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Plant host differences between *Cossus redtenbacheri* and *Cossus insularis*: insights from mechanical tests and molecular phylogeny

Fredd VERGARA[1], R. Craig EVERROAD[2], Guadalupe ANDRACA[3], Jun KIKUCHI[1,4,5,6], Hiroshi MAKIHARA[7]
[1]*Advance NMR Metabomics Research Team, RIKEN Plant Science Center, Yokohama, Japan*
[2]*RIKEN Advanced Science Institute, Wako, Japan*
[3]*Instituto de Ecología, Universidad Nacional Autónoma de México, Mexico*
[4]*Biomass Engineering Program, RIKEN, Yokohama, Japan*
[5]*Graduate School of Nanobiosciences, Yokohama City University, Japan*
[6]*Graduate School Bioagricultural Sciences, Nagoya University, Japan*
[7]*Forestry and Forest Products Research Institute, Tsukuba, Japan*

Abstract

Larvae of the family Cossidae are mostly associated with trees. *Cossus redtenbacheri* (Hammerschmidt) is exceptional in this family because its larvae live in agave. Two approaches were followed to better characterize this host difference. First, a mechanical test was performed to evaluate the gallery making ability of *C. redtenbacheri* and *Cossus insularis* (Staudinger) larvae transferred to non-natural hosts of differing tissue hardness. Second, the first molecular evolutionary analysis of *C. redtenbacheri* was performed to assess its relationship with selected wood-feeding Cossidae species. This phylogenetic reconstruction was based on partial sequences of mitochondrial cytochrome c oxidase subunit 1. The agave-feeding phenotype is confirmed as a derived phylogenetic character within the genus *Cossus*. The results show that the host difference is not due to mechanical constraints or to phylogentic distinctiveness. The possible types of ecological factors that might explain the host specialization of *C. redtenbacheri* are discussed.

Key words: 28S, borer insects, CO1, *Cossus redtenbacheri*, *Comadia redtenbacheri*, *Cossus insularis*.

Introduction

Cossus redtenbacheri (Hammerschmidt) (= *Comadia redtenbacheri*) is unusual among the Cossidea (Lepidoptera) because its larvae develop in Agavaceae species. The larvae of other species in this worldwide family develop in trees and shrubs where they usually bore galleries in branches and trunks (Davis *et al*., 2008). Anyway, regarding *Cossus cossus* L. (European goat moth), the larvae can bore artichokes (Inserra, 1962) and exceptionally sugar bet roots (Ugolini, 1962-63). It is possible also rear *C. cossus* on artificial diet (Gavioli and Baronio, 1987). Agave differs from trees in having soft rather than hard tissues and different nutrient content (Ortiz-Basurto *et al*., 2008). Agave also has different chemical defences (Simmons-Boyce and Winston, 2007). Thus the host-shift of *C. redtenbacheri* is an interesting ecological question.

There is an additional, applied, importance to understanding the mechanisms underlying the difference in cossid feeding niches. This is because both *C. redtenbacheri* and agave are culturally and economically important in Mexico. The larvae, commonly called *gusano rojo de maguey* (agave red worm), are used in cooking and the production of spirits (Ramos-Elorduy *et al*., 2011). Larvae for these purposes are currently collected from the wild. This reduces the natural population of the species and leads to the destruction of agave and the agave habitat. These negative effects would be alleviated by culturing *C. redtenbacheri* and culturing would be assisted by knowing how the species came to specialize on agave instead of trees.

A first step in identifying the correct mechanism behind the host differences within the Cossidae is therefore to test and, if possible, exclude some of the potential mechanisms. Consequently, we have initiated a project of investigating agave use by *C. redtenbacheri* and of feeding niches in the Cossidae. As part of this study we applied two approaches. The first of these was to examine the mechanical boring ability of larvae. If *C. redtenbacheri* is able to bore into wood, its specialization on agave is not due to a simple physiological or mechanical inability to bore into wood. Likewise, if *Cossus insularis* (Staudinger), a typical wood boring species, bores into agave, the wood-agave distinction cannot result from physiological or mechanical factors related to gallery creation. We therefore used a gallery making assay with *C. redtenbacheri* and *C. insularis*.

The second approach was to identify the phylogenetic position of *C. redtenbacheri* within *Cossus* using a molecular phylogenetic approach. The unique agave-feeding habit of this species would be no surprise if the species is not, in fact, closely related to other species in the genus. We therefore constructed the first molecular phylogeny of *Cossus* that includes *C. redtenbacheri* using partial sequences of the mitochondrial cytochrome c oxidase subunit 1 (CO1) from 13 Cossidae species. For additional information we also sequenced the nuclear 28S ribosomal subunit (28S) of *C. redtenbacheri* and *C. insularis*.

Both species were able to bore into wood and agave. Therefore, mechanical differences do not explain the host specialization. The molecular phylogeny situates

C. redtenbacheri well inside both the wood-boring *Cossidae* and the genus *Cossus* in particular. Thus, the phylogeny does not explain the host specialization. The agave specialization is thus an ecological feature that requires further explanation.

Materials and methods

Insects

C. redtenbacheri is found mainly in Mexico. Larvae were collected from agave plants in the federal state of Hidalgo, central Mexico, in July 2011. *C. insularis* is distributed entirely within Asia. Its larvae were collected from willow trees (*Salix nipponica* Franchet et Savatier) in Ibaraki prefecture, Japan, in June 2011.

Gallery making tests

In order to examine the mechanical ability of the two species, we tested *C. redtenbacheri* from agave in wood and *C. insularis* from wood in agave. *C. redtenbacheri* was tested in branches of pear trees obtained from a farm close to the site where *C. redtenbacheri* had been collected in Mexico. Pear was chosen because *C. insularis* can live in this tree in Japan (Nakanishi, 2005). Tunnels of approximately 10 mm diameter and 100 mm long were bored longitudinally in branches with a power drill. These dimensions are within the size range of tunnels bored naturally by *C. insularis*. Four tunnels were drilled in each of three branches. We removed all loose wood and sawdust from the tunnels so as to force the larvae to remove wood themselves. A single larva was then introduced into each tunnel, giving a total of 12 larvae. The tunnel openings were then sealed with caps of the same wood.

Agave americana L. plants were purchased from Nagashima Shokubutsuen (Kagoshima, Japan). Five leaves were removed from the stem and enclosed together with 15 late instar *C. insularis* larvae in a plastic box.

The pear branches and the agave leaves were then kept at room temperature for three weeks during which the activity of the larvae was observed.

Phylogenetic position

We examined the position of *C. redtenbacheri* within the cossid CO1 phylogeny. If its position does not fall within the clade of wood-boring cossid species then its feeding niche is phylogenetically determined. However, if the species lies within the phylogeny then the distinction in feeding niche is not phylogenetic but more likely to be ecological. The mitochondrial CO1 gene was chosen for a combination of reasons. It is practical because easily PCR-amplified from Lepidopteran DNA. It is useful because all insects possess the CO1 locus, enabling comparison of distant relatives. CO1 is frequently sequenced from insects in general (including cossids) because the Consortium for the Barcode of Life has adopted CO1 as the standard for DNA bar coding (http://www.barcoding.si.edu/protocols.html). CO1 has also been recently elected as a standard tool for molecular taxonomy and identification (Ratnasingham and Hebert, 2007). CO1 sequences are thus available for more cossid species than sequences of any other gene. Experimental data have shown that CO1 discriminates well between insect species and provides a good phylogenetic signal (Wilson, 2010). If insect species have dissimilar CO1 sequences they are very unlikely to be closely related (Smith *et al*., 2009).

In contrast to the mitochondrial CO1, 28S is a nuclear gene. Again, species with dissimilar 28S sequences are unlikely to be closely related. Species where neither CO1 nor 28S are dissimilar are very likely to be from the same genus and certainly within the same clade.

DNA extraction, amplification and sequencing

DNA was extracted from 10 larvae of *C. redtenbacheri* and 10 larvae of *C. insularis*. None of the larvae used for phylogenetics had been used in gallery making tests but were co-collected from the same natural populations at the same time and location. The larvae were first stunned by chilling at 4 °C for 10 minutes. Their bodies were cleaned by rubbing them against a tissue soaked with 70% ethanol to remove contaminating DNA. The larvae were then decapitated and their legs cut off. DNA was then immediately extracted only from the legs using the Qiagen DNEasy® blood & tissue kit (Maryland, USA, cat. 60504) following the manufacturers protocol for purifying total DNA from animal tissues based on a spin-column. Whole bodies were not extracted because Cossidae larvae have been reported to display entomophagy (Ichikawa and Ueda, 2009) and DNA from other insect species might be present in their guts. The extracted DNA was PCR-amplified using 0.5 µL of the forward and reverse primers (50 mM, Operon, table 1), 4 µL dNTPs (2.5 mM each, Takara, Japan, cat. 4030), 5 µL 10x ExTaq buffer (Takara, Japan, cat. 9152A), 0.25 µL ExTaq (5 U μL^{-1}, Takara, Japan, cat. RR01A), 1 µL of the DNA template solution and 38.5 µL sterile water. PCR thermocycling occurred at one cycle of 120 s at 94 °C, five cycles of 40 s at 94 °C, 40 s at 45 °C, and 60 s at 72 °C, followed by 36 cycles of 40 s at 94 °C, 40 s at 51 °C, and 60 s at 72 °C, with a final cycle of 300 s at 72 °C (Fisher and Smith, 2008). The PCR products were separated by electrophoresis on a 2.5% agarose gel and purified using the QIAquick® gel extraction kit (Qiagen, Hilden, Germany, cat. 28706) following the manufacturer's protocol for gel extraction using a microcentrifuge. The purified PCR products were bidirectionally sequenced using the original PCR primers with BigDye v3.1 on an ABI 3730xl DNA Analyzer (Applied Biosystems).

Table 1. Primer pairs used with *C. redtenbacheri* and *C. insularis* to amplify the mitochondrial gene CO1 and the nuclear 28S ribosomal RNA gene (Fisher and Smith, 2008).

Primer name	Primer sequence (5'-3')	Amplicon region
LepF1	ATTCAACCAATCATAAAGATATTGG	CO1
LepR1	TAAACTTCTGGATGTCCAAAAAATCA	CO1
D2B	GTCGGGTTGCTTGAGAGTGC	28S
D3Ar	TCCGTGTTTCAAGACGGGTC	28S

Nucleotide sequence editing, alignment, and phylogenetic reconstruction

Sequences were visually examined for quality using CodonCode Aligner (Codon Code Corporation, Dedham, MA). Poor-quality ends and primers were manually trimmed and ambiguous or low-quality bases (quality score <20) were edited. For each sample, forward and reverse reads were used to create a consensus sequence using BioEdit v.7 (Hall, 1999). These resulting sequences were aligned with ClustalX2.0.5 (Larkin *et al*., 2007). After masking positions within the alignment that contained gaps, similarity matrices for aligned CO1 and 28S rRNA gene sequences were obtained using BioEdit. An additional 14 CO1 nucleotide sequences from selected Cossidae were subsequently aligned to the study sequences using Clustal (table 2). These were subjected to phylogenetic analyses. Maximum parsimony was performed in PAUP* v.4 beta (Swofford, 2003), with 10 replicates of the heuristic search option with TBR branch-swapping and random sequence addition. Maximum likelihood (ML) was performed with PhyML online (Guindon and Gascuel, 2003; Guindon *et al*., 2010) with the ML tree taken from the best of five starting trees obtained by BIONJ (Gascuel, 1997) with NNI and SPR tree rearrangements and branch length and topology optimization. Bayesian analysis was performed using MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001). Two independent runs of four chains (one cold) of 500,000 Metropolis-coupled Markov chain Monte Carlo generations were run (until the deviation of split frequencies was <0.01), with sample trees taken every 100 generations. A majority rule consensus tree was constructed from these 5000 saved trees with a burn-in of 2000 trees. Both ML and Bayesian analyses were run with models conforming to GTR+I+G, with model parameters estimated by the respective program. Parsimony and ML analyses were bootstrap pseudo-replicated 100 times.

Results

C. redtenbacheri was able to bore into wood and *C. insularis* into agave. Furthermore the newly obtained *C. redtenbacheri* CO1 sequences formed their own clade within a larger monophyletic grouping of *Cossus* spp.

The late instar *C. redtenbacheri* larvae bored into pear branches producing wood fragments. They used these fragments to make their cocoons (figure 1a). *C. insularis* late instar larvae easily bored into agave leaves and stems. They were able to produce tunnels and, despite the softness of the agave tissue, keep the tunnels from collapsing. The agave leaves darkened considerably a few days after the larvae had started boring into them (figure 1b).

The CO1 sequences for *C. redtenbacheri* and *C. insularis* were highly similar. Based on the resulting 588 base-pair alignment, two unique sequences were identified from the *C. redtenbacheri* samples and one unique sequence was identified from the *C. insularis* samples. These *C. redtenbacheri* sequences were 87.0 and 86.7% similar to the *C. insularis* sequence, and were 99.6% similar to each other. Only one representative of each unique sequence was used in phylogenetic reconstruction.

The 28S rRNA gene sequences from the two species were also very similar. One A+G polymorphism was recorded at the aligned position 477 in *C. redtenbacheri*, Genbank accession number JN673374, and coded as R for downstream analyses. Identity matrix values for nucleotide sequences of this gene showed that there was only one genotype for each population. These were 85.0% similar to each other. Eight indels were recorded (table 3), for all of which alignment gaps were inserted into the *C. insularis* sequence. Excluding these gapped positions, the similarity between the two groups was 91.3%.

The Cossidae phylogeny based on partial CO1 sequences showed that *C. redtenbacheri* forms a monophyletic clade with the other *Cossus* species (figure 2).

Table 2. Accession numbers for gene sequences of the Cossidae species used in this study. New sequences are in bold.

Species	GenBank	Zoological State Collection, Munich
Archaeoses pentasema (Lower)	GU828800.1	
Cossus cossus 1	GU828604.1	
Cossus cossus 2		GWORL447-09
Cossus cossus 3		GWOR4165-09
Cossula coerulescens Schaus	GU828562.1	
***Cossus insularis* 28S**	JN673373	
***Cossus insularis* CO1**	JN673375	
***Cossus redtenbacheri* 28S**	JN673374	
***Cossus redtenbacheri* 1 CO1**	JN673376	
***Cossus redtenbacheri* 2 CO1**	JN673377	
Endoxyla secta Lucas	GU929764.1	
Givira mucida (Edwards)	GU828543.1	
Indarbela obliquifasciata Mell	GU828829.1	
Metarbelinae sp.	GU828771.1	
Phragmataecia castaneae (Hubner)	GU828623.1	
Prionoxystus macmurtrei (Guerin-Meneville)	GU087518.1	
Prionoxystus robiniae Peck 1	GU090140.1	
Prionoxystus robiniae 2	GU090139.1	
Trigonocyttara clandestina Turner	GU828808.1	

Figure 1. Gallery making: **a**, larvae of *C. redtenbacheri* (from agave) showing wood fragments created by the larvae boring into wood: **b**, larvae of *C. insularis* (from trees) bored easily into agave leaves and stems. Larval boring caused considerable darkening of the agave leaves, at right. For comparison, a leaf that remained untouched by the larvae is shown at left. The red arrow points to a larva that bored into the agave leaf.
(In colour at www.bulletinofinsectology.org)

Table 3. Indels detected by comparing the 28S sequences of *C. redtenbacheri* and *C. insularis*. *C. redtenbacheri* always showed the longest sequence.

Aligned position in *C. redtenbacheri* (Genbank accession number JN673374)	Indel sequence
172	GT
184	GA
202	CGCGCGT
305	ATCG
328	TCGT
340	AAGGTG
387	ATTGCTTCTTGC
504	T

Discussion

The different feeding niches are not the result of mechanical or physiological constraints because larvae of both species were able to bore successfully in both agave and in wood. Wood is much harder than agave tissue but nevertheless, larvae of the agave-feeding *C. redtenbacheri* had the mechanical ability to bore into pear wood. This was clear by the presence of wood fragments in the cocoons. These fragments must have been produced by the larvae because fragments left behind by the drill were carefully removed. The larvae were also able to survive in wood for three weeks and to pupate. Larvae of *C. insularis*, likewise, were able to bore in agave leaves and form suitable burrows. The duration of these experiments was sufficient to require boring by the larvae of both species. They may not have needed to bore for food as cossids may feed in nature on the fungal hyphae we observed growing in galleries. They may also feed on invertebrates in nature, as other *Cossus* species have been reported to do (Ichikawa and Ueda, 2009). Boring by the larvae themselves was adequately demonstrated by the wood fragments and gallery extensions. Given the ability of both species to bore and survive in the alternative host, other reasons must be invoked to explain the different feeding niches of these insects.

Because the CO1 sequences are very similar *C. redtenbacheri* is unlikely to be part of a separate, "agave-feeding" clade unrelated to wood-feeding *Cossus*. This phylogenetic reconstruction was not aimed at producing a general phylogeny of Cossidae. For this purpose use of a single gene would have been inadequate. It was aimed rather at demonstrating that *C. redtenbacheri* is not genetically distant from the wood boring *Cossus* species. For this purpose using CO1 is sufficient, especially given the lack of publicly available 28S sequences for close relatives of *C. redtenbacheri* and *C. insularis*. One possible alternative mechanism explaining the feeding niche is the difference in nutritive content of wood and agave. Such a mechanism is known in other groups (Kohyama *et al*., 2012). According to the slow growth/high mortality hypothesis insects feeding on low-quality hosts suffer higher enemy caused mortality owing to the longer period spent in vulnerable larval stages compared with conspecifics feeding on high-quality hosts (Häggström and Larsson, 1995). Wood has few free low molecular weight nutrients whereas agave has many. Agave is particularly rich in water soluble, low molecular weight sugars, mainly inulin and other fructans and amino acids (Michel-Cuello *et al*., 2008). Under laboratory conditions, *C. insularis* larvae feeding in wood need up to two years before pupating (Cheng *et al*., 2002). Under similar conditions larvae of *C. redtenbacheri* kept in agave pupate in about six months (Llanderal-Cázares *et al*., 2007). Future investigations should estimate the larval survival of *Cossus* species living in agave and trees to test if there is any correlation between food quality and mortality.

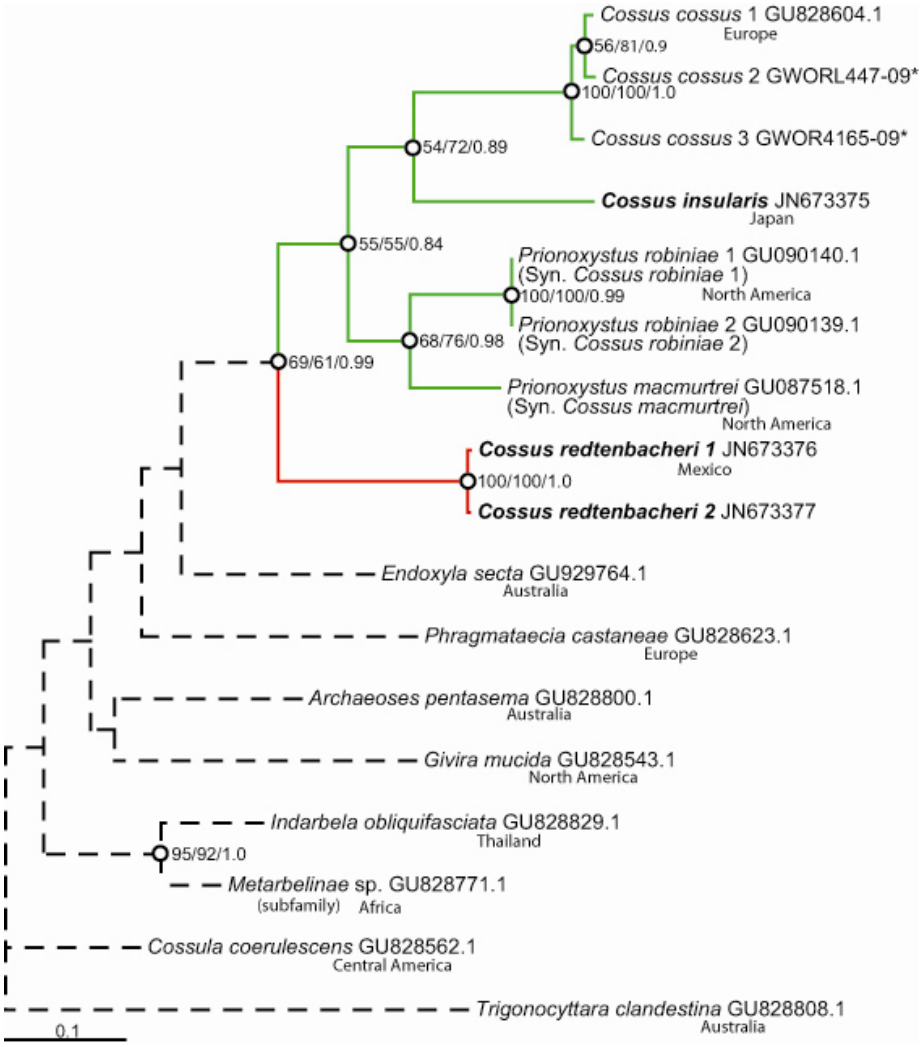

Figure 2. Unrooted maximum likelihood (ML) reconstruction of selected Cossidae based on a 588-nt fragment of available CO1 sequences. Bootstrap support of 50 for ML and of 50 for maximum parsimony (MP), and Bayesian posterior probabilities >/= 0.7, are only given at nodes supported by all three methods (o). Good phylogenetic resolution was not achieved for all taxa but the section of the tree with phylogenetic resolution (solid lines) supports a monophyletic relationship between the agave specialist *C. redtenbacheri* (in red) and the other members of the genus *Cossus* that specialize on trees (in green). New sequences are in bold. Accessions numbers after species names correspond to GenBank or the Zoological State Collection, Munich (*).
(In colour at www.bulletinofinsectology.org)

A further mechanism may be secondary plant chemistry. This is a well known cause of the evolution of different feeding niches (Janz, 2011). Steroidal saponins are well known constituents of agave plants (Simmons-Boyce and Winston, 2007). They, or other components, might be toxic to insects. The distinct feeding niche of *C. redtenbacheri* may thus have a basis in tuned detoxifying mechanisms that it has evolved against these plant chemicals. These mechanisms may be absent from other Cossidae. Both these alternatives, and others, require future investigation into the differences between feeding niches in the family Cossidae. The results in this paper, however, already exclude mechanical mechanisms related to tunnel creation. Further, the first molecular phylogenetic analysis of *C. redtenbacheri* (agave) with other *Cossus* (wood) confirms that these different feeding niches exist within the genus *Cossus*.

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Corresponding author: Fredd VERGARA (e-mail: fredd@psc.riken.jp; fredd@unam.mx), Advance NMR Metabomics Research Team, RIKEN Plant Science Center, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan.

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