Tracing the origin of the crayfish plague pathogen, *Aphanomyces astaci*, to the Southeastern United States

Laura Martín-Torrijos1,2, María Martínez-Ríos1, Gloria Casabella-Herrero3, Susan B. Adams2,4, Colin R. Jackson3,4 & Javier Diéguez-Uribeondo1,4

The oomycete *Aphanomyces astaci* is an emerging infectious pathogen affecting freshwater crayfish worldwide and is responsible for one of the most severe wildlife pandemics ever reported. The pathogen has caused mass mortalities of freshwater crayfish species in Europe and Asia, and threatens other susceptible species in Madagascar, Oceania and South America. The pathogen naturally coexists with some North American crayfish species that are its chronic carriers. Presumptions that *A. astaci* originated in North America are based on disease outbreaks that followed translocations of North American crayfish and on the identification of the pathogen mainly in Europe. We studied *A. astaci* in the southeastern US, a center of freshwater crayfish diversity. In order to decipher the origin of the pathogen, we investigated (1) the distribution and haplotype diversity of *A. astaci*, and (2) whether there are crayfish species-specificities and/or geographical restrictions for *A. astaci* haplotypes.

A total of 132 individuals, corresponding to 19 crayfish species and one shrimp species from 23 locations, tested positive for *A. astaci*. Mitochondrial rns and rnl sequences indicated that *A. astaci* from the southeastern US exhibited the highest genetic diversity so far described for the pathogen (eight haplotypes, six of which we newly describe). Our findings that *A. astaci* is widely distributed and genetically diverse in the region supports the hypothesis that the pathogen originated in the southeastern US. In contrast to previous assumptions, however, the pathogen exhibited no clear species-specificity or geographical patterns.

During the past few decades, fungal and fungal-like pathogens have caused several worldwide pandemics responsible for declines in wildlife populations—even causing extinctions1–5. Globalization facilitates these pandemics—usually consequences of the transport and introduction of exotic and invasive species6–8. Moreover, habitat alterations due to anthropogenic activity break down natural dispersal barriers, allowing invasive species (frequently carrying pathogens)9–15 to further expand their ranges. Climate change alters environmental conditions, further benefitting some invasive species and favoring the development and spread of disease2,4.

Fungal and fungal-like pathogenic species have impacted freshwater ecosystems particularly strongly, causing a global decline in freshwater biodiversity that is far greater than that seen in terrestrial ecosystems9,16,17. For example, the panzootic chytrid fungus *Batrachochytrium dendrobatidis* originated in Asia and spread globally due to amphibian trade, causing declines in more than 50 amphibian species over the past half-century18,19. Furthermore, fungal-like pathogens, such as *Saprolegnia diclina* and *Saprolegnia ferox* (Oomycetes), are also responsible for mass extinctions in amphibians20,21 and may be spread by the fish trade22. Another pathogenic oomycete, *Aphanomyces invadans*, causes epizootic ulcerative syndrome (EUS), affecting more than 100 fish species in Asia, Australia, North America and Africa23,24.

Similarly, *Aphanomyces astaci* causes the crayfish plague in native European, Asian and Australian crayfish species25–28 and has decimated crayfish populations in those continents5,6. This oomycete is a specialized pathogen in freshwater crayfish27,28,30, one third of which are threatened with extinction globally41. The pathogen coexists naturally with North American crayfish but can efficiently colonize non-North American crayfish, almost.

1Department of Mycology, Real Jardín Botánico-CSIC, Plaza Murillo 2, 28014 Madrid, Spain. 2USDA Forest Service, Southern Research Station, Center for Bottomland Hardwoods Research, 1000 Front Street, Oxford, MS 38655, USA. 3Department of Biology, University of Mississippi, University, MS 38677, USA. 4These authors contributed equally: Susan B. Adams, Colin R. Jackson and Javier Diéguez-Uribeondo. *email: lmtorrijos@rjb.csic.es
without resistance. In addition, *A. astaci* has spread rapidly throughout the world through translocations of North American chronic carriers. In non-North American crayfish, crayfish plague infections typically cause death within a few days. Presumptions about the origin of the crayfish plague were based on disease outbreaks that followed historical translocations of North American crayfish species to many countries for aquaculture, sport fishing, or aquarium pet trade. The first known introduction of a North American crayfish and subsequent crayfish plague outbreak was recorded in Europe in the nineteenth century. Later, additional large-scale introductions of North American crayfish species were made in European and non-European countries. Moreover, illegal translocations resulted in new crayfish plague outbreaks that decimated native crayfish populations in many countries (Fig. 1). Thus, *A. astaci* was listed among the 100 of the World’s Worst Invasive Alien Species.

Knowledge about the virulence and genetic variability of *A. astaci* has come primarily from studies of crayfish plague outbreaks in Europe and Asia. Specifically, mitochondrial DNA regions of *A. astaci* have been informative in assessing genetic diversity in both pure cultures and clinical samples of this clonally reproducing pathogen. To date, the mitochondrial DNA variability found in crayfish plague outbreaks in Europe and Japan has been allocated to six haplotypes (a, b, d1, d2, d3 and e-haplotypes) (Fig. 1) within two lineages. However, only three studies have confirmed the presence of the pathogen in North America, revealing only two of the previously described haplotypes: a and b-haplotypes.

Although evidence strongly supports a North American origin of *A. astaci*, our understanding of the crayfish plague pathogen in North America is still insufficient. A clearer understanding of the diversity and distribution of *A. astaci* within its native range is needed, not only to improve our comprehension of the evolution and epidemiology of pandemic pathogens, but also to determine future management and research directions. Similar questions have been faced when studying other emerging pathogens. For example, despite the occurrence of *Batrachochytrium dendrobatidis* in Asia, the lack of lethal outbreaks evidenced an endemic host–pathogen interaction in that region. Several studies have confirmed that the geographic origin of chytridiomycosis was in Asia, explaining the survival of Asian amphibian populations and stable host–pathogen dynamics.
Although more than 428 crayfish species are native to North America65, the diversity, distribution and prevalence of *A. astaci* there is still largely unknown. Within North America, the southeastern US harbors the highest number of endemic crayfish species. The region represents not only a center of diversity, but also one of the two distinct origins of freshwater crayfish6,52,66. The presence of *A. astaci* has not been investigated in this crayfish-rich region even though such knowledge would improve our understanding of the origin and diversity of the pathogen. Thus, the main aim of this study was to evaluate the southeastern US as the possible center of origin of the crayfish plague pathogen *A. astaci*. For this purpose, we tested key questions including: (1) what is the distribution and haplotype diversity of *A. astaci* in the southeastern US, and (2) are *A. astaci* haplotypes crayfish species-specific and/or geographically restricted. In order to perform this study, we isolated and analyzed the pathogen from 30 distinct crayfish populations comprising a total of 21 crayfish species and one shrimp from five states in the southeastern US.

**Results**

**Aphanomyces astaci detection.** We obtained a total of 391 crayfish from 30 locations in five states (Kansas, Kentucky, Louisiana, Mississippi and South Carolina) (Fig. 2). The crayfish represented six genera and 21 species: *Cambarellus shufeldtii*, *Cambarus latimanus*, *Cambarus striatus*, *Cambarus tenebrosus*, *Creaserinus fodiens*, *Creaserinus oryktes*, *Faxonius etnieri* species complex, *Faxonius sp.*, *Faxonius tricuspid*, *Faxonius wrighti*, *Lacunicambarus ludovicianus*, *Procambarus ablusus*, *Procambarus acutus*, *Procambarus clarkii*, *Procambarus hayi*, *Procambarus hybus*, *Procambarus pubescens*, *Procambarus raneyi*, *Procambarus troglodytes*, *Procambarus viaeviridis* and *Procambarus vioscai* (Supplementary Table 1). Additionally, one species of freshwater shrimp (*Palaemon kadiakensis*) was sampled and analyzed for the presence of *A. astaci*.

From 392 individuals, 132 crayfish and one shrimp tested positive for the *A. astaci* ITS region, 102 tested negative and 158 were not analyzed (i.e., the crayfish did not molt). *Aphanomyces astaci*-positive samples came from 23 locations and included 19 crayfish species: *C. shufeldtii*, *C. latimanus*, *C. striatus*, *C. fodiens*, *C. oryktes*, *F. etnieri* species complex, *Faxonius sp.*, *F. tricuspid*, *F. wrighti*, *P. ablusus*, *P. acutus*, *P. clarkii*, *P. hayi*, *P. hybus*, *P. pubescens*, *P. raneyi*, *P. troglodytes*, *P. viaeviridis* and *P. vioscai*) and one species of shrimp (*Palaemon kadiakensis*) (Fig. 2 and Supplementary Table 1). The ITS sequences (specific primers 42 and 640) for the 132 clinical samples were 99.82% identical to sequences of *A. astaci* available in GenBank (e.g., sequence FM999249-isolate SAP302) and identical to each other.
Sequence analyses and haplotyping of *A. astaci*. Twenty crayfish clinical samples (taken directly from crayfish) and 12 pure cultures from nine locations and three states (Kentucky, Mississippi and South Carolina) (Fig. 3) contained enough of the pathogen DNA for amplifying both mitochondrial regions (i.e., clinical samples often harbor low pathogen DNA concentration).

For the phylogenetic approximations [Bayesian Inference (BI) and Maximum likelihood (ML)] and diversity estimations, we included a total of 78 sequences [32 sequences from the present study, 43 obtained from GenBank as reported from previous studies33,52,62 and two sequences from new *A. astaci* and *A. fennicus* isolates (CCRJB-75 and CCRJB-76, respectively)57 with 476 and 355 bp fragments of rnnS and rnnL amplicons, respectively. The phylogenetic approximations (BI and ML) supported the differentiation of the two lineages previously described52 (Fig. 4). The genetic diversity analysis confirmed and supported the phylogenetic analysis. Although both mitochondrial ribosomal rnnS and rnnL regions were informative, there were differences between them (Fig. 5). We obtained five haplotypes for the rnnS subunit (Fig. 5a), represented by four segregating sites (S), with a haplotype diversity (Hd) of 0.703, a nucleotide diversity (π) of 0.0022 and 1.018 average nucleotide differences (k). On the other hand, we obtained ten haplotypes for the rnnL subunit (Fig. 5b), represented by13 segregating sites (S), with a haplotype diversity (Hd) of 0.786, a nucleotide diversity (π) of 0.009 and 3.16 average nucleotide differences (k). However, concatenating rnnS and rnnL regions we confirmed a total of 12 haplotypes represented by 17 segregating sites (S), where 13 of them were parsimony informative (Fig. 5c). The concatenated sequences presented a haplotype diversity (Hd) of 0.801 with a nucleotide diversity (π) of 0.005 and 4.178 average nucleotide differences (k).

The phylogenetic approximations and the haplotype network confirmed the presence of eight haplotypes for the rnnS and rnnL concatenated regions among the analyzed samples: a, d2, usa1, usa2, usa3, usa4, usa5 and usa6-haplotypes (Figs. 4, 5) (Table 1). Six of these eight haplotypes (usa1, usa2, usa3, usa4, usa5 and usa6-haplotypes) are described and reported here for the first time, bringing the total number of known *A. astaci* haplotypes to...
Moreover, these results were confirmed by an independent secondary molecular test (i.e., by repeating the amplification and sequencing of each of the haplotyped samples).

**Crayfish species, A. astaci haplotype diversity and distribution.** The haplotyping results for the 20 crayfish clinical samples included five haplotypes: usa1-haplotype (one *Faxonius tricuspid*), usa2-haplotype (one *Cambarus striatus*), a-haplotype (one *Procambarus hybus*, one *Procambarus acutus* and one *Faxonius etnieri*), d2-haplotype (ten *Cambarellus shufeldtii*, two *Procambarus clarkii* and one *Procambarus abiusus*) and usa6-haplotype (one *Cambarellus shufeldtii* and one *Procambarus clarkii*) (GenBank accession numbers MW346503-MW346522 for rnnS and MW346523-MW346542 for rnnL) (Table 1). The amount of infection in the remaining samples that tested positive for *A. astaci* (ITS region) was too low to obtain conclusive results for both rrnS and rrnL.

Additionally, the haplotyping results for the 12 pure cultures included four haplotypes: usa3-haplotype (one *Procambarus raneyi* culture), usa4-haplotype (one *Faxonius sp. culture*), usa5-haplotype (one *Procambarus raneyi culture*).
We report and describe for the first time the presence, distribution and genetic diversity of the crayfish plague pathogen, *A. astaci*, in its potential center of origin, the southeastern US68. Previous studies regarding the origin, diversity, and distribution of *A. astaci* have addressed different questions. In the current study, we have explored several of them, including (1) the distribution and diversity of *A. astaci* in the southeastern US, and (2) whether *A. astaci* haplotypes are crayfish species-specific66,69. Our results indicated that *A. astaci* is present and widely distributed in the southeastern US (e.g., it was present in 21 out of 23 sampling sites across the nine river basins we investigated).

**Discussion**

We found five scenarios relative to the distribution of *A. astaci* haplotypes among crayfish species at various locations: (1) one haplotype from one crayfish species (Locations 1, 4, 7 and 9), (2) two haplotypes from one crayfish species (Locations 3 and 23) (see below), (3) one haplotype from two crayfish species (Location 13), (4) two haplotypes from two crayfish species (Location 6) (see below), and (5) three haplotypes, one from each of three crayfish species (Location 11). At Location 23, the two haplotypes were recovered from one *P. raneyi* molt (i.e., we isolated the pathogen in two different pure cultures) and at Location 6, one haplotype was recovered of the pathogen’s native range52,62,63. Our results indicated that *A. astaci* is present and widely distributed in the southeastern US (e.g., it was present in 21 out of 23 sampling sites across the nine river basins we investigated).
and possesses the highest genetic diversity of A. astaci described from any region to date. Previously, only two haplotypes (a and b-haplotypes) had been found in the five North American crayfish species examined from California, Michigan and Pennsylvania (Pacifastacus leniusculus, Cambarus bartoni, Faxonius obscurus, Faxonius rusticus and Faxonius virilis)³³–³⁴. However, in the southeastern US, we examined 21 North American crayfish species and found a total of eight haplotypes, six of which are previously unreported. This represents almost 70% of the A. astaci haplotype diversity known globally. The genetic diversity we describe is comparable to that of other pathogenic oomycetes. For example, in the US, 13 Phytophthora infestans haplotypes were recently described using five mitochondrial loci⁶⁹. Moreover, we showed that A. astaci can chronically colonize 19 additional North American crayfish species and also one species of freshwater shrimp (Palaeon kadiakensis). Previously, the only known report of a wild shrimp carrying A. astaci was of a Macrobrachium lanchesteri population from Indonesia that co-occurred with P. clarkii⁷⁰. Only two (Cambarus tenebrosus and Lacunicamarus ludovicianus) of the 21 crayfish species that we sampled did not test positive for A. astaci. Thus, we confirm the presence of A. astaci in a wider distribution (Fig. 2) than previously described in North America⁵²–⁶⁴, confirming the origin of this pathogenic disease in North America.

Although the presence of the crayfish plague in North America was previously reported⁶²–⁶⁴, those studies examined a limited number of crayfish species and found haplotypes that had been previously isolated in Europe and Japan (i.e., a or b-haplotypes)⁵³,⁶²–⁶⁴. Likewise, we confirmed the presence in North America of two haplotypes (a- and d2-haplotypes) first described in Europe. In the case of the a-haplotype, it was described as the first haplotype introduced to Europe in the nineteenth century⁴⁸–⁵ⁱ, yet no North American carrier has been described in the literature for this introduction. Two recent studies described F. rusticus and F. obscurus as the only North American crayfish known to host the a-haplotype⁶⁸. We expanded the known range of this haplotype to include the southeastern US and added three additional native taxa as hosts: the F. etnieri species complex, P. clarkii and P. hybus. In the case of the d2-haplotype, the only two North American crayfish described as carriers so far in Europe are P. clarkii and Procambarus fallax virginalis. As we expected, our study shows for the first time the presence of A. astaci in native populations of P. clarkii⁵⁴, which carried the d2-haplotype and the newly discovered us6-haplotype.

We confirmed that at least some A. astaci haplotypes are neither host species-specific nor narrowly distributed. Broadening and deepening our knowledge of A. astaci haplotype diversity and distribution, we recovered the d2-haplotype from two species in different genera (Cambarellus and Procambarus) in one river basin (Locations 3, 4, 6 and 7) and from a third genus (Cambarus) in a different river basin (Location 11) (Table 1). Although the a-haplotype was previously thought to be restricted to F. rusticus and F. obscurus⁶²–⁶⁴, we found it not only in another congener, but also in another genus (Procambarus). In addition, we documented the a-haplotype in two river basins in the southeastern US, even though it was previously thought to be restricted to the northern US. We also documented the d2-haplotype from two river basins. Our results indicated that A. astaci haplotypes tend to be neither host species-specific nor restricted to small geographic areas.

We also found instances of multiple haplotypes of A. astaci occurring in one species, in one individual, and in one location. We isolated two pure cultures of two different, but closely related, A. astaci haplotypes (usa3 and usa5) from a single individual (SC32 MOLT in Location 23). Also, multiple A. astaci haplotypes often co-occurred in a location. Within Location 11, we found three haplotypes from two lineages: usa2-haplotype from Lineage 1 and d2-haplotype and usa4-haplotype from Lineage 2. We also recovered two closely related haplotypes (d2 and usa6) from Locations 3 and 6 and two others (usa3 and usa5) from Location 23. None of the phenomena described have been documented previously. Our analysis showed no clear haplotype distribution patterns with respect to crayfish host species or geography. Thus, the biogeographic distribution of A. astaci genetic diversity within North America needs further investigation.

The pathogen’s genetic diversity and observed lack of species-specificity may have implications within North America as well as on continents where A. astaci is introduced. Crayfish have been frequently translocated within North America. For example, although P. clarkii and F. virilis are native in parts of North America, both are invasive beyond their native range, including west of the Great Divide where all native crayfish belong to P. clarkii. The family Astacidae are invasive beyond their native range, including west of the Great Divide where all native crayfish belong to

In this study, we combined the sequencing of the ITS region (for identification of the Aphanomyces species)⁵⁹ and
the mtDNA (for identification of the *A. astaci* haplotype)\(^{52}\) in both clinical samples and pure cultures. Several studies have examined the genetic diversity of *A. astaci* using diverse methodologies (i.e., RAPD-PCR, the chitinase gene, AFLP, microsatellites)\(^{50,52,56,58-60,77}\). Although the mtDNA approximation\(^{52}\) requires a large concentration of the pathogen, it provides reliable results\(^{52}\) and enables detection of new diversity within *A. astaci* that might go undetected with other approximations\(^{59,60}\). Other approaches, such as eDNA monitoring\(^{8}\), could also be used to detect the presence of *A. astaci* in water samples and potentially to detect additional genetic diversity.

The uniquely high diversity of *A. astaci* haplotypes found in this study are an important step toward confirming the host–pathogen co-evolution between *A. astaci* and North American crayfish species. Our results suggest that further sampling in North America will reveal additional undiscovered *A. astaci* haplotype diversity vital to answering new host–pathogen co-evolutionary questions. Further, long-term monitoring might reveal emerging *A. astaci* diversity, depending on the rate of pathogen evolution.

**Methods**

**Sample collection.** We sampled 25 locations in five states between February and May 2019. Additionally, we included nine crayfish samples previously collected from five more locations and preserved in 95% ethanol. The specimens from Kansas were from an introduced population of *Procambarus clarkii* (Supplementary Table 1). Crayfish were captured by kick-seining and trapping and then held, separated by species and collection location, in the laboratory (US Forest Service, Southern Research Station, Center for Bottomland Hardwoods Research, in Oxford [Mississippi, USA]) until processed. Crayfish were kept individually in labeled, individual, plastic containers with chlorine-free water, aerators, gravel and medium size rocks. Each crayfish was kept alive until it molted and was subsequently preserved in 95% ethanol.

**Microscopic examination and *Aphanomyces astaci* isolation.** For the microscopic examination, the molts were carefully removed and handled individually due to the fragility of the samples. Molts were kept in individual petri dishes with distilled water at 4 °C in order to reduce bacterial growth and optimize the growth of potential mycelium\(^75\). After three days, each molt was examined with an inverted microscope to check for the presence of growing hyphae. Each sample was divided into two parts: one for molecular identification and one for the pathogen isolation.

Pieces of the molt intended for molecular identification were transferred into 1.5-ml tubes and were frozen at − 80 °C until the DNA extraction was carried out in the laboratory at the Department of Biology at the University of Mississippi (UM). Samples were subsequently homogenized by manual mechanical disruption. A DNeasy Blood & Tissue Kit (Qiagen, Valencia, California, USA) was used to isolate genomic DNA.

Pieces of the molt intended for culture isolation were grown in Peptone Glucose Agar (PGA) at 4 °C. A selected agar plug was cut out from the resulting mycelia and inserted within an aluminum ring placed on a new PGA plate to protect the growing isolate from bacterial growth\(^74\). Plates were incubated at 4 °C for seven days, and each isolate was transferred into new PGA media once the hyphae spread under the metal ring. This process was repeated until no bacterial growth was observed using an inverted microscope. Additionally, a selected agar plug containing mycelia was placed in a 9 mm Petri dish containing 10 mL of liquid Peptone Glucose (PG-1) and incubated at room temperature for 48 h in order to obtain material for molecular identification. The obtained mycelium was transferred into 1.5-ml tubes and frozen at −20 °C until the DNA extraction was carried out in the laboratory at UM. Samples were subsequently homogenized by manual mechanical disruption, and DNA extractions were carried out using an E.Z.N.A. Fungal DNA Mini Kit (Omega Biotek, Norcross, Atlanta, USA).

**Aphanomyces astaci** detection and haplotyping. To test for the presence of the *A. astaci* pathogen, a fragment of the internal transcribed spacer (ITS) region was amplified using the diagnostic primers 42 (5′-GCT TGTGCTGAAGATTCTTT-3′)\(^{53}\) and 640 (5′-CTATCCGACTCGGATTCTTG-3′)\(^{58-60}\) (which amplify ITS1, the 5.85 rDNA and ITS2) in a single round of amplification according to the assay described by\(^73\). DNA extracted from a pure culture of *A. astaci* was used as the positive control; sterile Milli-Q water was used as the negative control. Amplified products (3 μL of each reaction) were analyzed by electrophoresis in 2% agarose SB gels stained with ethidium bromide and then purified using magnetic beads. Sequencing of both strands of positive products was performed using an automated sequencer (Applied Biosystems 3730xl DNA Analyzer; DNA Analysis Facility at Yale, USA and Applied Biosystems 3730xl DNA, Macrogen, The Netherlands). Sequences were aligned and edited using the program Geneious 10.0.2\(^{80}\). A BLAST search (NCBI database) was performed to verify the identity of each sequence.

Genomic DNA samples that tested positive for the presence of *A. astaci* pathogen with diagnostic primers 42\(^{53}\) and 640\(^{58-60}\) were used to characterize the phylogenetic relationships and haplotypes. The mitochondrial ribosomal small (rnsS) and large (rnlL) subunits were amplified using the primer pairs AphSSUF/AphSSUR (5′-AGCATTCCGCTGAAGATTA-3′ and 5′-GGGCCGCGTTGTACCAAAGCTTC-3′)\(^{52}\) and AphLSUF/AphLSUR (5′-AGGCGGA AAGCTTACTATGTCCTG-3′ and 5′-CCAATCTCCTGCCACCTTC-3′), respectively, as described by\(^52\). Positive and negative controls were included. Amplified products were analyzed by electrophoresis, purified, sequenced and aligned as described above.

Phylogenetic approximations based on Bayesian inference (BI) and maximum likelihood (ML) were used to reconstruct relationships. The BI analysis was performed in MrBayes v.3.2.6\(^6\) using the MCMC method with 10 million generations, three runs (8 chains per run) with a burn-in of 25% and a standard deviation of split frequencies < 0.01. Nodes with posterior probability (pp) values ≥ 0.95 were considered supported. The ML analysis was performed using RaxML v.8.2\(^8\), as implemented in raxmlGUI v1.5b\(^1\), with 100 independent replicates and 1000 rapid bootstraps. Nodes with bootstrap values ≥ 75 were considered supported. The resulting trees from the BI and ML analyses were visualized with FigTree v1.4.2\(^8\). Sequences (57) corresponding to the mtDNA regions
rnS and rnlL of isolates analyzed in previous studies, available in GenBank, were also included in our analyses. Aphanomyces fennicus was used as the outgroup in both phylogenetic approximations. Analyses were performed with rnsS and rnlL individually, as well as with a concatenated rnsS and rnlL dataset, using the same parameters described above.

Genetic diversity was estimated by calculating the number of polymorphic (segregating) sites (S), the number of haplotypes, the haplotype diversity (Hd), the average number of nucleotide differences (k), and the nucleotide diversity (nπ) utilizing the program DNAsp v.5.10.01. Mutational changes between sequences in the most parsimonious haplotype network were estimated using TCS v.1.21, and the genealogical relationships were visualized with PopArt v.1.7.2.

**Ethics declarations.** All experimental procedures and animal manipulations, as well as field sampling, were performed according to the US legislation.

**Data availability** All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

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Author contributions

L.M.T. contributed to the design, with the laboratory work and writing of this manuscript. M.M.R. and G.C.H. contributed with the laboratory work and writing of this manuscript. S.B.A., C.R.J. and J.D.U. contributed to the supervision and writing of this manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to L.M.-T.

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