



FINAL REPORT

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

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1. Introduction



Fig. 1 Adult Aromia bungii specimen (https://www.boldsystems.org/pics/FBCOH/GBOL00002%2B1352 481226.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)



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 amplicons 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.

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

Table 1: Sample set for pretesting.

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/tRNALeu-R

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Russo et al. (2020)
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• 285 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 comparision. 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 avaible. GenBank also offered 5 sequence records for the complete mitochondiral 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).

Gene	GenBank	Q-Bank	Bold
СОІ	455	1	254
COII	1	0	0
ITS2	0	0	0
125	1	0	0
16S	2	0	0
185	2	0	0
285	2	0	0
complete genome (mitochondrium)	5	0	0
other genes	42	0	0

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.

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 occurences, 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.

Tree method	Max Seq Difference	Sequence Label	
Neighbor Joining 🗸	0.75 🗸 🕑	Sequence nue (n avail. 🕶 🦦	
		Mouse over an internal node for a subtree or alignment. Click on tree label to select sequence to download	Hide legend
E Find:	▼ all - 1	🚃 + TXT 🔍 🚼 🗘 🗘 🏠 🖓 🤔	Label color map
	Aromia moscha Aromia moscha Aromia moscha Aromia moscha Aromia moscha Aromia moscha Aromia bungii Th Aromia bungii Th Aromi Ar	ta voucher APHIS PPQ S&T Ois Laboratory, Massachusetts, USA-MII6 [2.01 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial toochata voucher APHIOS PIN>000676 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial oschata voucher MPO0381 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial chata voucher GBOL01728 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial chata voucher GBOL01728 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial chata voucher ZPMK-TIS-2003586 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial chata voucher ZPMK-TIS-2003586 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial chata voucher ZPMK-TIS-2003586 cytochrome oxidase subunit 1, partial cds, N-terminal side ina bungii GTa1-1 mitochondrial COX1 gene for cytochrome c oxidase subunit 1, partial cds, N-terminal side mia bungii voucher YCS cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial mia bungii voucher YCS cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial mia bungii voucher YCI cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial mia bungii voucher YCI cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial mia bungii voucher HA15 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial mia bungii voucher HA5 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial mia bungii voucher HA5 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial mia bungii voucher JA8 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial mia bungii voucher JA8 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial mia bungii voucher JJ3 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial mia bungii voucher JJ3 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial mia bungii voucher JJ3 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial mia	query from type material Blast names color map peetles unknown

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.

	Real-time PCR F	Rizzo et al., 2020			Barco	oding		
Assigned value of the samples	TaqMan	SYBR Green	EPPO 2021 <i>COI</i>	Lee 2021 (1)* <i>COI</i>	Lee 2021 (2)* <i>COI</i>	Lee 2021 (3)* <i>COI</i>	Tamura 2022 <i>COI</i>	Russo 2020 28S
Aromia bungii AnoL2100123	Positive	Positive	Positive	Negative	Positive	Positive	Negative	Positive
Aromia bungii 1771/18	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Aromia bungii 1772/18	Positive**	Positive**	Positive**	Positive**	Positive**	Positive**	(Positive)	Positive**

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

* (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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Date: December 13th, 2022

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Helga Reisenzein EURL Deputy Director

- Gould. Bulley

Richard Gottsberger Scientific Project Leader – Molecular Unit

9. Appendices

Appendix 1 - References

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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.

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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



Aromia bungii Ar_bungii_SnSm01 COX1 gene for cytochrome oxidase subunit I, partial sequence

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

beetles | 94 leaves

Fast Minimum Evolution tree for Abungii_285F



Fast Minimum Evolution tree for Abungii_484R

beetles 94 leaves
•
Aromia bungii voucher MK12 cytochrome c oxidase subunit I (COX1) gene, partial cds; mit
Aromia bungii voucher MK11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mit
Aromia bungii voucher MK10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mit
Aromia bungii voucher MK1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito
 Aromia bungii voucher JJY9 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
Aromia bungii voucher JJY8 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
Icl Query_44555

Fast Minimum Evolution tree for Abungii_309P

Aromia bungii voucher MK3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
 Aromia bungii voucher MK2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito...
 Aromia bungii voucher MK19 cytochrome c oxidase subunit I (COX1) gene, partial cds; mit...
 Aromia bungii voucher MK17 cytochrome c oxidase subunit I (COX1) gene, partial cds; mit...
 Aromia bungii voucher MK16 cytochrome c oxidase subunit I (COX1) gene, partial cds; mit...
 Aromia bungii voucher MK16 cytochrome c oxidase subunit I (COX1) gene, partial cds; mit...
 Aromia bungii voucher MK15 cytochrome c oxidase subunit I (COX1) gene, partial cds; mit...
 Aromia bungii voucher MK15 cytochrome c oxidase subunit I (COX1) gene, partial cds; mit...

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		TAACTTCCGTCTATTAGATGTA
Reverse primer		GCTAACTTGGTTGATTCG
Products on target t	emplate	25
>0L415177.1 Aromia	bungii	mitochondrion, complete genome
product length	- 157	
Forward primer	1	TAACTTCCGTCTATTAGATGTA 22
Template	3417	
Reverse primer	1	GCTAACTTGGTTGATTCG 18
Template	3573	
>OK393714.1 Aromia	a bungii	mitochondrion, complete genome
	-	
ppoduct longth	157	
Forward primer	1 1	TAACTTCCGTCTATTAGATGTA 22
Template	3417	3438
Reverse primer	1	GCTAACTTGGTTGATTCG 18
Template	3573	
>MW617355.1 Arom	ia bungi	ii mitochondrion, complete genome
	_	
ppoduct longth	157	
Forward primer	1 1	TAACTTCCGTCTATTAGATGTA 22
Template	3417	3438
Reverse primer	1	GCTAACTTGGTTGATTCG 18
Template	3573	
>JQ904852.1 Aromia	a bungii	tRNA-Leu gene, partial sequence; and cytochrome oxidase subunit II gene, partial cds; mitochondrial
nroduct length	- 157	
Forward primer	1	TAACTTCCGTCTATTAGATGTA 22
Template	436	457
Reverse primer	1	GCTAACTTGGTTGATTCG 18
Template	592	
>NC_053714.1 Arom	ia bungi	ii mitochondrion, complete genome
product length	= 157	
Forward primer	1	TAACTTCCGTCTATTAGATGTA 22
Template	3417	
Reverse primer	1	GCTAACTTGGTTGATTCG 18
rempiate	35/3	

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:

Stat Miniam Evolution tree for ABC_F1: New Wey State Provide State Amage Wey State Provide State </t beetles | 13 leaves

<text>

Fast Minimum Evolution tree for ABC_R3:

Aromia bungii voucher YY4 cytochrome c oxidase subunit I (COX1) gene, partial c	ds; mitoc
Aromia bungii voucher YY3 cytochrome c oxidase subunit I (COX1) gene, partial c	ds; mitoc
Aromia bungii voucher YY2 cytochrome c oxidase subunit I (COX1) gene, partial c	ds; mitoc
Aromia bungii voucher YY15 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 c	ds; mitoc
Aromia bungii voucher YW/ cytochrome c oxidase subunit I (COXI) gene, partial (eds; mito
Aromia bungii voucher YW5 cytochrome c oxidase subunit I (COX1) gene, partial (eds: 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 YI3 cytochrome c oxidase subunit I (COX1) gene, partial of	ds: mitoch
Aromia bungii voucher YD9 cytochrome c oxidase subunit I (COX1) gene, partial c	ds; mitoc
Aromia bungii voucher YD8 cytochrome c oxidase subunit I (COX1) gene, partial c	ds; mitoc
Aromia bungii voucher YD6 cytochrome c oxidase subunit I (COX1) gene, partial c	ds; mitoc
Aromia bungii voucher YD5 cytochrome c oxidase subunit I (COX1) gene, partial c	ds; mitoc
Aromia bungii voucher YD3 cytochrome c oxidase subunit I (COX1) gene, partial c	ds: mitoc
Aromia bungii voucher YD2 cytochrome c oxidase subunit I (COX1) gene, partial c	ds; 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 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 c	ds; mitoc
Aromia bungii voucher YC9 cytochrome c oxidase subunit I (COX1) gene, partial c	ds; mitoc
Aromia bungii voucher YC8 cytochrome c oxidase subunit I (COXI) gene, partial c	ds; mitoc
Aromia bungii voucher YC6 cytochrome c oxidase subunit I (COXI) gene, partial c	ds: mitoc
Aromia bungii voucher YC5 cytochrome c oxidase subunit I (COX1) gene, partial c	ds; mitoc
Aromia bungii voucher YC4 cytochrome c oxidase subunit I (COX1) gene, partial c	ds; mitoc
Aromia bungii voucher YC3 cytochrome c oxidase subunit I (COXI) gene, partial c	ds; mitoc
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
Anomia bungii voucher 10 stochrome e ovidase subunit I (COVI) gene, partial ede	de mitoo
\sim Alonna dunyii youcher 37 cytochronie c oxidane sudunit i (COAT) yene. Dartai cus	ds; mitoc
Aromia bungii voucher S8 cytochrome c oxidase subunit I (COXI) gene, partial cds	ds; mitoc ; mitocho ; mitocho
Aromia bungii voucher S8 cytochrome c'oxidase subunit ((COXI) gene, partial cds Aromia bungii voucher S8 cytochrome c oxidase subunit ((COXI) gene, partial cds	ds; mitoc ; mitocho ; mitocho ; mitocho
Aromia bungii voucher 39 cytochrome c oxidase subunit I (COXI) gene, partial cds Aromia bungii voucher S6 cytochrome c oxidase subunit I (COXI) gene, partial cds Aromia bungii voucher S6 cytochrome c oxidase subunit I (COXI) gene, partial cds Aromia bungii voucher S1 cytochrome c oxidase aubunit I (COXI) gene, partial cds	ds; mitoch ; mitocho ; mitocho ; mitocho ; mitocho ; mitocho
Aromia bungii voucher 39 cytochrome c oxidase subunit 1 (COX1) gene, partial cds Aromia bungii voucher S6 cytochrome c oxidase subunit 1 (COX1) gene, partial cds Aromia bungii voucher S6 cytochrome c oxidase subunit 1 (COX1) gene, partial cds Aromia bungii voucher S3 cytochrome c oxidase subunit 1 (COX1) gene, partial cds Aromia bungii voucher S16 cytochrome c oxidase subunit 1 (COX1) gene, partial cds Aromia bungii voucher S16 cytochrome c oxidase subunit 1 (COX1) gene, partial cds	ds; mitoch ; mitocho ; mitocho ; mitocho ; mitocho ls; mitocho ls; mitoch
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Aromia bungii voucher 35 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S6 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S15 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S15 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher S15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher S12 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher S12 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher S11 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher S10 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher S10 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P19 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P19 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P17 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P17 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P11 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P11 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher N15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii vo	ds; mitoc ; mitocho ; mitocho ; mitocho ; mitocho fs; mitocho fs; mitocho fs; mitocho fs; mitoch fs; mitoc fs; mitoc fs
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 Aromia bungii voucher 35 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S6 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S16 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S16 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S15 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S15 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S13 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher S11 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher S11 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher S11 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P19 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P19 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P17 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P17 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunitI	ds; mitoc ; mitocho ; mitocho ; mitocho fs; mitocho fs; mitocho fs; mitocho fs; mitoch fs; mitoc fs; mitoc fs
 Aromia bungi voucher 35 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S6 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P17 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P17 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P11 cytochrome c oxidase subunit I (COX1) g	ds; mitoc; ; mitocho; ; mitocho; s; mitocho; s; mitocho; fs; mitocho fs; mitocho fs; mitoch fs; mitoc fs; mitoc.
Aromia bungii voucher S5 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S6 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P10 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P10 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P17 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P17 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P17 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P17 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P15 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P12 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P13 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher P13 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher N14 cytochrome c oxidase subunit I (COX1) gene, partial cd Aromia bungii voucher N15 c	ds; mitoc; ; mitocho; ; mitocho; ; mitocho; fs; mitocho; fs; mitocho; fs; mitocho fs; mitoch fs; mitoch fs; mitocho fs; mitocho fs; mitocho fs; mitocho fs; mitocho fs; mitocho fs; mitoc fs; m
 Aromia bungii voucher 35 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S6 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S11 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher S10 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher PT9 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher PT9 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher PT9 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher PT9 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher PT9 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher PT5 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher PT5 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher PT1 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher PT1 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher PT1 cytochrome c oxidase subunit I (COX1) gene, partial cds Aromia bungii voucher PT1 cytochrome c oxidase	ds; mitoc ; mitocho ; mitocho ; mitocho fs; mitocho fs; mitoch fs; mitoc fs;

Fast Minimum Evolution tree for ABC_F4:

,	beetles 16 le	eaves	
ł	Aromia bungii	voucher	YY4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
1	Aromia bungii	voucher	YY3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
3	Aromia bungii Aromia bungii	voucher	YY15 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
4	Aromia bungii	voucher	YY14 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito
2	Aromia bungii	voucher	YY13 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito
2	Aromia bungii Aromia bungii	voucher	YY10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito YY10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito
4	Aromia bungii	voucher	YY1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito
9	Aromia bungii	voucher	YW7 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito
a	Aromia bungii	voucher	YW5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito
4	Aromia bungii	voucher	YW4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito
5	Aromia bungii	voucher	YW3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito
2	Aromia bungii Aromia bungii	voucher	YW2 cytochrome c oxidase subunit I (COXI) gene, partial cds; mito YW1 cytochrome c oxidase subunit I (COXI) gene partial cds; mito
4	Aromia bungii	voucher	YJ3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoch.
9	Aromia bungii	voucher	YD9 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
2	Aromia bungii Aromia bungii	voucher	YD8 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc YD6 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
ą	Aromia bungii	voucher	YD5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
5	Aromia bungii	voucher	YD4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
2	Aromia bungii Aromia bungii	voucher	YD3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc VD2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
ą	Aromia bungii	voucher	YD15 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito
9	Aromia bungii	voucher	YD14 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito
2	Aromia bungii	voucher	YD13 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito VD12 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito
3	Aromia bungii	voucher	YD11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito
9	Aromia bungii	voucher	YD10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito
2	Aromia bungii	voucher	YD1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc XC9 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
3	Aromia bungii	voucher	YC8 cytochrome c oxidase subunit I (COXI) gene, partial cds; mitoc
9	Aromia bungii	voucher	YC7 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
2	Aromia bungii	voucher	YC6 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
ą	Aromia bungii	voucher	YC4 cytochrome c oxidase subunit I (COXI) gene, partial cds; mitoc
9	Aromia bungii	voucher	YC3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
2	Aromia bungii	voucher	YC20 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito VC19 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito
4	Aromia bungii	voucher	YC18 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito
9	Aromia bungii	voucher	YC15 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito
2	Aromia bungii Aromia bungii	voucher	YC11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito VC10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito
4	Aromia bungii	voucher	YC1 cytochrome c oxidase subunit I (COXI) gene, partial cds; mito
2	Aromia bungii	voucher	S9 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitocho
2	Aromia bungii Aromia bungii	voucher	S8 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitocho S6 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitocho
4	Aromia bungii	voucher	S3 cytochrome c oxidase subunit I (COXI) gene, partial cds; mitocho
3	Aromia bungii	voucher	S16 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoch
3	Aromia bungii Aromia bungii	voucher	S15 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoch S14 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoch
4	Aromia bungii	voucher	S13 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoch
3	Aromia bungii	voucher	S12 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoch
3	Aromia bungii	voucher	S11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoch S10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoch
9	Aromia bungii	voucher	S1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitocho
2	Aromia bungii	voucher	PT9 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
3	Aromia bungii	voucher	PT7 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
9	Aromia bungii	voucher	PT6 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
2	Aromia bungii Aromia bungii	voucher	P15 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc PT4 cytochrome c oxidase subunit I (COX1) gene partial cds; mitoc
4	Aromia bungii	voucher	PT3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
9	Aromia bungii	voucher	PT2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
2	Aromia bungii Aromia bungii	voucher	PT11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito PT10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito
4	Aromia bungii	voucher	PT1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mito
9	Aromia bungii	voucher	OS2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
2	Aromia bungii Aromia bungii	voucher	OS1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc NS4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
4	Aromia bungii	voucher	NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
2	Aromia bungii	voucher	NS2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
3	Aromia bungii Aromia bungii	voucher	INST cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc MK9 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
ġ	Aromia bungii	voucher	MK8 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
2	Aromia bungii	voucher	MK7 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
ð	Aromia bungii Aromia bungii	voucher	MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
ġ	Aromia bungii	voucher	MK4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc.
9	Aromia bungii	voucher	MK3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
a	Aromia bungii	voucher	MK19 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc
9	Aromia bungii	voucher	MK17 cytochrome c oxidase subunit I (COX1) gene, partial cds; mit
4	Icl Query_117.	55	

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

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

Primer pair 1						Primer pair 1						
Forward primer Reverse primer	Sequence (5'->3') GGAATAGTAGGAACTTCTTTGAG AATTGGCAGTTCTGAGTATCTATG	Length 23 24	Tm 54.22 56.57	GC% 39.13 37.50	Self complementarity 5.00 5.00	Forward primer Reverse primer	Seque GGAA CTAC	nce (5'->3') TAGTAGGAACTTCTTTGAG AGTAAATATGTGATGAGCTC	Length 23 24	Tm 54.22 54.96	GC% 39.13 37.50	Self complementarity 5.00 6.00
Products on target >0L415177.1 Aromi	templates a bungii mitochondrion, complete genome					Products on target >LC617367.1 Aromi	templa a bungi	tes i Ar_bungii_SnSm03 COX1 gene for c	ytochrome	oxidase	subuni	I, partial sequence
product length Forward primer Template	= 1452 1 GGAATAGTAGGAACTTCTTTGAG 1498	23 1520				product length Forward primer Template	= 808 1 79	GGAATAGTAGGAACTTCTTTGAG 23	3 91			
Reverse primer Template	1 AATTGGCAGTTCTGAGTATCTATG 2949	24 2926				Reverse primer Template	1 886	CTACAGTAAATATGTGATGAGCTC	24 863			
>MW617355.1 Aron	nia bungii mitochondrion, complete genome					>LC617366.1 Aromi	a bungi	i Ar_bungii_SnSm01 COX1 gene for c	ytochrome	oxidase	e subunit	I, partial sequence
product length Forward primer Template	= 1452 1 GGAATAGTAGGAACTTCTTTGAG 1498	23 1520				product length Forward primer Template	= 808 1 79	GGAATAGTAGGAACTTCTTTGAG 23	3			
Reverse primer Template	1 AATTGGCAGTTCTGAGTATCTATG 2949	24 2926				Reverse primer Template	1 886	CTACAGTAAATATGTGATGAGCTC	24 863			
>NC_053714.1 Aron	nia bungii mitochondrion, complete genome					>OL415177.1 Arom	a bungi	i mitochondrion, complete genome				
product length Forward primer Template	= 1452 1 GGAATAGTAGGAACTTCTTTGAG 1498	23 1520				product length Forward primer Template	= 808 1 1498	GGAATAGTAGGAACTTCTTTGAG	23 1520			
Reverse primer Template	1 AATTGGCAGTTCTGAGTATCTATG 2949	24 2926				Reverse primer Template	1 2305	CTACAGTAAATATGTGATGAGCTC	24 2282			
>KF737790.1 Aromi	a bungii voucher HNAU.PP 011 cytochrome	oxidase sul	ounit I g	ene, co	mplete cds; mitochondrial	>OK393714.1 Arom	ia bungi	ii mitochondrion, complete genome				
product length Forward primer Template	= 1452 1 GGAATAGTAGGAACTTCTTTGAG 23 79 10	3 91				product length Forward primer Template	= 808 1 1498	GGAATAGTAGGAACTTCTTTGAG	23 1520			
Reverse primer Template	1 AATTGGCAGTTCTGAGTATCTATG 1530	24 1507				Reverse primer Template	1 2305	CTACAGTAAATATGTGATGAGCTC	24 2282			
>0K393714.1 Aromi	ia bungii mitochondrion, complete genome					>MW617355.1 Aron	nia bung	jii mitochondrion, complete genome				

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

	Sequence (5'->3')		Length	Tm	GC%	Self complementarity	Self 3' complementarit
Forward primer	AGAAGCCTTTGGCACT	CTCG	20	60.32	55.00	5.00	2.00
Reverse primer	AATTGGCAGTTCTGAG	TATCTATG	24	56.57	37.50	5.00	0.00
Products on target	templates						
>OL415177.1 Aromi	a bungii mitochondrion,	complete genome					
product length	= 736						
Forward primer	1 AGAAGCCTTT	GGCACTCTCG 20					
Template	2214	223	3				
Reverse primer	1 AATTGGCAGT	TCTGAGTATCTATG	24				
Template	2949		2926				
>MW617355.1 Aron	ia bungii mitochondrion	, complete genome					
product length	= 736						
Forward primer	1 AGAAGCCTTT	GGCACTCTCG 20					
Template	2214	223	3				
Reverse primer	1 AATTGGCAGT	TCTGAGTATCTATG	24				
Template	2949		2926				
>NC_053714.1 Aron	ia bungii mitochondrion	, complete genome					
product length	= 736						
Forward primer	1 AGAAGCCTTT	GGCACTCTCG 20					
Template	2214	223	3				
Reverse primer	1 AATTGGCAGT	TCTGAGTATCTATG	24				
Template	2949		2926				
>KF737790.1 Aromi	a bungii voucher HNAU.I	PP 011 cytochrome	oxidase su	ıbunit I g	jene, coi	mplete cds; mitochondrial	
product length	= 736						
Forward primer	1 AGAAGCCTTTG	GCACTCTCG 20					
Template	795	814					
Reverse primer	1 AATTGGCAGT	TCTGAGTATCTATG	24				
Template	1530		1507				

In silico data PCR assay 7

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.

Search was restricted to Aromia bungii

Fast Minimum Evolution tree for CO1-Croz:

setles | 15 leaves

1	
	Aromia bungii voucher YW5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
	Aromia bungii voucher YW4 cytochrome c oxidase subunit I (COX1) gene, partial cds: mitochondrial
4	Aromia bungii youcher YW3 cytochrome c oxidase subunit I (COX1) gene, partial cds: mitochondrial
- 2	Aromia bungii youchar XW2 outochrome c ovidase subunit I (COX1) gene, partial edge mitochondrial
- 2	Adoma bungi voucher I w2 cytochronie c oxidase subunit (COAT) gene, partial cus, mitochondrial
	Aromia bungii voucher Y w1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
	Aromia bungii voucher YJ3 cytochrome c oxidase subunit I (COXI) gene, partial cds; mitochondrial
-	Aromia bungii voucher YD9 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
	Aromia bungii voucher YD8 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
- (Aromia bungii voucher YD6 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
4	Aromia bungii youcher YD5 cytochrome c oxidase subunit I (COX1) gene, partial cds: mitochondrial
4	Aromia bungii youcher YD4 cytochrome c oxidase subunit I (COX1) gene, partial cds: mitochondrial
- 2	Aromia bungai voucher VD3 attechrome c oxidase subunit I (COVI) gene partial ede; mitochondrial
- 2	A single burger volume in the solution of the
- 2	Aromia bungii voucher 1D2 cytochrome c oxidase subunit 1 (COX1) gene, partiai cos; mitochondriai
- 2	Aromia bungii voucher YD15 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
	Aromia bungii voucher YD14 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
1	Aromia bungii voucher YD13 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
- (Aromia bungii voucher YD12 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
- 6	Aromia bungii voucher YD11 cytochrome c oxidase subunit I (COX1) gene, partial cds: mitochondrial
4	Aromia bungii youcher YD10 cytochrome c oxidase subunit L (COX1) gene, partial cds: mitochondrial
4	Aromia bungii youcher YD1 cytochrome c oxidase subunit I (COX1) gene partial cds; mitochondrial
- 2	Aromia bungii voucher IDT estechnome e oxidase subunit I (COXT) gene, partial eds; mitcehondrial
- 2	Alonia bulgi volcher 1C4 cytochrone c oxidase submit 1 (COAT) gene, partial cis, intochondrial
	Aromia bungii voucher YC3 cytochrome c oxidase subunit I (COXI) gene, partial cds; mitochondrial
	Aromia bungii voucher YC20 cytochrome c oxidase subunit I (COXI) gene, partial cds; mitochondrial
1	Aromia bungii voucher YC19 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
1	Aromia bungii voucher YC11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
- 1	Aromia bungii voucher YC10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
- (Aromia bungii voucher S9 cvtochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
4	Aromia bungii youcher S8 cytochrome c oxidase subunit I (COX1) gene, partial cds: mitochondrial
4	Aromia bungii youcher S6 cytochrome c oxidase subunit I (COX1) sene, partial cdc, mitchondrial
- 2	Aromia bungii youdha S2 attachang a condace gibunit I (COVI) gana partial das mitashandhial
- 2	Aromia bungii voucher 55 cytoenome e oxidase subunit ((COXI) gene, partai cus, intocholidiai
- 2	Aroma bungi voucher 516 cytochrome c oxidae subunit I (COXI) gene, partial cds, mitochondrial
- 2	Aromia bungii voucher S15 cytochrome c oxidase subunit I (COXI) gene, partial cds; mitochondrial
- 3	Aromia bungii voucher S14 cytochrome c oxidase subunit I (COXI) gene, partial cds; mitochondrial
- 2	Aromia bungii voucher \$15 cytochrome c oxidase subunit I (COXI) gene, partial cds; mitochondrial
	Aromia bungii voucher \$12 cytochrome c oxidase subunit I (COXI) gene, partial cds; mitochondrial
1	Aromia bungii voucher S11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
	Aromia bungii voucher S10 cytochrome c oxidase subunit I (COX1) gene, partial cds; nutochondrial
1	Aromia bungii voucher S1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
1	Aromia bungii voucher PT9 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
1	Aromia bungii voucher PT8 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
- 1	Aromia bungii voucher PT7 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
1	Aromia bungii voucher PT6 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
	Aromia bungii voucher PT5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
1	Aromia bungii voucher PT4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
	Asomia hungii youshas DT2 aitashsama a ayidasa suhunit L(COV1) aona nagtial ada, mitashandsial
1	Aroma bungii voucher P15 cytochronie c oxidase subunit I (COA1) gene, partial cus; mitochondrial
1	Aromia bungii voucher PT3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
	Aromia bungii voucher PT2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
	Aromia bungii voucher PTS cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PTI cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PTI cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PTI cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial
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	Aromia bungii voucher PT12 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher OS1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher OS1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher OS1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK9 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK7 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK7 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK7 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK19 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK19 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aro
	Aromia bungii voucher PT2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher OS1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher OS1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK9 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK8 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK8 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK6 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK6 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK6 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Arom
	Aromia bungii voucher PT2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher OS2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher OS2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher OS1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK17 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK17 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK16 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aro
	Aromia bungii voucher PT2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher OS1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher OS1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK9 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK19 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK19 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK16 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aro
	Aromia bungii voucher PT2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher OS2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher OS1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS9 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS9 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK6 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK6 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK6 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK17 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK17 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK17 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aro
	Aromia bungii voucher PT2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher OS1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher OS1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK7 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK7 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK7 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK19 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK16 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Arom
	Aromia bungii voucher PT2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher OS1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher OS1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK6 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK6 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK19 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK19 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Arom
	Aromia bungii voucher PT2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher OS1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher OS1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher OS1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK7 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK7 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK16 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK16 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Arom
	Aromia bungii voucher PT2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher OS1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher OS1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS4 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS15 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia
	Aromia bungii voucher PT2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT11 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT10 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher PT1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher OS1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher OS1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher NS3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS9 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS9 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MS9 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK6 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK6 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK6 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK12 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK12 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK12 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aromia bungii voucher MK16 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial Aro

Aromia bungii mitochondrion, complete genome

Fast Minimum Evolution tree for tRNALeu-R:

Aromia bungii mitochondrion, complete genome
Aromia bungii mitochondrion, complete genome
 • Aromia bungii mitochondrion, complete genome
Aromia bungii tRNA-Leu gene, partial sequence; and cytochrome oxidase subunit II gene, partial eds; mitochondrial
Aromia bungii OHa1-4 mitochondrial COX1 gene for cytochrome c oxidase subunit 1, partial cds, C-terminal side
 Aromia bungii OHa1-1 mitochondrial COX1 gene for cytochrome c oxidase subunit 1, partial cds, C-terminal side
Aromia bungii ATo1-1 mitochondrial COX1 gene for cytochrome c oxidase subunit 1, partial cds, C-terminal side
Aromia bungii SSo1-1 mitochondrial COX1 gene for cytochrome c oxidase subunit 1, partial cds, C-terminal side
Aromia bungii GTa2-2 mitochondrial COX1 gene for cytochrome c oxidase subunit 1, partial cds, C-terminal side
Aromia bungii GTa2-1 mitochondrial COX1 gene for cytochrome c oxidase subunit 1, partial cds, C-terminal side
Aromia bungii GTa1-3 mitochondrial COX1 gene for cytochrome c oxidase subunit 1, partial cds, C-terminal side
Aromia bungii GTa1-2 mitochondrial COX1 gene for cytochrome c oxidase subunit 1, partial cds, C-terminal side
Aromia bungii GTa1-1 mitochondrial COX1 gene for cytochrome c oxidase subunit 1, partial cds, C-terminal side
vlcl[Query_36137
Aromia bungii odorant binding protein 1 (OBP1) mRNA, complete cds
Aromia bungii odorant binding protein 12 (OBP12) mRNA, complete cds
Aromia bungii Ar_bungii_SnSm03 COX1 gene for cytochrome oxidase subunit I, partial sequence
Aromia bungii Ar_bungii_SnSm01 COX1 gene for cytochrome oxidase subunit I, partial sequence
Aromia bungii odorant binding protein 8 (OBP8) mRNA, complete cds
Aromia bungii odorant binding protein 2 (OBP2) mRNA, complete cds
Aromia bungii odorant receptor 1 (OR1) mRNA, partial cds
Aromia bungii odorant binding protein 24 (OBP24) mRNA, complete cds
Aromia bungii odorant binding protein 16 (OBP16) mRNA, complete cds
Aromia bungii TIt1-1 mitochondrial COX1 gene for cytochrome c oxidase subunit 1, partial cds, N-terminal side
Aromia bungii OHa1-4 mitochondrial COX1 gene for cytochrome c oxidase subunit 1, partial cds, N-terminal side
Aromia bungii OHa1-1 mitochondrial COX1 gene for cytochrome c oxidase subunit 1, partial cds, N-terminal side
Aromia bungii GTa2-2 mitochondrial COX1 gene for cytochrome c oxidase subunit 1, partial cds, N-terminal side
Aromia bungii GTa2-1 mitochondrial COX1 gene for cytochrome c oxidase subunit 1, partial cds, N-terminal side
Aromia bungii GTa1-3 mitochondrial COX1 gene for cytochrome c oxidase subunit 1, partial cds, N-terminal side
Aromia bungii GTa1-2 mitochondrial COX1 gene for cytochrome c oxidase subunit 1, partial cds, N-terminal side
Aromia bungii GTa1-1 mitochondrial COX1 gene for cytochrome c oxidase subunit 1, partial cds, N-terminal side

Aromia bungii mitochondrion, complete genome

In silico PCR CO1-Croz/ tRNALeu-R, search was restricted to Aromia

Forward primer Reverse primer	Sequence (5'->3') CAACATTTATTTTGATTTTTGGTCA GGGGTTTAAATCCATTGCAC		Length 26 20	Tm 54.75 55.18	GC% 23.08 45.00	Self complementarity 6.00 8.00	Self 3' complementarity 2.00 2.00
Products on target	template	S					
>OK393714.1 Arom	ia bungii	mitochondrion, complete genome					
product length Forward primer Template	= 884 1 2113	CAACATTTATTTTGATTTTTTGGTCA	26 2138				
Reverse primer Template	1 2996	GGGGTTTAAATCCATTGCAC 20 GG 2977					
>MW617355.1 Aror	nia bungi	i mitochondrion, complete genome					
product length Forward primer Template	= 884 1 2113	CAACATTTATTTTGATTTTTTGGTCA	26 2138				
Reverse primer Template	1 2996	GGGGTTTAAATCCATTGCAC 20 GG. 2977					
>0L415177.1 Arom	ia bungii	mitochondrion, complete genome					
product length Forward primer Template	= 884 1 2113	CAACATTTATTTTGATTTTTTGGTCA	26 2138				
Reverse primer Template	1 2996	GGGGTTTAAATCCATTGCAC 20 GG 2977					
>NC_053714.1 Aror	nia bungi	ii mitochondrion, complete genome					
product length Forward primer Template	= 884 1 2113	CAACATTTATTTTGATTTTTTGGTCA	26 2138				

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 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 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

Icl|Query 49983

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

Forward primer	Seq	uence (5'->3')		Length	Tm 54.75	GC%	Self complementarity	Self 3' complementarity
Reverse primer GGGGTTTAAATCCATTGCAC			20	55.18	55.18 45.00	8.00	2.00	
Products on target	templat	25						
•OK393714.1 Aromi	a bungii	mitochondrion, complete genome						
product length	= 884							
orward primer	1	CAACATTTATTTTGATTTTTTGGTCA	26					
ſemplate	2113	CA	2138					
Reverse primer	1	GGGGTTTAAATCCATTGCAC 20						
emplate	2996	GG 2977						
MW617355.1 Aron	nia bung	i mitochondrion, complete genome						
product length	- 884							
orward primer	1	CAACATTTATTTTGATTTTTTGGTCA	26					
emplate	2113	CA	2138					
Reverse primer	1	GGGGTTTAAATCCATTGCAC 20						
Template	2996	GG 2977						
OL415177.1 Aromi	a bungii	mitochondrion, complete genome						
product length	= 884							
orward primer	1	CAACATTTATTTTGATTTTTTGGTCA	26					
Template	2113	C	2138					
averse primer	1	GGGGTTTAAATCCATTGCAC 20						
Template	2996	GG 2977						
NC_053714.1 Aron	nia bung	ii mitochondrion, complete genome						
product length	= 884							
orward primer	1	CAACATTTATTTTGATTTTTTGGTCA	26					
Template	2113	C	2138					
Reverse primer	1	GGGGTTTAAATCCATTGCAC 20						
cover be primer	÷	GGGGTTTAAATCCATTOCAC 20						

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 μ l	
Water	6	
Mastermix	2	1x
Primer1:	0,5	0,5μM
Primer2:	0,5	0,5μΜ
Σ	9	
DNA	1	

	°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'- CAGCAGTTCTTCTTTATTATC -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 µl	
Water	2	
Mastermix	5	1x
Primer1:	0.5	0.5μΜ
Primer2:	0.5	0.5μΜ
Probe	1	0.1µM
Σ	9	
DNA	1	

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

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 µl	
Water	6	
Mastermix	2	1x
Primer1:	0,5	0.5μΜ
Primer2:	0,5	0.5μΜ
Σ	9	
DNA	1	

Step	°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°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:

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	0,5μΜ
Primer2:	0,5	0,5μΜ
Σ	9	
DNA	1	

	°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' tRNALeu-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:

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	0,5μΜ
Primer2:	0,5	0,5μΜ
Σ	9	
DNA	1	

	°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 CGG 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:

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
Σ	9	
DNA	1	

	°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	8	

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/tRNALeu-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