primer pair: CO1-Croz/tRNA^{Leu}-R

Russo *et al.* (2020)

- 28S sequencing primer pair: D2f/r

6. 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 comparison. Not only the number, but also the genetic and geographic diversity of the records and the quality are potential issues that should be taken into account.

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 were checked and, if reasonable, the search was extended to other gene loci.

6.1 Results

In all three consulted databases sequence records for *A. bungii* on the *COI* locus are available. GenBank also offered 5 sequence records for the complete mitochondrial genome, as well as limited sequences of several other loci (e.g. *COII*, ribosomal DNA loci like the 28S locus, etc.). None of the three databases offered any records on the *ITS* locus (query date October 2022).

Table 2: Number of sequence records per gene for each database (data accessed November 2022). Sequence records relevant for tests included in this pretesting are highlighted in red.

Gene	GenBank	Q-Bank	Bold
<i>COI</i>	455	1	254
<i>COII</i>	1	0	0
<i>ITS2</i>	0	0	0
12S	1	0	0
16S	2	0	0
18S	2	0	0
28S	2	0	0
complete genome (mitochondrium)	5	0	0
other genes	42	0	0

6.2 Detailed information

NCBI GenBank and Bold database hold sequence records on the *COI* locus for two species: *A. bungii* and *A. moschata*. In the EPPO Q-Bank only one *COI* barcode for *A. bungii* is deposited. Two 28S sequences are available in GenBank.

Out of 261 *A. bungii* specimen found in the Bold database, 137 records had sequences of at least 500 bp. The total *Aromia* genus had 173 records with barcodes out of 304 specimen records.

The geographic variation of the sequence records for *A. bungii* in Bold is limited to only two regions in Europe (10 records from Italy, one from Germany), where the pest is known to have been present with restricted distribution and few occurrences, respectively. Otherwise all records are from Japan. The sole record found in EPPO Q-Bank is a EURL sequence from an adult female, which was sampled in China.

6.3 Tree-based identification

To evaluate the species divergences within the genus, a Neighbor Joining (NJ) tree of distance were constructed using NCBI GenBank (max. seq. diff. of 0.75) and *COI* sequences. Figure 3 revealed two clusters: *A. moschata* and *A. bungii*, that showed a clear separation between these two species.

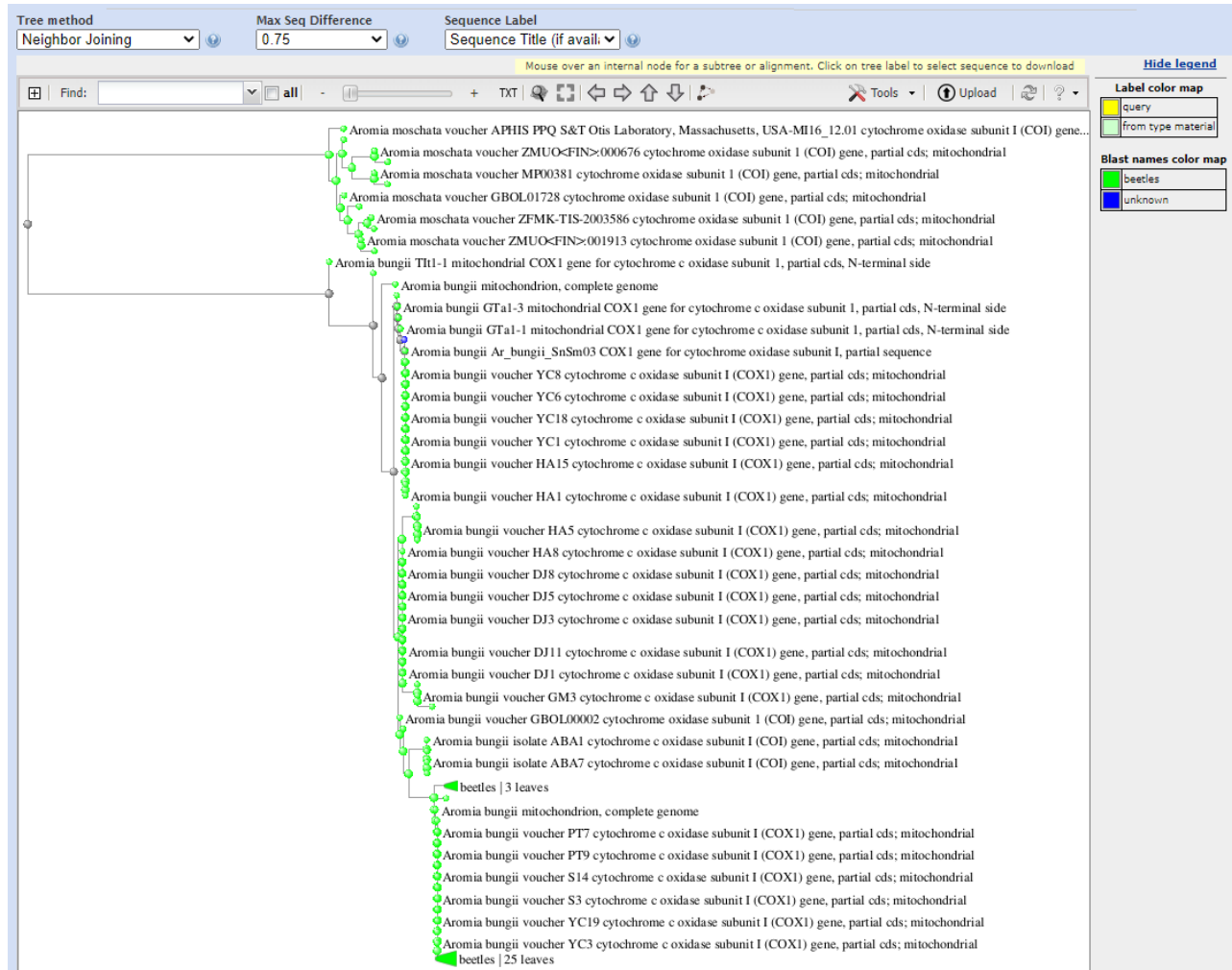


Fig 3: Neighbor joining tree for *A. bungii* on the *COI* locus

7. Results of pretesting

The real-time PCR tests on the sample set were suitable to identify *A. bungii* samples correctly (see Tab. 3). There was no deviation from the assigned values, except for the frass sample, whose lower dilutions seemed to inhibit the PCR reactions.

For the frass and non-destructively extracted larval fragment sample, sequencing was at times impossible due to low sequence quality, while all six sequencing methods tested resulted in positive results for the fresh adult leg sample. This suggests, that the type of sample is crucial, with some sequencing primer pairs being more susceptible to these differences in DNA quality than others (see Tab. 3). Especially when comparing the three PCRs according to Lee *et al.* (2021), the expected amplicon size seems to be an important factor.

Table 3: Results of pretesting with the sample set; results for *Aromia bungii* according to assigned values

Assigned value of the samples	Real-time PCR Rizzo et al., 2020		Barcoding					
	TaqMan	SYBR Green	EPPO 2021 COI	Lee 2021 (1)* COI	Lee 2021 (2)* COI	Lee 2021 (3)* COI	Tamura 2022 COI	Russo 2020 28S
<i>Aromia bungii</i> Anol2100123	Positive	Positive	Positive	Negative	Positive	Positive	Negative	Positive
<i>Aromia bungii</i> 1771/18	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
<i>Aromia bungii</i> 1772/18	Positive**	Positive**	Positive**	Positive**	Positive**	Positive**	(Positive)	Positive**

* (1): ABC_F1/R2; (2): ABC_F1/R3; (3): ABC_F4/R2

** inhibition in undiluted/ 1:20 sample

Detailed information see Appendix 4-6.

8. Conclusions and discussion

For molecular identification of *A. bungii* five different *COI* barcoding primer sets, one *28S* primer set and two real-time PCRs were pretested in this study.

In silico studies (database inventory, primer blast, including fast minimum evolution tree analysis, and *in silico* PCR) were conducted and showed suitability of the selected tests.

All tests could be successfully applied to the fresh adult leg specimen in our sample set (sample 1771/18), while the fragmented larval and frass samples (Anol2100123 and 1772/18) led to some issues with PCR inhibition and occasionally low sequence quality. The *28S* sequence analysis was successful for all three samples. As seen in this pretesting, DNA extracted from frass samples can cause inhibition during PCR reactions; the inclusion of higher dilutions of the samples allows accurate interpretation of negative results. Specimen type and DNA quality is a highly important criterium to consider when evaluating molecular tests. In this pretesting, especially the *COI* primers according to Tamura *et al.* (2022) did not yield amplicons or sequences of sufficient quality in two of the three samples tested. Similarly, the *COI* primer pair ABC_F1/ABC_F2 according to Lee *et al.* (2021) did not achieve a positive sequencing result for the larval fragment sample. In this case, the large expected amplicon size of 1452bp might factor in as well.

Therefore, a selection of primer pairs for *COI* sequencing as well as *28S* sequence analysis will be compared with real-time PCRs according to Rizzo *et al.* (2020) when conducting the validation study. The sample set for this study will be extended to more *A. bungii* specimens and closely related non-target Cerambycidae specimens. The intention is also to study the detection of this species in further frass samples, regarded this material will be available after the sample acquisition by the EURL.

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 Technical staff/ Operators: Claudia Heiss, Chiara Pohn

Date: December 13th, 2022



Helga Reisenzein
 EURL Deputy Director



Richard Gottsberger
 Scientific Project Leader – Molecular Unit

9. Appendices

Appendix 1 - References

- 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.
- Rizzo, D., Taddei, A., Da Lio, D., Nugnes, F., Barra, E., Stefani, L., Bartolini L., Griffo R. V., Spigno P., Cozzolino L., Rossi E. & Garonna, A. P. (2020). Identification of the red-necked longhorn beetle *Aromia bungii* (Faldermann, 1835)(Coleoptera: Cerambycidae) with real-time PCR on frass. Sustainability, 12(15), 6041.
- 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.
- Lee, S., Cha, D., Nam, Y., & Jung, J. (2021). Genetic diversity of a rising invasive pest in the native range: Population genetic structure of *aromia bungii* (coleoptera: Cerambycidae) in South Korea. Diversity, 13(11), 582.
- Tamura, S., & Shoda-Kagaya, E. (2022). Genetic Differences among Established Populations of *Aromia bungii* (Faldermann, 1835)(Coleoptera: Cerambycidae) in Japan: Suggestion of Multiple Introductions. Insects, 13(2), 217.
- Campbell, B. C., Steffen-Campbell, J. D., & Werren, J. H. (1994). Phylogeny of the *Nasonia* species complex (Hymenoptera: Pteromalidae) inferred from an internal transcribed spacer (ITS2) and 28S rDNA sequences. Insect molecular biology, 2(4), 225-237.
- Russo, E., Nugnes, F., Vicinanza, F., Garonna, A. P., & Bernardo, U. (2020). Biological and molecular characterization of *Aromia bungii* (Faldermann, 1835)(Coleoptera: Cerambycidae), an emerging pest of stone fruits in Europe. Scientific Reports, 10(1), 7112.
- Armstrong, K. F., & Ball, S. L. (2005). DNA barcodes for biosecurity: invasive species identification. Philosophical Transactions of the Royal Society B: Biological Sciences, 360(1462), 1813-1823.

Appendix 2 – *In silico* data

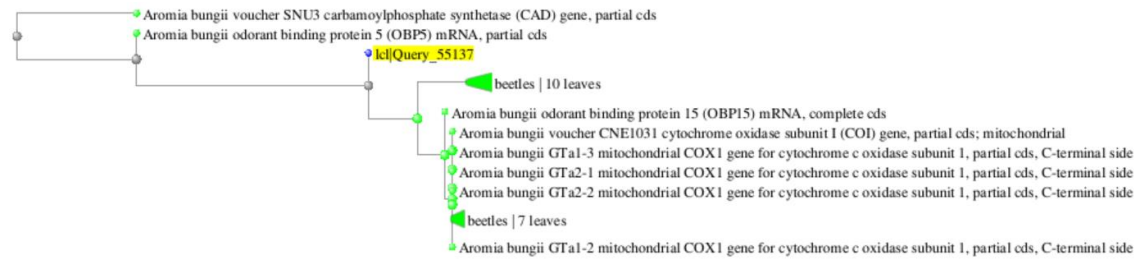
In silico data PCR assay 1

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.

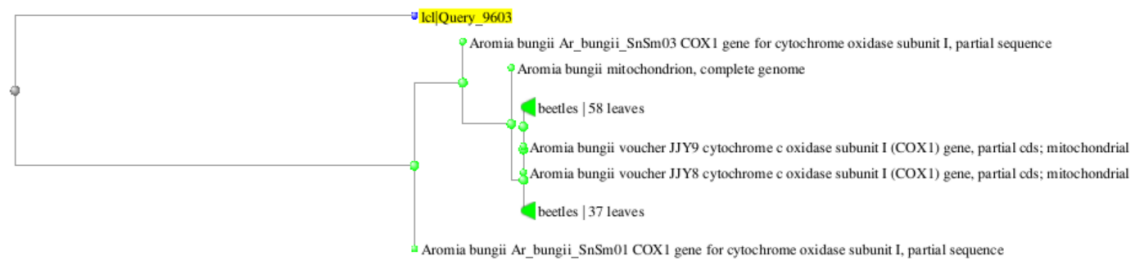
Hajibabaei, M., Janzen, D. H., Burns, J. M., Hallwachs, W., & Hebert, P. D. (2006). DNA barcodes distinguish species of tropical Lepidoptera. Proceedings of the National Academy of Sciences, 103(4), 968-971.

Search was restricted to *Aromia bungii*

Fast Minimum Evolution tree for LepF



Fast Minimum Evolution tree for LepR

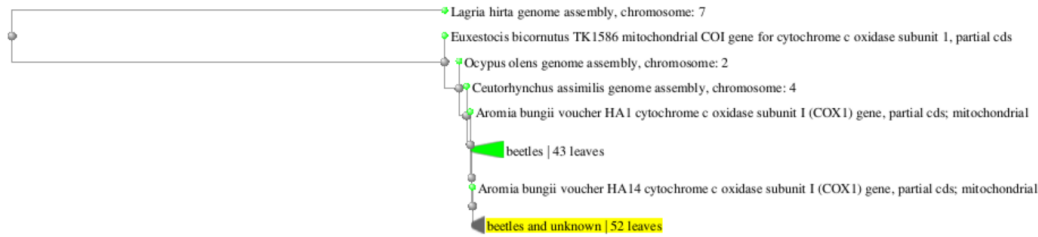


In silico data PCR assay 2 and 3

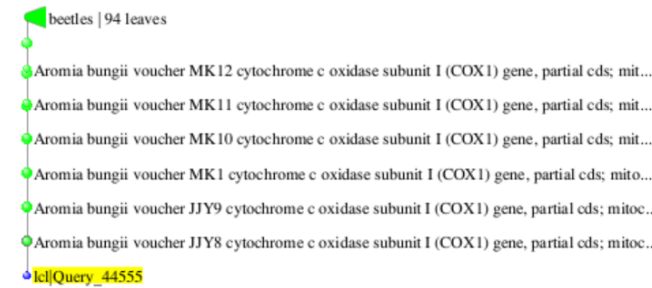
Rizzo, D., Taddei, A., Da Lio, D., Nugnes, F., Barra, E., Stefani, L., Bartolini L., Griffo R. V., Spigno P., Cozzolino L., Rossi E. & Garonna, A. P. (2020). Identification of the red-necked longhorn beetle *Aromia bungii* (Faldermann, 1835)(Coleoptera: Cerambycidae) with real-time PCR on frass. Sustainability, 12(15), 6041.

Search limited to Coleoptera

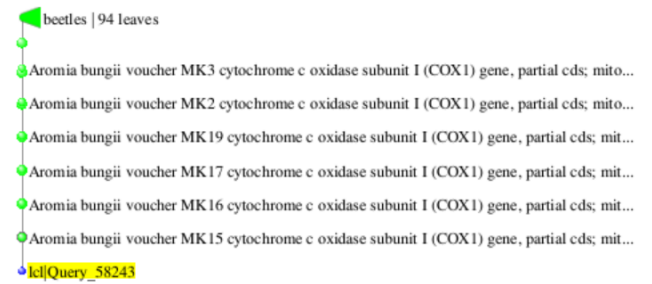
Fast Minimum Evolution tree for Abungii_285F



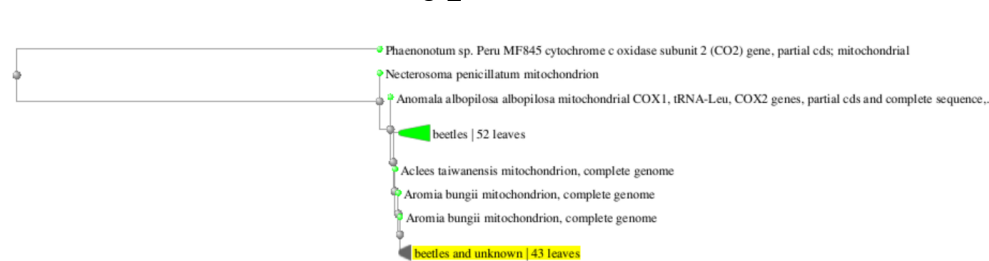
Fast Minimum Evolution tree for Abungii_484R



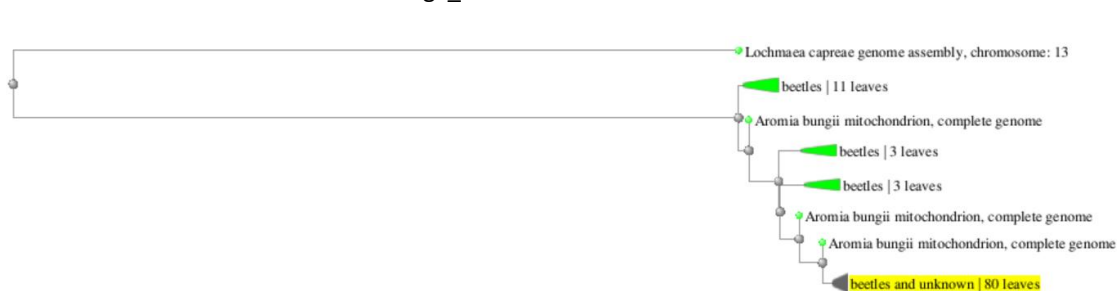
Fast Minimum Evolution tree for Abungii_309P



Fast Minimum Evolution tree for Abungii_436F



Fast Minimum Evolution tree for Abungii_592R



In silico PCR Abungii_436F/Abungii_592R

Primer pair 1

	Sequence (5'→3')
Forward primer	TAACTTCGGTCTATTAGATGTA
Reverse primer	GCTAACTTGGTTGATTTCG

Products on target templates

>OL415177.1 Aromia bungii mitochondrion, complete genome

product length = 157
Forward primer 1 TAACTTCGGTCTATTAGATGTA 22
Template 3417 3438

Reverse primer 1 GCTAACTTGGTTGATTTCG 18
Template 3573 3556

>OK393714.1 Aromia bungii mitochondrion, complete genome

product length = 157
Forward primer 1 TAACTTCGGTCTATTAGATGTA 22
Template 3417 3438

Reverse primer 1 GCTAACTTGGTTGATTTCG 18
Template 3573 3556

>MW617355.1 Aromia bungii mitochondrion, complete genome

product length = 157
Forward primer 1 TAACTTCGGTCTATTAGATGTA 22
Template 3417 3438

Reverse primer 1 GCTAACTTGGTTGATTTCG 18
Template 3573 3556

>JQ904852.1 Aromia bungii tRNA-Leu gene, partial sequence; and cytochrome oxidase subunit II gene, partial cds; mitochondrial

product length = 157
Forward primer 1 TAACTTCGGTCTATTAGATGTA 22
Template 436 457

Reverse primer 1 GCTAACTTGGTTGATTTCG 18
Template 592 575

>NC_053714.1 Aromia bungii mitochondrion, complete genome

product length = 157
Forward primer 1 TAACTTCGGTCTATTAGATGTA 22
Template 3417 3438

Reverse primer 1 GCTAACTTGGTTGATTTCG 18
Template 3573 3556

In silico data PCR assay 4 to 6

Lee, S., Cha, D., Nam, Y., & Jung, J. (2021). Genetic diversity of a rising invasive pest in the native range: Population genetic structure of *Aromia bungii* (coleoptera: Cerambycidae) in South Korea. *Diversity*, 13(11), 582.

Search was restricted to *Aromia bungii*

Fast Minimum Evolution tree for ABC_F1:

- beetles | 13 leaves
- Aromia bungii GTa1-3 mitochondrial COX1 gene for cytochrome c oxidase subunit I, partial...
- Aromia bungii GTa1-2 mitochondrial COX1 gene for cytochrome c oxidase subunit I, partial...
- Aromia bungii GTa1-1 mitochondrial COX1 gene for cytochrome c oxidase subunit I, partial...
- Aromia bungii mitochondrion, complete genome
- Aromia bungii voucher HNAU.PP 011 cytochrome oxidase subunit I gene, complete cds; mitoc...
- Aromia bungii mitochondrion, complete genome
- Aromia bungii isolate ABA7 cytochrome c oxidase subunit I (COI) gene, partial cds; mitoch...
- Aromia bungii isolate ABA6 cytochrome c oxidase subunit I (COI) gene, partial cds; mitoch...
- Aromia bungii cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
- Aromia bungii voucher CW7 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii odorant binding protein 6 (OBP6) mRNA, complete cds
- Aromia bungii odorant binding protein 4 (OBP4) mRNA, partial cds
- Aromia bungii voucher YY8 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher YY7 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher YY6 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher YY4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher YY3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher YY2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher YY15 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher YY14 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher YY13 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher YY11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher YY10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher YY1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher YW7 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher YW5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher YW4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher YW3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher YW2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher YW1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher YJ3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoch...
- Aromia bungii voucher YD9 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher YD8 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher YD6 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher YD5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher YD4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher YD3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher YD2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher YD15 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher YD14 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher YD13 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher YD12 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher YD11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher YD10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher YD1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher YC9 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher YC8 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher YC7 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher YC6 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher YC5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher YC4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher YC3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher YC20 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher YC19 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher YC18 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher YC15 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher YC11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher YC10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher YC1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher S9 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoch...
- Aromia bungii voucher S8 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoch...
- Aromia bungii voucher S6 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoch...
- Aromia bungii voucher S3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoch...
- Aromia bungii voucher S16 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoch...
- Aromia bungii voucher S15 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoch...
- Aromia bungii voucher S14 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoch...
- Aromia bungii voucher S13 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoch...
- Aromia bungii voucher S12 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoch...
- Aromia bungii voucher S11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoch...
- Aromia bungii voucher S10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoch...
- Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoch...
- Aromia bungii voucher PT9 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher PT8 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher PT7 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher PT6 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher PT5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher PT4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher PT3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher PT2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher PT11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher PT10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
- Aromia bungii voucher PT1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher OS2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- Aromia bungii voucher OS1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...
- klQuery_19705

In silico PCR ABC_F1/R2, search was restricted to Aromia

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity
Forward primer	GGAATAGTAGGAACTTCTTTGAG	23	54.22	39.13	5.00
Reverse primer	AATTGGCAGTTCTGAGTATCTATG	24	56.57	37.50	5.00

Products on target templates

>OL415177.1 Aromia bungii mitochondrion, complete genome

product length = 1452
 Forward primer 1 GGAATAGTAGGAACTTCTTTGAG 23
 Template 1498 1520
 Reverse primer 1 AATTGGCAGTTCTGAGTATCTATG 24
 Template 2949 2926

>MW617355.1 Aromia bungii mitochondrion, complete genome

product length = 1452
 Forward primer 1 GGAATAGTAGGAACTTCTTTGAG 23
 Template 1498 1520
 Reverse primer 1 AATTGGCAGTTCTGAGTATCTATG 24
 Template 2949 2926

>NC_053714.1 Aromia bungii mitochondrion, complete genome

product length = 1452
 Forward primer 1 GGAATAGTAGGAACTTCTTTGAG 23
 Template 1498 1520
 Reverse primer 1 AATTGGCAGTTCTGAGTATCTATG 24
 Template 2949 2926

>KF737790.1 Aromia bungii voucher HNAU.PP 011 cytochrome oxidase subunit I gene, complete cds; mitochondrial

product length = 1452
 Forward primer 1 GGAATAGTAGGAACTTCTTTGAG 23
 Template 79 101
 Reverse primer 1 AATTGGCAGTTCTGAGTATCTATG 24
 Template 1530 1507

>OK393714.1 Aromia bungii mitochondrion, complete genome

In silico PCR ABC_F1/R3, search was restricted to Aromia

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity
Forward primer	GGAATAGTAGGAACTTCTTTGAG	23	54.22	39.13	5.00
Reverse primer	CTACAGTAAATATGTGATGAGCTC	24	54.96	37.50	6.00

Products on target templates

>LC617367.1 Aromia bungii Ar_bungii_SnSm03 COX1 gene for cytochrome oxidase subunit I, partial sequence

product length = 808
 Forward primer 1 GGAATAGTAGGAACTTCTTTGAG 23
 Template 79 101
 Reverse primer 1 CTACAGTAAATATGTGATGAGCTC 24
 Template 886 863

>LC617366.1 Aromia bungii Ar_bungii_SnSm01 COX1 gene for cytochrome oxidase subunit I, partial sequence

product length = 808
 Forward primer 1 GGAATAGTAGGAACTTCTTTGAG 23
 Template 79 101
 Reverse primer 1 CTACAGTAAATATGTGATGAGCTC 24
 Template 886 863

>OL415177.1 Aromia bungii mitochondrion, complete genome

product length = 808
 Forward primer 1 GGAATAGTAGGAACTTCTTTGAG 23
 Template 1498 1520
 Reverse primer 1 CTACAGTAAATATGTGATGAGCTC 24
 Template 2305 2282

>OK393714.1 Aromia bungii mitochondrion, complete genome

product length = 808
 Forward primer 1 GGAATAGTAGGAACTTCTTTGAG 23
 Template 1498 1520
 Reverse primer 1 CTACAGTAAATATGTGATGAGCTC 24
 Template 2305 2282

>MW617355.1 Aromia bungii mitochondrion, complete genome

In silico PCR ABC_F4/R2, search was restricted to Aromia

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AGAAGCCTTTGGCACTCTCG	20	60.32	55.00	5.00	2.00
Reverse primer	AATTGGCAGTTCTGAGTATCTATG	24	56.57	37.50	5.00	0.00

Products on target templates

>OL415177.1 Aromia bungii mitochondrion, complete genome

product length = 736
 Forward primer 1 AGAAGCCTTTGGCACTCTCG 20
 Template 2214 2233
 Reverse primer 1 AATTGGCAGTTCTGAGTATCTATG 24
 Template 2949 2926

>MW617355.1 Aromia bungii mitochondrion, complete genome

product length = 736
 Forward primer 1 AGAAGCCTTTGGCACTCTCG 20
 Template 2214 2233
 Reverse primer 1 AATTGGCAGTTCTGAGTATCTATG 24
 Template 2949 2926

>NC_053714.1 Aromia bungii mitochondrion, complete genome

product length = 736
 Forward primer 1 AGAAGCCTTTGGCACTCTCG 20
 Template 2214 2233
 Reverse primer 1 AATTGGCAGTTCTGAGTATCTATG 24
 Template 2949 2926

>KF737790.1 Aromia bungii voucher HNAU.PP 011 cytochrome oxidase subunit I gene, complete cds; mitochondrial

product length = 736
 Forward primer 1 AGAAGCCTTTGGCACTCTCG 20
 Template 795 814
 Reverse primer 1 AATTGGCAGTTCTGAGTATCTATG 24
 Template 1530 1507

Fast Minimum Evolution tree for tRNA^{Leu}-R:



In silico PCR CO1-Croz/ tRNA^{Leu}-R, search was restricted to Aromia

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CAACATTATTTTGATTTTTTGGTCA	26	54.75	23.08	6.00	2.00
Reverse primer	GGGGTTTAAATCCATTGCAC	20	55.18	45.00	8.00	2.00

Products on target templates

>OK393714.1 Aromia bungii mitochondrion, complete genome

product length = 884

Forward primer 1 CAACATTATTTTGATTTTTTGGTCA 26
 Template 2113C.....A.. 2138

Reverse primer 1 GGGGTTTAAATCCATTGCAC 20
 Template 2996G....G..... 2977

>MW617355.1 Aromia bungii mitochondrion, complete genome

product length = 884

Forward primer 1 CAACATTATTTTGATTTTTTGGTCA 26
 Template 2113C.....A.. 2138

Reverse primer 1 GGGGTTTAAATCCATTGCAC 20
 Template 2996G....G..... 2977

>OL415177.1 Aromia bungii mitochondrion, complete genome

product length = 884

Forward primer 1 CAACATTATTTTGATTTTTTGGTCA 26
 Template 2113C.....A.. 2138

Reverse primer 1 GGGGTTTAAATCCATTGCAC 20
 Template 2996G....G..... 2977

>NC_053714.1 Aromia bungii mitochondrion, complete genome

product length = 884

Forward primer 1 CAACATTATTTTGATTTTTTGGTCA 26
 Template 2113C.....A.. 2138

Reverse primer 1 GGGGTTTAAATCCATTGCAC 20
 Template 2996G....G..... 2977

In silico data PCR assay 8

Russo, E., Nugnes, F., Vicinanza, F., Garonna, A. P., & Bernardo, U. (2020). Biological and molecular characterization of *Aromia bungii* (Faldermann, 1835)(Coleoptera: Cerambycidae), an emerging pest of stone fruits in Europe. *Scientific Reports*, 10(1), 7112.
Campbell, B. C., Steffen-Campbell, J. D., & Werren, J. H. (1994). Phylogeny of the *Nasonia* species complex (Hymenoptera: Pteromalidae) inferred from an internal transcribed spacer (ITS2) and 28S rDNA sequences. *Insect molecular biology*, 2(4), 225-237.

Search was restricted to *Aromia bungii*

Fast Minimum Evolution tree for 28S-D2 forward:



Fast Minimum Evolution tree for 28S-D2 reverse:

- Aromia bungii voucher SNU3 large subunit ribosomal RNA gene, partial sequence
- Aromia bungii voucher CNE1031 28S ribosomal RNA gene, partial sequence
- Aromia bungii odorant binding protein 13 (OBP13) mRNA, complete cds
- Aromia bungii mitochondrion, complete genome
- Aromia bungii mitochondrion, complete genome
- Aromia bungii mitochondrion, complete genome
- Aromia bungii mitochondrion, complete genome
- Aromia bungii voucher SNU3 small subunit ribosomal RNA gene, partial sequence
- Aromia bungii odorant binding protein 22 (OBP22) mRNA, complete cds
- Aromia bungii odorant binding protein 8 (OBP8) mRNA, complete cds
- Aromia bungii odorant binding protein 7 (OBP7) mRNA, complete cds
- Aromia bungii tRNA-Leu gene, partial sequence; and cytochrome oxidase subunit II gene, partial cds; mitocho
- Aromia bungii odorant binding protein 18 (OBP18) mRNA, complete cds
- Aromia bungii odorant binding protein 15 (OBP15) mRNA, complete cds
- Aromia bungii odorant binding protein 5 (OBP5) mRNA, partial cds
- Aromia bungii isolate ABA10 large subunit ribosomal RNA gene, partial sequence
- Aromia bungii isolate ABA9 large subunit ribosomal RNA gene, partial sequence
- Aromia bungii isolate ABA8 large subunit ribosomal RNA gene, partial sequence
- Aromia bungii isolate ABA7 large subunit ribosomal RNA gene, partial sequence
- Aromia bungii isolate ABA6 large subunit ribosomal RNA gene, partial sequence
- Aromia bungii isolate ABA5 large subunit ribosomal RNA gene, partial sequence
- Aromia bungii isolate ABA4 large subunit ribosomal RNA gene, partial sequence
- Aromia bungii isolate ABA3 large subunit ribosomal RNA gene, partial sequence
- Aromia bungii isolate ABA2 large subunit ribosomal RNA gene, partial sequence
- Aromia bungii isolate ABA1 large subunit ribosomal RNA gene, partial sequence
- Aromia bungii beta-actin mRNA, complete cds
- Aromia bungii odorant binding protein 20 (OBP20) mRNA, complete cds
- Aromia bungii odorant binding protein 10 (OBP10) mRNA, complete cds
- Aromia bungii odorant binding protein 4 (OBP4) mRNA, partial cds
- Aromia bungii odorant binding protein 1 (OBP1) mRNA, complete cds
- Aromia bungii voucher CW7 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
- Aromia bungii odorant binding protein 11 (OBP11) mRNA, partial cds
- Aromia bungii odorant binding protein 3 (OBP3) mRNA, partial cds
- [Ic|Query_49983](#)

In silico PCR 28S-D2 forward/reverse, search was restricted to Aromia

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CAACATTTATTTTGATTTTTGGTCA	26	54.75	23.08	6.00	2.00
Reverse primer	GGGGTTTAAATCCATTGCAC	20	55.18	45.00	8.00	2.00

Products on target templates

>OK393714.1 Aromia bungii mitochondrion, complete genome

product length = 884

Forward primer 1 CAACATTTATTTTGATTTTTGGTCA 26
 Template 2113C.....A.. 2138

Reverse primer 1 GGGGTTTAAATCCATTGCAC 20
 Template 2996G....G..... 2977

>MW617355.1 Aromia bungii mitochondrion, complete genome

product length = 884

Forward primer 1 CAACATTTATTTTGATTTTTGGTCA 26
 Template 2113C.....A.. 2138

Reverse primer 1 GGGGTTTAAATCCATTGCAC 20
 Template 2996G....G..... 2977

>OL415177.1 Aromia bungii mitochondrion, complete genome

product length = 884

Forward primer 1 CAACATTTATTTTGATTTTTGGTCA 26
 Template 2113C.....C.....A.. 2138

Reverse primer 1 GGGGTTTAAATCCATTGCAC 20
 Template 2996G....G..... 2977

>NC_053714.1 Aromia bungii mitochondrion, complete genome

product length = 884

Forward primer 1 CAACATTTATTTTGATTTTTGGTCA 26
 Template 2113C.....C.....A.. 2138

Reverse primer 1 GGGGTTTAAATCCATTGCAC 20
 Template 2996G....G..... 2977

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'- TAAACTTCTGGATGTCCAAAAAATCA-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:

Thermocycler 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	0,5 μ M
Primer2:	0,5	0,5 μ M
Σ	9	
DNA	1	

PCR conditions:

	$^{\circ}$ 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 (TaqMan real-time PCR)

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

Abungii_285F: 5'- CAGCAGTTCTTCTTTTATTATC -3'

Abungii_484R: 5'- GGTGTCCAAAGAATCAAA -3'

Abungii_309P: FAM- TACCAGTATTAGCAGGAGCCATTACG -TQ2*

* TQ2: Tide Quencher™ 2 phosphoramidite

Literature: Rizzo, D., Taddei, A., Da Lio, D., Nugnes, F., Barra, E., Stefani, L., Bartolini L., Griffo R. V., Spigno P., Cozzolino L., Rossi E. & Garonna, A. P. (2020). Identification of the red-necked longhorn beetle *Aromia bungii* (Faldermann, 1835)(Coleoptera: Cerambycidae) with real-time PCR on frass. *Sustainability*, 12(15), 6041.

Fragment length: 199bp

PCR - Parameters:

Analytic Jena qTower³ G (230 V) with accompanying software, Bio Molecular Systems Magnetic Induction Cycler (MIC) with accompanying software.

Mastermix: PerfeCTa qPCR ToughMix® Quanta Bio. Contains AccuStart II Taq DNA polymerase, AccuVue plate loading dye, MgCl₂, dNTPs

Composition:		Final concentration:
	Volume per reaction μ l	
Water	2	
Mastermix	5	1x
Primer1:	0.5	0.5 μ M
Primer2:	0.5	0.5 μ M
Probe	1	0.1 μ M
Σ	9	
DNA	1	

PCR conditions:

Step	°C	Duration (min., sec.)	Nr. of Cycles
Start	95	10 min	1
Denaturation	95	15 sec	45
Annealing/Extension and fluorescence reading	60	60 sec	

Specification of the PCR Assay 3 (SYBR Green real-time PCR)

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

Abungii_436F: 5'- TAACTTCCGTCTATTAGATGTA-3'

Abungii_592R 5'- GCTAACTTGGTTGATTCG-3'

Literature: Rizzo, D., Taddei, A., Da Lio, D., Nugnes, F., Barra, E., Stefani, L., Bartolini L., Griffo R. V., Spigno P., Cozzolino L., Rossi E. & Garonna, A. P. (2020). Identification of the red-necked longhorn beetle *Aromia bungii* (Faldermann, 1835)(Coleoptera: Cerambycidae) with real-time PCR on frass. *Sustainability*, 12(15), 6041.

Fragment length: 157bp

PCR - Parameters:

Analytic Jena qTower³ G (230 V) with accompanying software, Bio Molecular Systems Magnetic Induction Cycler (MIC) with accompanying software.

Mastermix: HOT FIREPol[®] EvaGreen[®] qPCR Mix (Solis Biodyne)

Composition:		Final concentration:
	Volume per reaction μ l	
Water	6	
Mastermix	2	1x
Primer1:	0,5	0.5 μ M
Primer2:	0,5	0.5 μ M
Σ	9	
DNA	1	

PCR conditions:

Step	$^{\circ}$ C	Duration (min., sec.)	Nr. of Cycles
Start	95	12 min	1
Denaturation	95	15 sec	45
Annealing	55	30 sec	
Extension and fluorescence reading	72	30 sec	
Melting curve	72-95	0.1 $^{\circ}$ C/s	Stepwise increment

Specification of the PCR Assay 4-6 (COI Barcoding according to Lee et al. 2021)

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

ABC_F1: 5'-GGA ATA GTA GGA ACT TCT TTG AG-3'

ABC_R2: 5'-AAT TGG CAG TTC TGA GTA TCT ATG-3'

ABC_R3: 5'-CTA CAG TAA ATA TGT GATGAG CTC-3'

ABC_F4: 5'-AGA AGC CTT TGG CAC TCT CG-3'

Literature: Lee, S., Cha, D., Nam, Y., & Jung, J. (2021). Genetic diversity of a rising invasive pest in the native range: Population genetic structure of *aromia bungii* (coleoptera: Cerambycidae) in South Korea. *Diversity*, 13(11), 582.

Fragment length ABC_F1/ABC_R2: 1452bp

Fragment length ABC_F1/ABC_R3: 808bp

Fragment length ABC_F4/ABC_R2: 736bp

PCR - Parameters:

Thermocycler 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	0,5 μ M
Primer2:	0,5	0,5 μ M
Σ	9	
DNA	1	

PCR conditions:

	$^{\circ}$ C	Duration (min., sec.)	Nr. of Cycles
Start	95	15 min	1
Denaturation	95	30 sec	39
Annealing	55	30 sec	
Extension	72	45 sec	
Final extension	72	7 min	1
Cooling	15	∞	

Specification of the PCR Assay 7 (COI Barcoding according to Tamura et al. 2022)

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

CO1-Croz: 5'- CAA CAT TTA TTT TGAT TTT TTG GTC A-3'

tRNA^{Leu}-R: 5'- GGG GTT TAA ATC CAT TGC AC-3'

Literature: Tamura, S., & Shoda-Kagaya, E. (2022). Genetic Differences among Established Populations of *Aromia bungii* (Faldermann, 1835)(Coleoptera: Cerambycidae) in Japan: Suggestion of Multiple Introductions. *Insects*, 13(2), 217.

Fragment length: 762bp

PCR - Parameters:

Thermocycler 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	0,5 μ M
Primer2:	0,5	0,5 μ M
Σ	9	
DNA	1	

PCR conditions:

	$^{\circ}$ C	Duration (min., sec.)	Nr. of Cycles
Start	95	15 min	1
Denaturation	95	30 sec	39
Annealing	45	30 sec	
Extension	72	45 sec	
Final extension	72	7 min	1
Cooling	15	∞	

Specification of the PCR Assay 8 (285)

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

D2 forward: 5'-AGT CGT GTT GCT TGA TAG TGC AG-3'

D2 reverse: 5'-TTG GTC CGT GTT TCA AGA CCG G-3'.

Literature:

Russo, E., Nugnes, F., Vicinanza, F., Garonna, A. P., & Bernardo, U. (2020). Biological and molecular characterization of *Aromia bungii* (Faldermann, 1835)(Coleoptera: Cerambycidae), an emerging pest of stone fruits in Europe. *Scientific Reports*, 10(1), 7112.

Campbell, B. C., Steffen-Campbell, J. D., & Werren, J. H. (1994). Phylogeny of the *Nasonia* species complex (Hymenoptera: Pteromalidae) inferred from an internal transcribed spacer (ITS2) and 28S rDNA sequences. *Insect molecular biology*, 2(4), 225-237.

Fragment length: 445bp

PCR - Parameters:

Thermocycler 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	0,5 μ M
Primer2:	0,5	0,5 μ M
Σ	9	
DNA	1	

PCR conditions:

	$^{\circ}$ C	Duration (min., sec.)	Nr. of Cycles
Start	95	15 min	1
Denaturation	95	30 sec	39
Annealing	48	30 sec	
Extension	72	30 sec	
Final extension	72	5 min	1
Cooling	15	∞	

Appendix 4 – Results of the real-time PCRs according to Rizzo *et al.* (2020)

Sample	Ct TaqMan	Ct SYBR Green
Anol 2100 123 1:20	22.80	19.81
Anol 2100 123 1:200	26.32	23.09
Anol 2100 123 1:2.000	29.85	26.63
Anol 2100 123 1:20.000	33.63	30.79
Anol 2100 123 1:200.000	37.32	-
Anol 2100 123 1:2.000.000	-	40.47
1771/18	12.84	10.04
1771/18 1:20	18.11	14.46
1771/18 1:200	21.95	18.22
1771/18 1:2000	25.15	21.69
1772/18	-	-
1772/18 1:20	21.52	-
1772/18 1:200	25.16	22.09
1772/18 1:2000	28.57	25.28

Appendix 5 – Results of the conventional PCRs according to Hajibabaei *et al.* (2006) Lee *et al.* (2021), Tamura *et al.* (2022), and Russo *et al.* (2020)

Sample	Hajibabaei <i>et al.</i> (2006) LepF/R	Lee <i>et al.</i> (2021) ABC_F1/R2	Lee <i>et al.</i> (2021) ABC_F1/R3	Lee <i>et al.</i> (2021) ABC_F4/R2	Tamura <i>et al.</i> (2022) CO1-Croz/tRNA ^{Leu} -R	Russo <i>et al.</i> (2020) D2f/r
1771/18	+	+	+	+	+	+
1771/18 1:20	+	+	+	+	+	+
1772/18	-	-	-	-	-	-
1772/18 1:20	+	+	+	+	+	+
Anol2100123	+	(+)	+	+	-	+
Anol2100123 1:20	+	-	(+)	+	-	+

Appendix 6 – Barcoding results according to Lee *et al.* (2021), Tamura *et al.* (2022), and Russo *et al.* (2020)

PCR	Sample	contig	length	HQ	result	% identity	Accession
COI 1 (Lee)	1771/18	yes	1360	96.5	A. bungii	99.71	LC617367.1
	1772/18	yes	1362	81.4	A. bungii	99.49	LC617367.1
	AnoL2100123	no	1300 r	81.7	A. bungii	97.03	LC617367.1
COI 2 (Lee)	1771/18	yes	761	100	A. bungii	99.61	LC617367.1
	1772/18	yes	741	92.8	A. bungii	99.46	LC617367.1
	AnoL2100123	yes	777	94.6	A. bungii	100	LC617367.1
COI3 (Lee)	1771/18	yes	702	99.7	A. bungii	99.86	LC617367.1
	1772/18	yes	704	99.3	A. bungii	99.86	LC617367.1
	AnoL2100123	yes	695	100	A. bungii	100	LC574291.1
COI4 (Tamura)	1771/18	yes	842	100	A. bungii	99.76	MW617355.1
	1772/18	yes	857	58.5	A. bungii	98.23	MW617355.1
28S (Russo)	1771/18	yes	585	99.8	A. bungii	100	HQ832606.1
	1772/18	yes	585	100	A. bungii	100	HQ832606.1
	AnoL2100123	yes	585	100	A. bungii	100	HQ832606.1