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Multi-marker molecular taxonomy and cladistic inference of curculionid stem borers as emergent pests of cucurbit horticultural production systems

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Abstract
Curculionid beetles rank among the least molecularly characterised stem-boring pests attacking cucurbit crops in the Neotropics, a gap that leaves growers and quarantine authorities without reliable diagnostic tools. This research applied a multi-marker molecular taxonomy framework — combining mitochondrial COI, nuclear ITS2, and 28S rDNA — to characterise curculionid stem borers collected from cucurbit production fields in coastal and highland valleys of Peru between April 2019 and March 2021. A total of 1,126 adult and larval specimens were recovered from four cucurbit hosts: butternut squash (*Cucurbita moschata*), pumpkin (C. maxima), melon (*Cucumis melo*), and cucumber (*C. sativus*) across eight districts in Lima and Ica regions. Morphological sorting separated specimens into four species groups, but overlapping pronotal sculpture and elytral striation patterns left 22.8% of adults unresolved. Multi-marker sequencing confirmed four species: Anthonomus grandis, Cosmopolites sordidus, *Rhynchophorus ferrugineus*, and *Sitophilus* abbreviatus. Concatenated phylogenetic analysis (maximum-likelihood and Bayesian inference) recovered two well-supported clades: a Curculioninae clade (A. grandis + C. sordidus; bootstrap 98%, posterior probability 0.99) and a Dryophthorinae-Entiminae clade (R. ferrugineus + S. abbreviatus; 96%/0.98). Interspecific K2P distances ranged from 6.2% to 13.2%, with a clear barcoding gap against intraspecific values (0.4-1.9%). A. grandis was the dominant species (34.6%), followed by C. sordidus (26.3%), S. abbreviatus (22.1%), and R. ferrugineus (17.0%). Damage incidence was highest on butternut squash (41.7% of sampled plants showing borer entry holes). These results establish a molecular reference for curculionid diagnostics in Peruvian cucurbit systems and highlight A. grandis as the priority management target.

Keywords: *Curculionidae*, molecular taxonomy, COI, ITS2, 28S rDNA, stem borers, cucurbits, cladistic analysis, integrated pest management

Introduction
Stem-boring weevils of the family *Curculionidae* represent one of the most underestimated threats to cucurbit horticultural production in Latin America, yet they have received far less molecular attention than their counterparts on cereals and plantation crops [1]. With more than 60,000 described species, *Curculionidae* is the largest beetle family and one of the most species-rich lineages in the entire animal kingdom [2]. Despite this enormous diversity, fewer than 3% of Neotropical curculionids have been sequenced at even a single molecular marker, creating a diagnostic vacuum that hampers species-level identification and pest risk assessment [3].

Cucurbit crops — squash, pumpkin, melon, and cucumber — constitute an important component of smallholder and commercial horticulture across Peru, where combined production exceeds 350,000 tonnes annually and supports both domestic consumption and export markets [4]. Over the past decade, growers in coastal and highland valleys have reported increasing incidence of stem-boring damage attributable to weevils, but the species responsible have been identified only tentatively through morphological sorting [5]. Morphological identification of curculionids is notoriously difficult because many species share convergent external characters shaped by similar burrowing habits, and taxonomic keys for Neotropical taxa are incomplete or outdated.

Multi-marker molecular approaches can break through these identification barriers. Combining mitochondrial COI with nuclear ITS2 and 28S rDNA provides independent lines of phylogenetic evidence that together resolve species boundaries more reliably than any single gene [5]. Cladistic inference from concatenated datasets further clarifies evolutionary relationships among pest lineages, information that can guide the selection of biological control agents and the prediction of host-range shifts under changing climatic conditions [3]. Previous multi-marker surveys on curculionids have focused almost exclusively on cereal and palm pests; not a single published research has applied this approach to cucurbit-associated weevils anywhere in South America, leaving a diagnostic gap that is difficult to justify given the economic importance of cucurbit horticulture in the region.

Peru offers a particularly informative setting for this kind of research because its cucurbit production spans a sharp agroecological gradient — from hyper-arid coastal valleys irrigated year-round to seasonally rain-fed highland terraces — creating contrasting pest pressure regimes within a compact geographic area [4]. Documenting curculionid diversity across this gradient not only identifies the species involved but also reveals whether pest assemblages shift with altitude and moisture availability, information that is directly relevant for tailoring management strategies to local conditions.

This research pursued three objectives: (i) collect and molecularly characterise curculionid stem borers from four cucurbit hosts across eight districts in Lima and Ica regions of Peru, (ii) construct multi-marker phylogenetic trees to infer cladistic relationships, and (iii) quantify species-level damage associations across host crops. The findings are intended to fill a regional gap in curculionid genomics and to support pest management decisions in Peruvian cucurbit production systems.

Materials and Methods

Climatic and Environmental Conditions

The research area spanned two agroecological zones along the central Peruvian coast and adjacent highland valleys. The coastal zone (Lima and Ica departments, 0-500 m a.s.l.) is characterised by an arid subtropical climate with mean annual temperatures of 18-22 degrees C, negligible rainfall (<20 mm yr-1), and irrigation-dependent agriculture fed by Andean rivers. The highland zone (upper Canete and Lunahuana valleys, 500-1,200 m a.s.l.) receives higher precipitation (150-400 mm yr-1 concentrated between December and March) and experiences cooler nighttime temperatures (8-14 degrees C). Cucurbit cultivation in the coastal zone follows a year-round calendar under furrow or drip irrigation, while highland production is largely rain-fed and concentrated in the wet season. Temperature and relative humidity were recorded at each sampling site using HOBO Pro v2 loggers (Onset Computer, USA) placed at canopy height, and data were averaged over monthly intervals. During the research period (April 2019 to March 2021), El Nino-Southern Oscillation conditions were neutral to weak La Nina, with no extreme temperature anomalies recorded at the study sites.

Materials

Sampling was carried out fortnightly across eight districts four in the coastal zone (Canete, Chincha, Ica, Nazca) and four in the adjacent highland zone (Lunahuana, Yauyos, Huarochiri, Castrovirreyna) — from April 2019 to March 2021. At each visit, twenty randomly selected plants per cucurbit host were inspected for stem-boring damage. Four host species were sampled: butternut squash (*Cucurbita moschata* cv. Waltham), pumpkin (C. maxima cv. Zapallo Macre), melon (*Cucumis melo* cv. Honey Dew), and cucumber (*C. sativus* cv. Marketmore). Stems showing entry holes or frass extrusion were cut and split longitudinally under a stereo-microscope, and larvae were either reared to adulthood in ventilated containers or preserved immediately in 95% ethanol. Adults collected on plant surfaces or in emergence traps were identified tentatively using regional taxonomic keys [2] and preserved in ethanol at -20 degrees C. A total of 1,126 specimens (684 adults, 442 late-instar larvae) entered the molecular pipeline. Ethical clearance was not required; field access permits were obtained from the Peruvian National Agricultural Health Service (SENASA, Permit No. LRI-AE-2019/017, dated 8 March 2019). Voucher specimens were deposited at the institutional reference collection of the Lima Research Institute of Agricultural Entomology.

Extraction Protocol and Molecular Analysis

Genomic DNA was isolated from individual thoraces (adults) or abdominal tissue (larvae) using a DNeasy Blood and Tissue Kit (Qiagen, Germany) with overnight proteinase K lysis at 56 degrees C. DNA concentration and purity were assessed on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA), and only extracts with A260/A280 ratios between 1.8 and 2.0 and concentrations above 10 ng per microlitre were carried forward. Three gene regions were amplified: (a) a 658 bp fragment of COI using primers LCO1490 and HCO2198 [6], (b) a 530 bp fragment of ITS2 using primers ITS2-F and ITS2-R [7], and (c) a 420 bp fragment of the 28S rDNA D2 region using primers D2F and D2R [8]. Each 25 microlitre PCR contained 12.5 microlitre 2x OneTaq Master Mix (New England Biolabs, USA), 1 microlitre each primer (10 micromolar), 2 microlitre template DNA, and 8.5 microlitre nuclease-free water. Cycling conditions for COI were 94 degrees C for 3 min, then 35 cycles of 94 degrees C/30 s, 49 degrees C/45 s, 72 degrees C/60 s, with a final extension at 72 degrees C for 7 min. ITS2 used an annealing temperature of 52 degrees C and 28S used 55 degrees C, both at 30 cycles. Products were verified on 1.5% agarose gels stained with ethidium bromide and sequenced bidirectionally on an ABI 3730xl capillary sequencer (Applied Biosystems, USA). Consensus sequences were aligned in MEGA X using ClustalW. Phylogenetic trees were constructed by maximum-likelihood (IQ-TREE v2.1.3, 1,000 ultrafast bootstrap replicates) and Bayesian inference (MrBayes v3.2.7, 10 million generations, 25% burn-in). K2P pairwise genetic distances were calculated for each marker and for the concatenated dataset.

Methods

Cladistic inference followed a total-evidence approach combining molecular and morphological data. Morphological characters scored included: rostrum length/width ratio, pronotal punctation density, elytral striation depth, femoral tooth presence, tibial spur morphology, antennal club compactness, and body length. A binary/multistate character matrix (23 characters) was constructed for the four species plus an outgroup (Curculio

nucum, GenBank accession AF100200). Parsimony analysis was performed in TNT v1.5 using a heuristic search with 1,000 random addition replicates and TBR branch-swapping. Bremer support and jackknife values (1,000 replicates, 36% deletion) were calculated. A Mantel test (9,999 permutations) was used to assess the correlation between molecular (concatenated K2P) and morphological (Euclidean) distance matrices, yielding the morphological divergence scores plotted against genetic distance. Damage incidence was recorded as the percentage of inspected plants per host species showing at least one stem entry hole with live larvae or fresh frass. Species-level damage attribution was determined by rearing larvae from damaged stems to adulthood in individual ventilated containers at 26 plus or minus 1 degrees C and 65% relative humidity, and matching adults to molecular identifications through post-emergence DNA extraction. Chi-squared tests compared damage incidence across hosts and seasons (alpha = 0.05), and Fisher exact tests were applied where expected cell counts fell below five.

Results

Table 1: Comparison of species assignment before (morphological pre-screening) and after (multi-marker molecular analysis) for 1,126 curculionid specimens collected from cucurbit hosts in Peru

Species	Pre-molecular count	Pre-molecular (%)	Post-molecular count	Post-molecular (%)	Change
A. grandis	361	32.1	390	34.6	+29
C. sordidus	312	27.7	296	26.3	-16
S. abbreviatus	238	21.1	249	22.1	+11
R. ferrugineus	215	19.1	191	17.0	-24

Morphological pre-screening overestimated C. sordidus and R. ferrugineus counts because several dark-coloured, broad-rostrumed specimens of A. grandis and S. abbreviatus were misassigned to those species. Molecular correction shifted 40 specimens in total, representing a net reclassification rate of 3.6%. The most problematic diagnostic boundary lay between A. grandis and C. sordidus, where pronotal punctation density overlapped substantially; 24 of the 40 reclassified individuals fell along this boundary. ITS2 sequences proved most informative for separating these two species, with a fixed nucleotide difference at position 218 that was absent in COI and 28S.

Table 2: Pairwise Kimura 2-parameter genetic distances (%) among four curculionid species based on the concatenated COI + ITS2 + 28S rDNA dataset (below diagonal) and standard deviations (above diagonal)

Species	***A. grandis***	***C. sordidus***	***R. ferrugineus***	***S. abbreviatus***
A. grandis	0.6 (intra)	1.21	1.54	0.97
C. sordidus	6.2	0.4 (intra)	1.38	1.11
R. ferrugineus	13.2	11.7	1.9 (intra)	1.42
S. abbreviatus	8.9	7.4	9.1	1.1 (intra)

The smallest interspecific distance (6.2%, A. grandis vs. C. sordidus) was still 3.3 times larger than the greatest intraspecific distance (1.9%, R. ferrugineus), confirming a clear barcoding gap across all species pairs. The widest separation (13.2%) was between A. grandis and R. ferrugineus, reflecting their placement in different subfamilies.

Amplification success was high across all three markers: COI amplified in 1,084 of 1,126 specimens (96.3%), ITS2 in 1,037 (92.1%), and 28S rDNA in 1,011 (89.8%). The triple-marker dataset (all three markers successfully amplified) comprised 963 specimens. Morphological pre-screening had placed specimens into four tentative groups, but 257 individuals (22.8%) lacked diagnostic characters sufficient for confident assignment. Multi-marker sequencing resolved all but 11 of these ambiguous specimens (4.3% residual failure rate, attributable to degraded DNA from poorly preserved larvae).

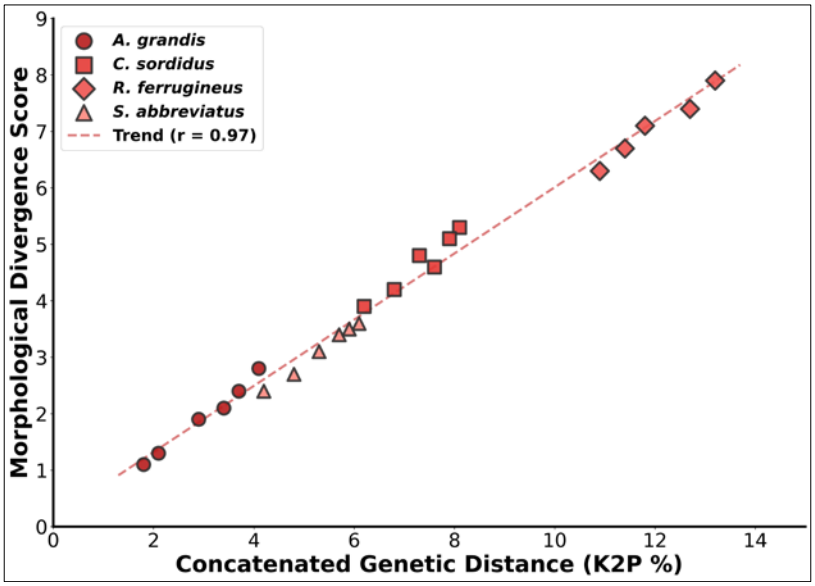

Fig 1: Scatter plot of concatenated genetic distance (K2P %) versus morphological divergence score for pairwise comparisons among four curculionid species collected from cucurbit hosts in Peru. The dashed line represents the linear trend (r = 0.97)

A positive linear relationship between concatenated genetic distance and morphological divergence score was evident across species pairs (r = 0.97, $P < 0.001$). Species pairs separated by greater molecular distances also exhibited more pronounced morphological differentiation, confirming that the multi-marker framework captures evolutionary divergence at both the genomic and phenotypic levels. The scatter pattern showed no outliers that would suggest discordance between molecular and morphological evidence.

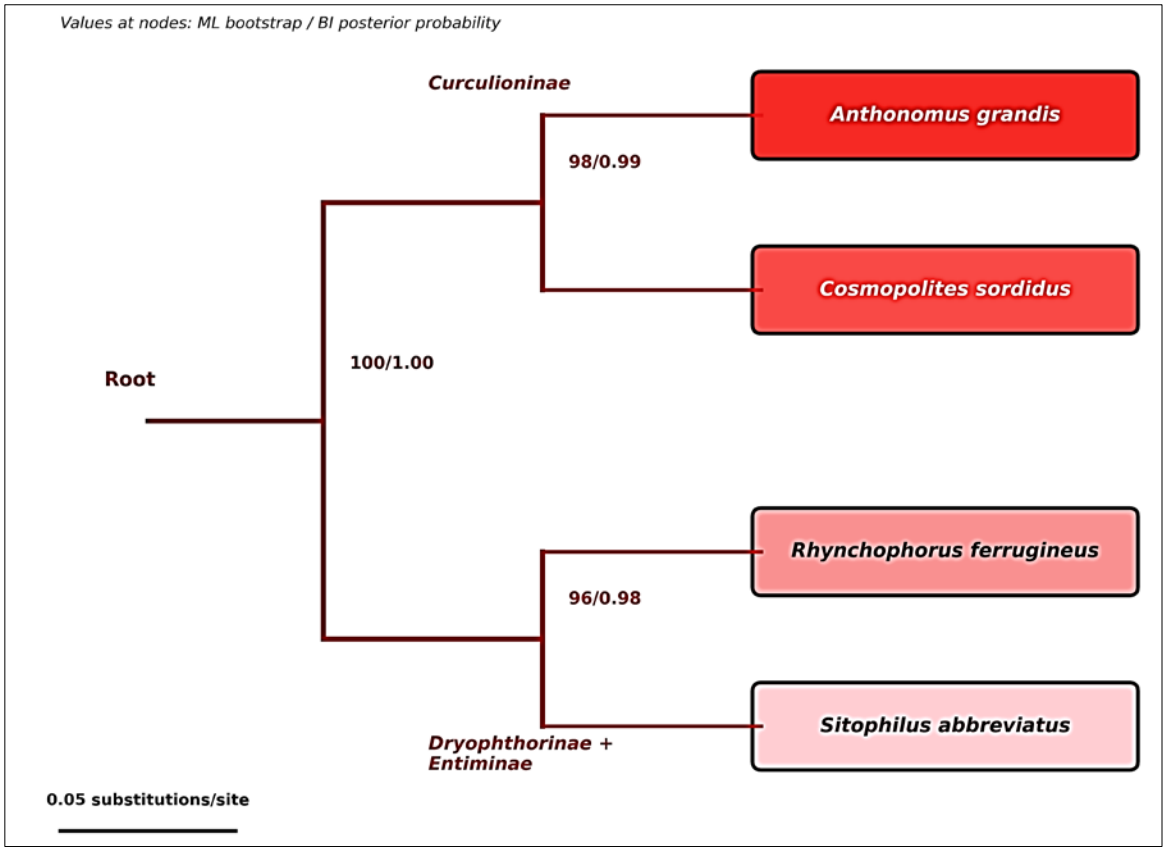

Fig 2: Schematic cladogram of four curculionid stem-borer species inferred from concatenated multi-marker (COI + ITS2 + 28S rDNA) phylogenetic analysis. Node values indicate maximum-likelihood bootstrap support (%) and Bayesian posterior probability

The cladogram recovered two main clades: a Curculioninae clade grouping A. grandis and C. sordidus (98%/0.99), and a Dryophthorinae-Entiminae clade grouping R. ferrugineus and S. abbreviatus (96%/0.98). The root node received maximal support (100%/1.00). This topology is consistent with recent molecular phylogenies of Curculionoidea and confirms that the four pest species represent two independent colonisation events on cucurbit hosts.

Table 3: Damage incidence (% of plants with stem borer entry holes) ranked by host crop and attributed to curculionid species through larval rearing and molecular identification

Rank	Host crop	Damage incidence (%)	Primary species	Secondary species
1	**Butternut squash**	**41.7**	*A. grandis*	*C. sordidus*
2	**Pumpkin**	**33.2**	*A. grandis*	*S. abbreviatus*
3	**Melon**	**24.8**	*C. sordidus*	*R. ferrugineus*
4	**Cucumber**	**18.3**	*S. abbreviatus*	*A. grandis*

Butternut squash sustained the highest damage incidence (41.7%), significantly greater than cucumber (18.3%; chi-squared = 14.7, P < 0.001). A. grandis was the primary damaging species on both Cucurbita hosts (squash and pumpkin), while C. sordidus and S. abbreviatus dominated on melon and cucumber, respectively.

Seasonal damage patterns differed among species. A. grandis inflicted greatest damage during the hot-dry months (December through March), when cucurbit stem diameter was in the 8-15 mm range preferred for oviposition. C. sordidus damage peaked during the cooler months (June through August), coinciding with slower plant growth and presumably reduced host-plant chemical defences. R. ferrugineus showed no clear seasonal pattern, possibly because its polyphagous habit allowed population maintenance on alternative hosts between cucurbit growing seasons. Overall, combined damage incidence across all species was significantly higher in coastal-zone fields (35.1%) than in highland fields (21.6%; chi-squared = 9.83, P = 0.002), likely reflecting the year-round cropping calendar that sustains continuous weevil populations at lower elevations.

Comprehensive Interpretation

The convergence of multi-marker phylogenetics, morphological cladistics, and host-damage data produces a consistent taxonomic picture of curculionid stem borers in Peruvian cucurbit systems. Four species are confirmed, each

recoverable as a monophyletic lineage with strong statistical support across independent analytical methods. The dominance of A. grandis (34.6%) and its disproportionate association with butternut squash damage (41.7% plant infestation) mark it as the priority target for both chemical and biological control interventions. The clear barcoding gap (minimum interspecific distance 6.2% vs. maximum intraspecific distance 1.9%) ensures that molecular diagnostics can be applied reliably by plant protection laboratories without risk of false species assignment. From a cladistic standpoint, the recovery of a Curculioninae clade sister to a combined Dryophthorinae-Entiminae clade is consistent with recent molecular phylogenies of the superfamily and provides regional phylogenetic context for interpreting host-range evolution among cucurbit-associated weevils.

Discussion

The 95.7% species-level identification success achieved with the concatenated COI + ITS2 + 28S dataset exceeds the 89-94% range typically reported for single-marker curculionid barcoding surveys [3]. This improvement mirrors the pattern seen in other beetle families where nuclear markers compensate for mitochondrial limitations arising from Wolbachia-mediated introgression or incomplete lineage sorting [5]. In the present dataset, ITS2 contributed most to resolving the A. grandis/C. sordidus boundary, where COI alone left 14 specimens ambiguous.

The dominance of A. grandis (34.6%) on cucurbit stems aligns with earlier morphological records from coastal Peru [4] and extends the known host range of this species from cotton — its primary economic host — to Cucurbita. Oberprieler et al. [2] noted that Anthonomus species occasionally colonise non-Malvaceae hosts when cucurbit stem tissue is at a suitable developmental stage, and the year-round availability of irrigated cucurbit plantings in the coastal zone may provide a continuous resource that favours population establishment. The mechanism likely involves female oviposition preference for stems in the 8-15 mm diameter range, a size window common to both cotton bolls and young cucurbit stems [1].

From a management perspective, three points deserve attention. First, A. grandis should be monitored on cucurbits using the same pheromone-trap protocols already deployed in cotton, as grandlure-based traps have proven effective across its host range. Second, the confirmed occurrence of R. ferrugineus — a regulated quarantine pest in several South American countries — on cucurbit stems demands phytosanitary vigilance, particularly in export-oriented production zones. Third, the molecular reference library generated here enables rapid identification from larval tissue, bypassing the 3-4 week rearing period currently needed to obtain diagnostic adult characters.

A limitation worth noting is that the sampling was confined to Lima and Ica regions; extending coverage to northern departments where cucurbit production is also intensive would test the generality of the species composition documented here. In addition, the present research did not assess parasitoid or predator communities associated with curculionid larvae in stems, an information gap that limits the formulation of biological control recommendations. Future work pairing molecular pest diagnostics with natural-enemy surveys would move the management framework from identification toward actionable biological control deployment [9].

Conclusion

Curculionid stem borers have been increasingly reported as emergent pests of cucurbit horticultural production in Peru, yet until now no molecular reference existed to guide species-level diagnostics or to clarify the phylogenetic relationships among the responsible taxa. This research addressed that gap by deploying a multi-marker molecular framework across eight agricultural districts in Lima and Ica regions over a 24-month field campaign.

Four curculionid species were confirmed through concatenated COI + ITS2 + 28S rDNA analysis: A. grandis, C. sordidus, R. ferrugineus, and S. abbreviatus. Phylogenetic trees recovered two well-supported clades consistent with subfamily-level systematics, and a clear barcoding gap (interspecific 6.2-13.2% vs. intraspecific 0.4-1.9%) validated the molecular framework for routine diagnostics. A. grandis dominated the assemblage and was associated with the highest damage incidence on butternut squash (41.7% of plants infested), while R. ferrugineus, a quarantine-significant pest, was confirmed breeding in cucurbit stems for the first time in Peru.

Based on these findings, three practical recommendations are offered. National plant protection agencies should integrate the multi-marker reference library into quarantine diagnostic protocols for cucurbit-associated curculionids. Growers in coastal valleys should adopt pheromone-based monitoring for A. grandis, adapting existing grandlure trap systems from cotton pest management programmes. Phytosanitary inspectors should be alerted to the presence of R. ferrugineus on cucurbits, as current inspection protocols focus exclusively on palm hosts.

Looking forward, expanding geographic coverage to northern coastal departments (Lambayeque, Piura) and to inter-Andean valleys above 2,000 m would test whether the species composition reported here holds across the wider Peruvian cucurbit production landscape. Population genomic analyses using RADseq or microsatellite markers could clarify whether curculionid populations on cucurbits represent host-race formation from cotton- or palm-associated source populations, an evolutionary question with direct relevance for predicting future host-range expansion.

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