



## A reverse-ecology framework to uncover the potential metabolic interplay among '*Candidatus Liberibacter*' species, citrus hosts and psyllid vector

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### ABSTRACT

*Candidatus Liberibacter* species have developed a dependency on essential nutrients and metabolites from the host cell, as a result of substantial genome reduction. Still, it is difficult to state which nutrients they acquire and whether or not they are metabolically reliant. We used a reverse-ecology model to investigate the potential metabolic interactions of '*Ca Liberibacter*' species, *Citrus*, and the psyllid *Diaphorina citri* in the huanglongbing disease pyramid. Our findings show that hosts (citrus and psyllid) tend to support the nutritional needs of '*Ca. Liberibacter*' species, implying that the pathogen's metabolism has become tightly linked to hosts, which may reflect in the parasite lifestyle of this important genus.

### 1. Introduction

The citrus tree is a member of the Rutaceae family and is one of the major fruit trees in the world, including important crops such as oranges (*C. sinensis*), lemons (*C. limon*), grapefruits (*C. paradisi*), pomelos (*C. maxima*), and limes (*C. aurantiifolia*). In addition, several biotic and abiotic issues have challenged the production and quality of citrus fruits across the world, with huanglongbing (HLB), or greening disease, standing out for its ability to have a significant impact on *Citrus* trees. HLB is caused by a group of Gram-negative bacteria '*Candidatus Liberibacter*' spp., which include the '*Candidatus Liberibacter asiaticus*' (CLas), '*Candidatus Liberibacter americanus*' (CLam), '*Candidatus Liberibacter africanus*' (CLaf) (Jagoueix et al., 1994). Together, these species are phloem-colonizing, psyllid-transmitted fastidious bacteria, classified in the Rhizobiaceae (Class: Alphaproteobacteria) (Fagen et al., 2014). '*Ca. Liberibacter*' spp. is transmitted in the hemolymph and salivary glands of psyllids *Diaphorina citri*, and since psyllids feed on sap, this allows bacteria to enter the plant's phloem (Nadarasarah and Stavrinides, 2011). They cause significant metabolic and regulatory changes in the plant, causing damage to the transport system, affecting the plant's defensive mechanisms, and altering the chemical and sensory properties

of the fruit. Blotchy mottle leaves, stunted growth, reduced fruit size, premature fruit drop, corky veins, and root decline are also observed (Baldwin et al., 2010; Bové and Barros, 2006; Dala Paula et al., 2018; Wang et al., 2017).

'*Ca. Liberibacter*' spp. are obligate host-associated bacteria with specialized living environments in the host plant (i.e., phloem) or vector (specific psyllid cells, tissues, and organs). As a result, their genomes undergo extensive gene and DNA loss, resulting in a small genome (approximately 1.2 Mb) with few genes and correspondingly restricted metabolic capacities that rely on the regular supply of certain energy substrates from hosts (Moran, 2002; Thapa et al., 2020). Although several studies have addressed the interaction of '*Ca. Liberibacter*' spp., citrus, and psyllids (Mafra et al., 2013; Ramsey et al., 2020; de Francesco et al., 2022), it is still unclear which nutrients they acquire and whether there is metabolic dependency. Therefore, we employed a reverse ecology analysis to obtain insights into the potential metabolic interplay of '*Ca. Liberibacter*' spp., *C. sinensis* and the psyllid *D. citri*. This approach is based on several computational tools to translate high-throughput genetic data into large-scale ecological data, which potentially turns ecology into a high-throughput field (Levy and Borenstein, 2012).

Reverse ecology approaches have been used in different fields,

**Abbreviations:** HLB, huanglongbing; CLas, *Candidatus Liberibacter asiaticus*; CLam, *Candidatus Liberibacter americanus*; CLaf, *Candidatus Liberibacter africanus*; KEGG, Kyoto Encyclopedia of Genes and Genomes; BSS, biosynthetic support score; MCI, metabolic complementarity index.

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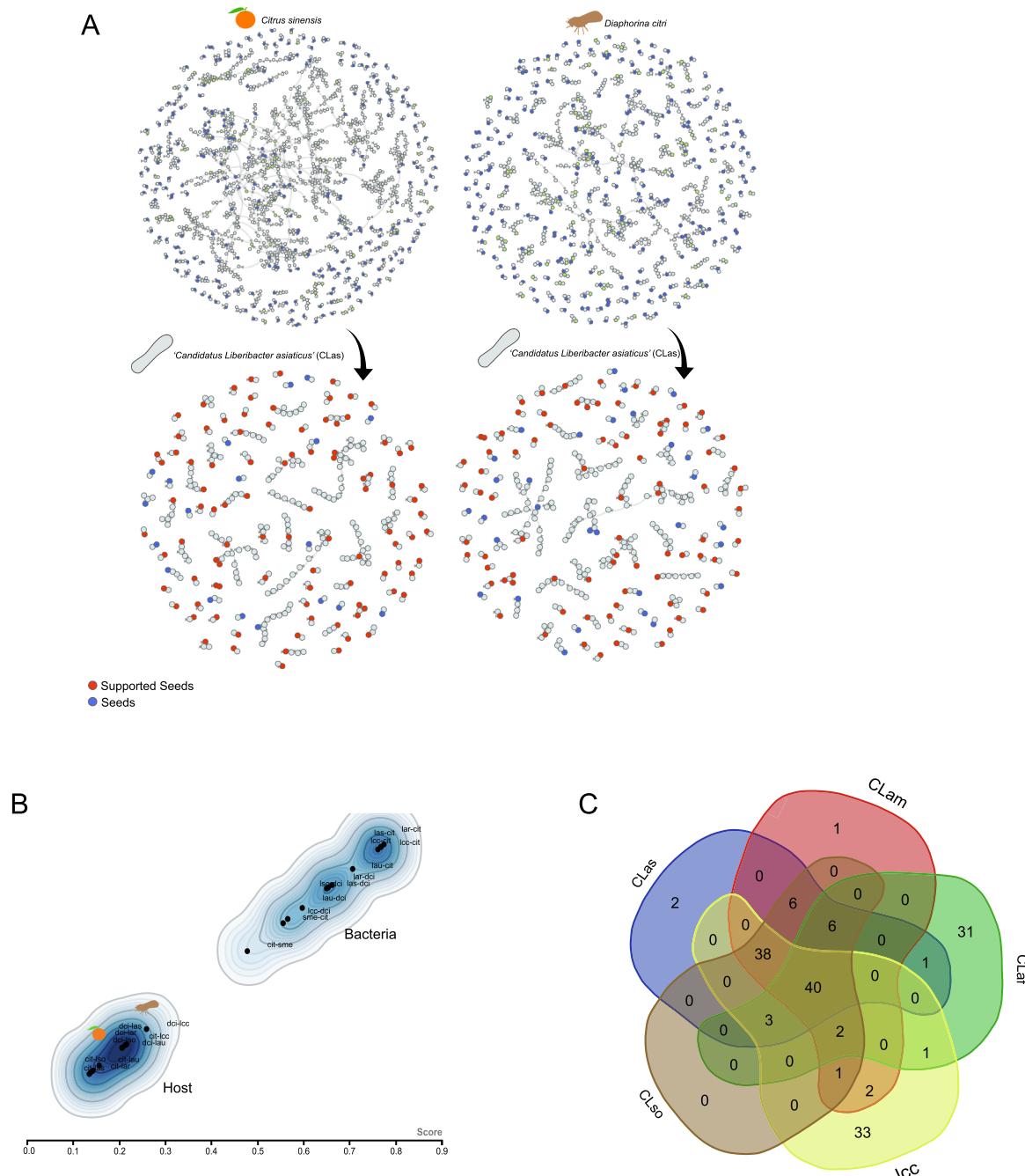
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including microbial-plant interactions to uncover metabolic environments (Karpinets et al., 2014; Ofaim et al., 2017); in human health, to quantify microbes' biosynthetic capabilities across the human oral microbiome (Bernstein et al., 2019) and to identify immune-beneficial infant gut bacteria by mining their metabolism for prebiotic feeds (Michelini et al., 2018). Here, we investigated the potential metabolic interplay among '*Ca. Liberibacter*' spp., *C. sinensis*, and *D. citri* using reverse ecology analysis.

## 2. Material and methods

Public metabolic data were retrieved from the KEGG (Kyoto Encyclopedia of Genes and Genomes) database using the function *getOrgMetabolicData* in the Package RevEcoR (Cao et al., 2016). The following '*Ca. Liberibacter*' spp. was employed in this study: CLas, CLam, CLaf, and '*Ca. Liberibacter solanacearum*' CLso-ZC1 (CLso-ZC1 - *lso*). In addition, we included *Liberibacter crescens* BT-1 (*lcc*), a culture representative surrogate for plant pathogen '*Ca. Liberibacter*' (Jain et al., 2019), and



**Fig 1.** The potential interplay among 'Ca Liberibacter' species, *C. sinensis*, and the psyllid *D. citri* depicted through reverse ecology. **A**, The metabolic networks of 'Ca' *L. asiaticus* (CLas), *Citrus*, and *D. citri*. Nodes represent compounds and edges connecting substrates to products. Each network highlights which metabolites were identified as seeds (blue), which of these seeds were identified as being potentially supported by the second species (red), and the corresponding supporting metabolites in the second network (green). Only the giant components are considered here. **B**, the distribution of the biosynthetic support score. **C**, Diagram Ven showing shared compounds among the 'Ca' Liberibacter species. Abbreviations: '*Candidatus Liberibacter asiaticus*' (CLas); '*Candidatus Liberibacter americanus*' (CLam); '*Candidatus Liberibacter africanus*' (CLaf); '*Candidatus Liberibacter solanacearum*' CLso-ZC1 (CLso); *Liberibacter crescens* BT-1 (lcc); *Sinorhizobium meliloti* 1021 (sme). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

*Sinorhizobium meliloti* 1021 (*sme*), a closely related model bacterium to *Liberibacter*. We also retrieved metabolic data from the hosts *C. sinensis* (*cit*) and *D. citri* (*dci*). Each KEGG Organism code is displayed in the front of the species name in parenthesis.

Next, a matrix was created including the substrate and product for each species, and then NetCooperate was applied for inferring host-microbe cooperation (cooperative and competitive potential) (Levy et al., 2015). The biosynthetic support score (BSS), and metabolic complementarity index (MCI) were measured for species interactions. The compounds were then annotated in KEGG compounds with biological roles and the Phytochemical compounds Database (<https://www.genome.jp/kegg/compound/>). In addition, NetMet (<https://freilich-lab-tools.com/>) (Tal et al., 2020) was used for predicting the metabolic performances of microbes and their corresponding combinations in user-defined environments. For that, we took the lists of species-specific enzymatic reactions (EC numbers) in the Joint Genome Institute (JGI, <https://genome.jgi.doe.gov/portal/>) for the '*Ca. Liberibacter*' spp. and used the *C. sinensis* and *D. citri* compounds from RevEcoR output as environmental input (nutritional resources).

### 3. Results and discussion

We first calculate the Biosynthetic Support Score (BSS), which evaluates a host species' capacity to provide the nutritional requirements of a parasitic or commensal species (Levy et al., 2015). According to network analysis (Fig. 1A), the majority of '*Ca. Liberibacter*' spp. metabolic pathways were supported by metabolic pathways of *C. sinensis* and *D. citri*, as shown by the support seeds (red dots), indicating that the bacteria obtain compounds exogenously from the hosts. These seed sets were proven to be compatible with the lifestyles of many bacteria and typically correspond with various basic aspects of the species' surroundings, as well as biological observations of key adaptations (Borenstein et al., 2008). Additionally, the distribution of BSS values of '*Ca. Liberibacter*' spp. against *C. sinensis* and *D. citri* ranged from approximately 0.1 to 0.8 (Fig. 1B), and there was a significant difference in BSS scores between bacteria and hosts (Fig. 1B). Our findings suggest that CLam and CLas are more dependent on *D. citri* than CLaf and CLso-ZC1, but the same evidence was not observed for *C. sinensis* (Fig. 1B). In addition, we revealed that the '*Ca. Liberibacter*' spp. and closely related species *L. crescens* and *S. meliloti* have a similar score, despite the fact that *S. meliloti* has low score support from the host (Fig. 1B).

Furthermore, we seek to provide which pathways and compounds are involved in these interactions in both directions. In the '*Ca. Liberibacter*' spp. – *C. sinensis* interaction, we mapped a total of 1702 compounds grouped into 17 pathways, while in the '*Ca. Liberibacter*' spp. – *D. citri* interaction, we mapped 1141 compounds grouped into 14 pathways (Fig. S1, Supplementary Table S1). These pathways were involved mostly in metabolic pathways, biosynthesis of secondary metabolites, biosynthesis of cofactors, and biosynthesis of amino acids. We observe that the hosts may supply some compounds involved in specific pathways for '*Ca. Liberibacter*' spp., *S. meliloti*, and *L. crescens*, such as compounds in D-amino acid metabolism in the *C. sinensis*-CLam interaction; and compounds in fatty acid and pyrimidine metabolism in the *C. sinensis*-CLas/ *D. citri*-CLas interaction (Fig. S2, Fig. S3).

In general, most of these compounds were involved in metabolic pathways, including ATP, UDP-glucose, L-Alanine, 5-Phospho-alpha-D-ribose 1-diphosphate, and Folinic acid, following biosynthesis of secondary metabolites. Details of the compounds that were found in the hosts-pathogen interaction can be found in Supplementary Table S2. Other key pathways for bacterial survival have been found, including amino acid biosynthesis, carbon metabolism, and fatty acid metabolism. It has been proposed that very-long-chain fatty acids play an important role in the axenic growth of pathogenic *Liberibacter* spp., but this gene is missing in all '*Ca. Liberibacter*' spp. (Leonard et al., 2012). Remarkably, we only mapped this compound in the interaction among *S. meliloti*, a closely related model bacterium, *C. sinensis* and *D. citri* (Supplementary

Table S2). Besides, CLas lacks the ability to synthesize phosphatidyl-choline, which is associated with the fluidity, permeability, and potential of bacterial membranes, but encodes a predicted ABC transporter system for choline, indicating that it is capable of utilizing extracellular choline (Li et al., 2012). We found evidence that *D. citri* may support this compound for CLas (Supplementary Table S2).

The compounds involved in the BSS were then contracted against each '*Ca. Liberibacter*' spp. We found that *L. crescens* and CLaf had the most exclusive compounds mapped, 33 and 31, respectively (Fig. 1C). We mapped *L. crescens*, compounds with biological roles in the classes of organic acids (2-oxoisovalerate), carbohydrates (d-mannose), peptides (l-ornithine), and vitamins and cofactors (phylloquinone, menaquinone, and nicotinamide). In addition, for CLaf, we found organic acids (malate), nucleic acids (dAMP), vitamins and cofactors (pyridoxamine, UDP-glucose, biotin) (Supplementary Table S3).

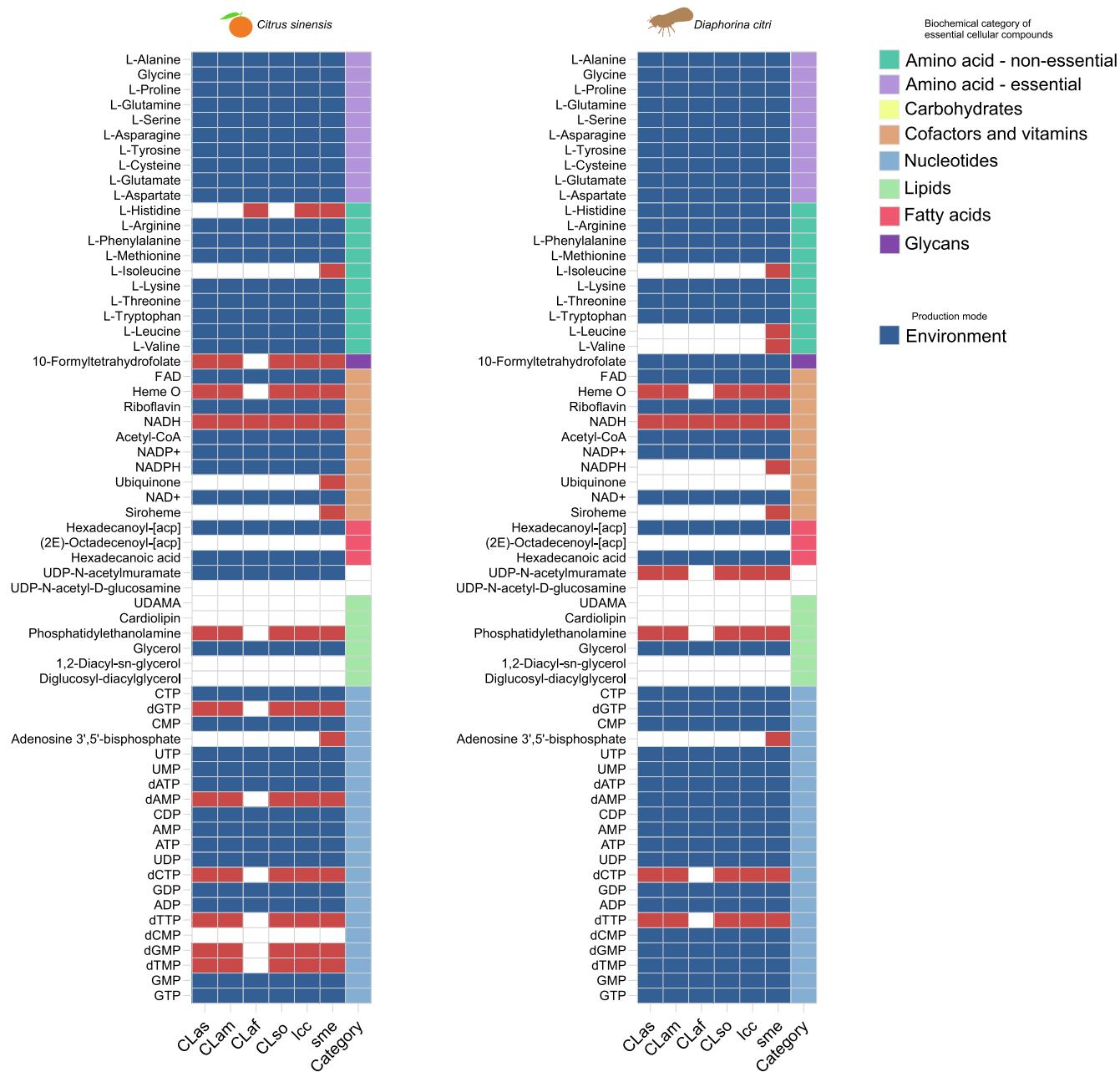
Finally, we used NetMet (Tal et al., 2020), a tool for predicting the metabolic performances of microorganisms and their environments, which we defined as a compilation of nutrients provided by the hosts citrus and psyllid (Fig. 2). Our analysis shows that the hosts may provide practically all needed cellular components, however, some categories are host-dependent and/or produced individually by the strain, such as a few essential amino acids, cofactors and nucleotides (Fig. 2).

Throughout the longitudinal study, our *in-silico* approach was also congruent with experimental data of metabolite extracts obtained from leaf samples taken from CLas and control grafted lemon plants (Ramsey et al., 2020). The authors profiled 25 metabolites using 1H NMR spectroscopy, including sugars, amino acids, and other primary and secondary metabolites. Interestingly, adenosine, along with many amino acids (proline, arginine, and the branched-chain amino acids (BCAA), was among the metabolites with the significant concentration changes between fruit taken from CLas<sup>+</sup> trees and those collected from CLas trees (Slisz et al., 2012). Similar metabolites were also reported by other studies using different *Citrus* varieties (Chin et al., 2014; Liu et al., 2020).

Fan et al., (2012) observed different transcriptional changes in host responses to CLas in highly susceptible *C. sinensis* and tolerant rough lemon (*C. jambhiri* Lush.) throughout the time course, including lipid metabolism and hormone metabolisms, using microarray analysis. Furthermore, the authors demonstrated that pathways such as glucose metabolism, cell wall metabolism, and stress response were completely changed in disease development. Overall, these results indicate that several metabolites are available during the infection of '*Ca. Liberibacter*' spp. in *Citrus* trees may be used by the pathogen to thrive in the plant environment.

Here, we demonstrated that the main common compounds abundantly available in the phloem sap and hemolymph environments, such as organic acids, and amino acids, vitamins, saccharides, and fatty acids (Duan et al., 2009; Killiny, 2017; Killiny et al., 2017; Merfa et al., 2019), including the metabolism of purines and pyrimidines, which CLas lacks essential enzymes (Hartung et al., 2011), may be provided by the hosts for '*Ca. Liberibacter*' spp. In addition, several studies have shown that '*Ca. Liberibacter*' spp. obtain essential nutrients from microbial communities other than citrus and psyllid hosts ((Zuñiga et al., 2020; Hu et al., 2021).

Taken together, our findings show that hosts (citrus and psyllid) tend to support the nutritional requirements of '*Ca. Liberibacter*' spp. This suggests that the pathogen's metabolism has become tightly linked to hosts, which may reflect in the parasite lifestyle and the complexity to isolate in axenic culture '*Ca. Liberibacter*' spp. in an artificial medium. Understanding the interaction between the host and pathogen could aid in the development of new disease-control strategies, as well as the design of sustainable media culture that supports the growth of the bacteria.



**Fig 2.** Profiles of the predicted production of cellular building blocks by the *C. sinensis* and *D. citri*. X-axis: 'Candidatus Liberibacter asiaticus' (CLas); 'Candidatus Liberibacter americanus' (CLam); 'Candidatus Liberibacter africanus' (CLaf); 'Candidatus Liberibacter solanacearum' CLso-ZC1 (CLso); 'Liberibacter crescens' BT-1 (lcc); 'Sinorhizobium meliloti' 1021 (sme).

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## CRediT authorship contribution statement

**Osiel Silva Gonçalves:** Conceptualization, Software, Data curation, Visualization, Investigation, Writing – original draft. **Ralph Bonandi Barreiros:** Conceptualization, Investigation, Writing – original draft. **Sumaya Martins Tupy:** Software, Data curation. **Mateus Ferreira Santana:** Conceptualization, Supervision, Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.gene.2022.146679>.

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