Ultrastructural and genomic characterization of a second banchine polydnavirus confirms the existence of shared features within this ichnovirus lineage

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INTRODUCTION

Currently, the virus family Polydnaviridae comprises two taxa: the bracoviruses (BVs) and the ichnoviruses (IVs), named for the wasp families Braconidae and Ichneumonidae, respectively, that carry them (Francki et al., 1991). In terms of morphology, these entities are very distinct: while BV particles consist of one or more cylindrical nucleocapsids surrounded by a single unit membrane envelope, IV particles have lenticular nucleocapsids surrounded by two envelopes (Stoltz & Vinson, 1979). Most IV isolates described to date were obtained from wasps belonging to the subfamilies Campopleginae and Banchinae displaying strikingly different virion morphologies and genomic features. However, the current description for banchine IVs is based on the characterization of a single species, namely that of the Glypta fumiferanae IV (GfIV). Here we provide an ultrastructural and genomic analysis of a second banchine IV isolated from the wasp Apophua simplicipes, and we show that this virus shares many features with GfIV, including a multi-nucleocapsid virion, an aggregate genome size of ~300 kb, genome segments <5 kb, an impressively high degree of genome segmentation and a very similar gene content (same gene families in both viruses). Altogether, the data presented here confirm the existence of shared characteristics within this banchine IV lineage.

The GenBank/EMBL/DDBJ accession numbers for the sequences of contigs >500 bp determined in this study are KC752207–KC752432. A supplementary table and five supplementary figures are available with the online version of this paper.

Polydnaviruses (PDVs) are symbiotic viruses carried by endoparasitic wasps and transmitted to caterpillar hosts during parasitization. Although they share several features, including a segmented dsDNA genome, a unique life cycle where replication is restricted to the wasp host, and immunodepressive/developmental effects on the caterpillar host, PDVs carried by ichneumonid and braconid wasps (referred to as ichnoviruses and bracoviruses, respectively) have different evolutionary origins. In addition, ichnoviruses (IVs) form two distinct lineages, with viral entities found in wasps belonging to the subfamilies Campopleginae and Banchinae displaying strikingly different virion morphologies and genomic features. However, the current description for banchine IVs is based on the characterization of a single species, namely that of the Glypta fumiferanae IV (GfIV). Here we provide an ultrastructural and genomic analysis of a second banchine IV isolated from the wasp Apophua simplicipes, and we show that this virus shares many features with GfIV, including a multi-nucleocapsid virion, an aggregate genome size of ~300 kb, genome segments <5 kb, an impressively high degree of genome segmentation and a very similar gene content (same gene families in both viruses). Altogether, the data presented here confirm the existence of shared characteristics within this banchine IV lineage.

In order to further explore this question, a PDV isolate from Glypta fumiferanae was recently characterized in some detail (Lapointe et al., 2007; Cusson et al., 2012). The study identified a number of differences between this virus and others.
(originally referred to as GfV) and typical IVs. First, as previously reported (Stoltz et al., 1981), each particle contained several smaller nucleocapsids. In addition, nucleocapsid surface substructure could be much more readily visualized. Interestingly, the packaged viral genome was observed to display an unusually high degree of segmentation (≥ 105 genome segments) and was shown to encode a family of proteins that had not previously been associated with any PDV genome, namely the NTPase-like family. Like all BV genomes thus far sequenced, the G. fumiferanae viral genome was found to contain a number of protein tyrosine phosphatase (PTP) genes, which had never been found in other IVs. Taken together, the differences observed between the *Glypta* virus and the better-known campoplegine IVs were deemed to be fairly significant (Lapointe et al., 2007).

While the BVs are known to derive from a nudiviral ancestor (Bézier et al., 2009), IV origins have remained obscure. Recent work suggests that the IV lineage likely derives from the acquisition by an ichneumonid parasitoid of a viral genome not as yet represented in extant databases, or else is now extinct (Volkoff et al., 2010). Specifically, IV structural polypeptides were shown to be encoded by unusual regions of the wasp genome referred to as ‘Ichnovirus Structural Protein Encoding Regions’, or IVSPERs. IVSPERs differ significantly from typical wasp genomic sequences in that they exhibit a higher exon density and contain genes made of a single exon, indicating that they likely encode unspliced mRNAs. Accordingly, IVSPERs are considered to be almost certainly viral in origin (Volkoff et al., 2010, 2012). A recent analysis of transcripts isolated from *G. fumiferanae* ovaries revealed similarities between some transcripts and IVSPER genes (M. Cusson and others, unpublished; see also Cusson et al., 2010), suggesting a common origin for both campoplegine and banchine PDVs. For this reason, the *Glypta* virus is currently considered to be an IV, and so will hereafter be referred to as GfIV. Since only a single banchine PDV species has as yet been characterized, we thought it important to describe an additional isolate, if such indeed existed, from the Banchinae.

Here we report the discovery of a second banchine IV (AsIV), from *Apophua simplicipes*, a parasitoid of the oblique-banded leafroller, *Choristoneura rosaceana* (Cossentine et al., 2004). We present a transmission electron microscopy (TEM) characterization of AsIV virion morphology and an SDS-PAGE comparison of its polypeptide profile with that of GfIV. We also provide an electrophoretic and genomic characterization of its segmented, circular dsDNA genome. Finally, we discuss these findings in the context of polydnavirus phylogenomics.

**RESULTS AND DISCUSSION**

**Virion structure and morphogenesis**

The morphology of the AsIV virion appears very similar to that of the only other banchine PDV thus far studied, namely GfIV (Lapointe et al., 2007; Cusson et al., 2012). Like GfIV particles, the AsIV virion consists of several nucleocapsids surrounded by two unit membranes, the inner of which is assembled de novo within the nuclei of calyx epithelial cells, with the latter being acquired by budding through the apical plasma membrane (Fig. 1). As with GfIV, the AsIV subvirion (inner membrane and enclosed nucleocapsids) exits through the nuclear envelope (Fig. 1) and then rapidly loses both of the two additional membranes acquired as a result of this nuclear exit strategy; interestingly, while nuclear egress is readily observed in the case of other ichnoviruses, including GfIV, it was only very rarely observed with AsIV. AsIV subvirions often appeared in membrane protrusions of the basal plasma membrane, but successful budding from that region of the cell surface was never observed. Particles traversing the cytoplasm often appeared to be aligned with profiles of the rough endoplasmic reticulum (not shown), suggesting either specific recognition events or, alternatively, a transient physical impediment to free diffusion within the cytosol. Subvirions associated with the putative virogenic stroma were roughly spherical in shape, while those apparently separated from this material, or which had reached the cytoplasm, were typically elongated (Figs 1 and 2), and so it is difficult to know whether there is a real size/volume difference between intranuclear and cytosolic subvirions; that said, it is clear that nucleocapsids are more tightly packed together in the case of the latter. In addition, AsIV nucleocapsid diameter appeared to undergo a marked reduction during particle morphogenesis: nucleocapsids associated with the virogenic stroma appear wider than...
those observed in what are assumed to represent more mature particles (Figs 1 and 2). Similar though much more subtle changes of this nature have been seen in the case of several other IVs (D. Stoltz, unpublished). Large aggregates, possibly representing excess nucleocapsids, were very common in A. simplicipes calyx epithelial cells (Fig. 2). These have also been observed in Glypta material, though less frequently (unpublished observations). We have not as yet been able to obtain sufficient material with which to examine nucleocapsid structure by negative staining. As far as can be determined from sectioned material, however, AsIV nucleocapsids appear very similar to those seen in sectioned G. fumiferanae calyx tissue. As has been observed in the case of G. fumiferanae ovaries (D. Stoltz, unpublished), rickettsia-like endosymbionts are present in calyx epithelial cells (not shown), but were not seen in calyx fluid itself. Thus, overall AsIV virion structure and morphogenesis match very closely those described for GfIV (Lapointe et al., 2007; Cusson et al., 2012).

Since very few wasps were available to us, we were not able to adequately address the question of when AsIV morphogenesis actually begins, but we assume that it probably occurs in the late pupal stage, as virus was plentiful in the ovaries of the 1-week-old female examined. The calyx tissue of the 3-week-old female was identical in terms of ultrastructure to that of the younger female. Virion morphogenesis, as in other IVs, appeared to proceed continuously. Budding at apical cell surfaces also appeared to be ongoing, and of course provides a non-lytic strategy for virus release. We believe it reasonable to assume that the calyx epithelium likely maintains its integrity throughout the adult wasp lifespan.

Electrophoretic fractionation of AsIV virion proteins and DNA

In spite of the high degree of ultrastuctural similarity between AsIV and GfIV virions, the proteins extracted from these two viruses displayed very different SDS-PAGE profiles, both in terms of polypeptide size and relative abundance (Fig. 3a). However, these observations do not necessarily reflect large differences in terms of homologous relationships between AsIV and GfIV virion proteins, given that structural proteins obtained from different campoplegine IVs also tend to display distinct electrophoretic profiles (D. Stoltz and M. Cusson, unpublished data) while many of these proteins clearly have a common ancestor (Volkoff et al., 2010).

Agarose gel electrophoresis (AGE) analysis of AsIV DNA (Fig. 3b) revealed a range of genome segment sizes (~1–4 kb) similar to that observed for GfIV (~1.5–5.2 kb) and, therefore, much lower than that reported for campoplegine IVs (see Lapointe et al., 2007).

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**Fig. 2.** Nucleocapsid (arrows) diameter appears to shrink as the subvirion moves away from the virogenic stroma (VS). Nucleocapsid aggregates (NC agg.) were commonly observed within the nuclei of calyx epithelial cells. N, nucleus; C, cytoplasm. Bar, 200 nm.

**Fig. 3.** Electrophoretic fractionation of AsIV virion proteins and DNA. (a) SDS-PAGE fractionation of AsIV virion proteins, as compared with those of GfIV. Positions of molecular markers (kDa) are shown on the left side of the gel. (b) AGE fractionation of AsIV DNA (third lane). The first and second lanes show the positions of a 1 kb DNA ladder and covalently closed circular (ccc) DNA markers (Invitrogen), with sizes (kb) shown on the left and right sides of the figure, respectively.
Genomic analysis of AsIV

To characterize the AsIV genome, we used a next-generation sequencing approach that is fundamentally different from the strategy employed to sequence the GfIV genome. Whereas we cloned individual GfIV genome segments prior to submitting them to Sanger sequencing (Lapointe et al., 2007), here we first amplified the DNA extracted from AsIV virions and then submitted it to Roche 454 (GS-FLX+) sequencing, after fragmentation and library preparation. Newbler assembly of the 454 reads generated 347 unique contigs, 132 of which fell within the predicted size range of AsIV genome segments (i.e. 1–4 kb; see Fig. 3b), with the largest one being 3720 bp in length (GenBank accession numbers for contigs >500 bp: KC752207–KC752432). These contigs were assumed to represent individual genome segments, although some of them likely correspond to partial sequences that have failed to assemble with small contigs, including those <1 kb. The circular nature of the putative AsIV genome segments was apparent in 32 contigs that contained a portion of a single ORF at each of the 5’ and 3’ ends, implying that circularization of the sequence is necessary for the gene to be functional (see for examples, contig000002, contig00010 or contig00026). We did not attempt to confirm the correspondence between contigs and whole genome segments, but alignment of some of them with homologous GfIV genome segments pointed to a high degree of similarity between the overall segment length (see Fig. S1, available in JGV Online, for examples), suggesting that these segments were adequately assembled. Thus, it is clear from the present data that the AsIV genome displays a very high degree of segmentation, similar to that reported for GfIV (105 segments; Lapointe et al., 2007), if not higher. Based on all contigs available, the aggregate genome size of AsIV was estimated to be ~304 kb, similar to the ~291 kb reported for GfIV (Lapointe et al., 2007).

To identify AsIV genes, all contigs >500 bp were scanned for putative ORFs, 168 of which were detected. Strikingly, BLAST analysis against the NCBI non-redundant database revealed exactly the same gene families identified in GfIV (Lapointe et al., 2007; Cusson et al., 2012), represented by 69 ORFs in AsIV (Table 1). These included PTPs, viral ankyrins (Vanks), NTPase-like proteins, BV-like proteins and recombinase-like proteins (MULE transposase domains). In all analyses, the closest relatives were GfIV proteins, pointing to an unequivocal homologous relationship between the genes of these two viruses. Interestingly, we could not detect ORFs in a large portion of the contigs analysed (90/227 or 40%). Although the latter value is most likely an overestimate resulting from the presence of ORF-free contigs that should have assembled with contigs containing ORFs, an unusually high proportion of GfIV genome segments (30%) was also observed to contain no detectable ORFs (Lapointe et al., 2007), pointing to another genomic feature shared by AsIV and GfIV.

As observed for GfIV, the PTPs represent the largest AsIV gene family, with 11 more members than in the GfIV genome (Table 1). PTPs are also an important gene family in the bracoviruses (Provost et al., 2004), but banchive IV homologues constitute a distinct lineage (Lapointe et al., 2007). Here, a phylogenetic analysis of AsIV and GfIV PTPs pointed to a high degree of similarity between the two groups of proteins, including potential orthologous relationships for 12 GfIV–AsIV protein pairs (Fig. 4). Their functions in banchive IVs have not been examined yet, but some BV PTPs have been shown to display phosphatase activity (Provost et al., 2004; Eum et al., 2010) and to have antiphagocytic properties (Pruijssers & Strand, 2007) through induction of apoptosis of phagocytic immune cells (Suderman et al., 2008). They may also be involved in the disruption of ecdysone biosynthesis in prothoracic glands of infected hosts (Falabella et al., 2006). Among the AsIV PTP sequences reported here, some of which appear to be incomplete, only four contain the catalytic cysteine residue considered essential for enzymic activity (see Eum et al., 2010). Whether enzymically inactive PTPs play a role during parasitism remains to be determined.

The viral ankyrins represent the only PDV gene family shared by BVs and both IV lineages (Lapointe et al., 2007). CLUSTAL W alignment of the nine AsIV Vank proteins reported here (Table 1) with those of GfIV revealed a notable degree of sequence similarity between the two protein sets (Fig. S2), again pointing to the relatedness of these two viruses. In contrast, an earlier phylogenetic analysis indicated a relatively high level of sequence divergence between IV and BV Vank copies (Lapointe et al., 2007). In host–parasitoid relationships involving BV-carrying wasps, Vank proteins have been shown to block the activity of NF-kB transcription factors, thereby interfering with the activation of immune genes (Thoetkiattikul et al., 2005; Falabella et al., 2007). Although Vank proteins from both IV lineages likely play a similar role, this hypothesis has not yet been tested.

Table 1. Number of ORFs as a function of gene family identified in AsIV and GfIV

<table>
<thead>
<tr>
<th></th>
<th>PTP</th>
<th>Vank</th>
<th>NTPase-like</th>
<th>BV-like</th>
<th>MULE</th>
<th>Unassigned</th>
</tr>
</thead>
<tbody>
<tr>
<td>AsIV</td>
<td>34</td>
<td>9</td>
<td>11</td>
<td>16</td>
<td>1</td>
<td>115</td>
</tr>
<tr>
<td>GfIV*</td>
<td>23</td>
<td>4</td>
<td>9</td>
<td>4</td>
<td>1</td>
<td>60</td>
</tr>
</tbody>
</table>

*From Lapointe et al. (2007).
The NTPase-like proteins identified earlier in the GfIV genome represented a new PDV gene family, apparently unique to the banchine IV lineage (Lapointe et al., 2007). Although these proteins varied greatly in size, the family was observed to contain some of the largest PDV-encoded proteins reported to date (e.g. 972 amino acids for C20-1; Cusson et al., 2012) and found on the largest GfIV genome segments (Lapointe et al., 2007). A similar situation was observed for AsIV NTPase-like proteins, 11 of which were identified in the present study (Table 1). The coding region of the largest member of this family (853 aa) was identified on the largest AsIV contig (contig1-ORF1), thus presumably on the largest genome segment. In BLASTP searches against the NCBI non-redundant database, all NTPase-like proteins identified here had GfIV NTPase-like proteins as their closest relatives, with e-values close to zero in some cases (see Table S1). Interestingly, however, a phylogenetic analysis of AsIV and GfIV NTPase-like proteins (Fig. 5) revealed a greater degree of divergence between these two groups of proteins than that observed for PTPs (Fig. 4). Indeed, the analysis suggested few, if any, potential orthologous relationships for AsIV–GfIV protein pairs, indicating that these genes may be under stronger positive selection than the PTPs.

Banchine IV NTPase-like genes appear to have been acquired through the horizontal transfer of a viral primase/helicase gene from an ascovirus to the genome of an ancestor of *A. simplicipes* and *G. fumiferanae*. Evidence for this phenomenon was first reported by Bigot et al. (2008) following the sequencing of the *Diadromus pulchellus* ascovirus 4a (DpAV4a), from which one of the encoded proteins displayed much higher similarity to GfIV NTPase-like proteins than to any other
known protein. Sequencing of the genome of another ascovirus (*Heliothis virescens* ascovirus 3g; HvAV3g; Huang et al., 2012) has recently revealed the existence of another ascoviral primase/helicase whose amino acid sequence is even closer to those of the banchine NTPase-like proteins (Table 2), giving additional support to the ascovirus–ichnovirus horizontal gene transfer hypothesis.

The function of the banchine IV NTPase-like proteins is currently unknown, but a recent analysis of the primary sequence of some GfIV representatives suggested that they have lost most or all of the primase domain and that the NTPase/helicase domain is catalytically inactive (Cusson et al., 2012). The NTPase-like genes are among the most highly expressed GfIV genes during parasitism of *Choristoneura fumiferana*, which includes a 26-week host diapause (M. Cusson, unpublished data). It has been suggested that the function of NTPase-like proteins may be related to the uncommon life history strategy of the univoltine *G. fumiferanae* (Lapointe et al., 2007; Cusson et al., 2012), whose larvae spend the winter as first instars within their second instar host (Brown, 1946). However, the present study casts some doubt on this hypothesis since *A. simplicipes* can successfully parasitize larvae of both summer and winter generations of its bivoltine *Choristoneura rosaceana* host (Cossentine et al., 2004), which would leave the AsIV NTPase-like proteins in search of a function during the summer generation. A comparison of AsIV NTPase-like gene transcript abundance between summer and winter hosts could help shed some light on this issue.

The four GfIV BV-like proteins, thus named because of their homology to a protein encoded by the *Cotesia vestalis* (Cotesia plutellae) BV (GenBank accession number AAZ04291), were found to have 16 homologues in AsIV (Table 1 and Figs 2 and S3), indicating a very significant expansion of this gene family in AsIV and suggesting that it may play an important role in this host–parasitoid system. No specific domain is recognized in these proteins and their function is unknown. We also identified an AsIV protein showing similarity to another BV-related protein that was identified in GfIV (Lapointe et al., 2007), a recombinase-like protein with a MULE domain similar to two *Chelonus inanitus* BV proteins (GenBank accession numbers CAA91234 and CAC82011; see Fig. S4). Again, the exact function of these proteins is unknown.

Finally, among the 115 AsIV ORFs that could not be assigned to known families (Table 1), we identified 10 clusters containing two or more related proteins, including seven with GfIV homologues. The largest of these families has two AsIV and four GfIV members (Fig. S5), none of which have recognizable domains. Nonetheless, the presence of families like this one in both viruses suggests that they may serve a function during parasitism.

In conclusion, most AsIV features described in the present study match very closely those reported earlier for GfIV (Lapointe et al., 2007; Cusson et al., 2012), providing unequivocal evidence for the common origin of these two viruses. The data presented herein thus confirm the existence of shared characteristics within this banchine IV lineage, the description of which had until now been based on a single species, namely GfIV. It is worth noting that *A. simplicipes* and *G. fumiferanae* are two very closely related species that belong to the same tribe, Glyptini, while the subfamily Banchinae contains two additional tribes, Banchini and Atrophini. Although a wasp from the latter tribe (*Lissosoma* sp.) has been identified as carrying a putative PDV (Stoltz & Whitfield, 1992), its similarity and relatedness to AsIV and GfIV have not yet been assessed.

### Table 2. BLASTP e-values for comparisons between NTPase-like proteins of AsIV and GfIV, and ATPases from the ascoviruses HvAV3g (GenBank accession number AFV50347) and DpAV4a (CCA61447)

<table>
<thead>
<tr>
<th></th>
<th>AsIV NTPase-like 12-1</th>
<th>GfIV NTPase-like C20-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>HvAV3g-ATPase</td>
<td>2 e–54</td>
<td>5 e–61</td>
</tr>
<tr>
<td>DpAV4a-ATPase</td>
<td>3 e–47</td>
<td>4 e–38</td>
</tr>
</tbody>
</table>

Values shown here are for the closest AsIV and GfIV relatives only.
Given that there is little evidence that each of the three banchine tribes is reciprocally monophyletic (Quicke et al., 2009), an investigation of putative PDVs carried by Banchini and Atropophini wasps would help to determine whether the features described here apply to viruses potentially found in all three banchine tribes.

METHODS

Insects. A. simplicipes wasps came from a laboratory colony maintained by J. Cossentine (Agriculture and Agri-Food Canada, Summerland, BC, Canada) on Choristoneura rosaceana larvae. G. fumiferanae wasps were obtained from field-collected Choristoneura fumiferanae hosts as described (Lapointe et al., 2007).

TEM. Ovaries from two adult wasps (aged 1 and 3 weeks) were dissected out into the primary fixative (3% glutaraldehyde plus 1% acrolein in 50 mM sodium cacodylate buffer containing 250 mM sucrose) and left at room temperature for 2 h. While in the fixative, the calyx region was separated from other parts of the ovaries. Post-fixation was in 2% osmium tetroxide in the same buffer, followed by overnight staining in 0.1% uranyl acetate. Embedment was in TAAB resin, following dehyrdration in acetone. Micrographs were taken with a JEOL JEM-1250 TEM.

SDS-PAGE fractionation of AsIV virion proteins. Ovaries of 10 A. simplicipes wasps were dissected in PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ and gently teased open to release the calyx fluid. The latter was collected with a glass pipette and centrifuged at 2375 g to pellet the virus. The AsIV pellet was resuspended in 12 µl insect saline. After boiling with 5× sample buffer (0.22 M Tris-HCl, pH 6.8, 49% glycerol, 5.5% SDS, 0.005% bromophenol blue, 0.25 M DTT, 5 µl was fractionated in a 10% SDS-PAGE gel. The proteins were then submitted to silver staining. For comparative purposes, a GIV virion extract was obtained using a similar procedure and run on the same gel next to the AsIV sample.

AsIV DNA extraction and AGE fractionation. Calyx fluid was obtained from eight A. simplicipes wasps using the method described above, and partially purified through a 0.45 µm nitrocellulose filter. The viral DNA was extracted as described (Béliveau et al., 2000; Stoltz et al., 1986), treated with RNase I, taken up in 20 µl water and quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Viral DNA (250 ng) was fractionated in a 1.2% agarose gel at 75 V for ~45 min.

Sequencing of the packaged AsIV genome and genomics analyses. DNA extracted from AsIV virions (~45 ng) was amplified using the REPLI-g Mini kit (Qiagen) according to the manufacturer’s instructions. One microgram of amplified DNA was fragmented using a nebulizer from the Rapid Library kit (Roche) and used for Roche 454 Rapid Library construction according to procedures provided by Roche. Following quantification and recommended quality controls, the library was sequenced on a GS-FLX+ (Roche) at the Plateforme d’Analyses Génomiques de l’Institut de Biologie Intégrative et des Systèmes (Université Laval, Canada) according to Roche procedures, and at a coverage of ~20×. Genome assembly was performed with the gAssembler module of Newbler v2.5.3, using default parameters.

Contigs >500 bp in length were submitted to ORF Finder (http://www.ncbi.nlm.nih.gov/projects/orfl/) and to BLASTX analyses (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify putative ORFs. Only protein products with >100 amino acid residues were considered in these analyses, although some smaller ones identified as belonging to established gene/protein families were also included. The deduced amino acid sequences were then subjected to various analyses, including BLASTP against the NCBI non-redundant database and local BLASTP searches against all known AsIV and GIV ORFs.

To reconstruct FPT and NTpase-like phylogenetic trees, relevant AsIV and GIV nucleotide sequences were imported into MEGA5 (Tamura et al., 2011), where they were conceptually translated into proteins. Alignments were performed on the amino acid sequences using CLUSTAL W, as implemented by MEGA5, with default settings except for penalties relating to multiple alignment gap openings and extensions, which were set to 3.0 and 1.8, respectively (Hall, 2008). The back-translated nucleotide alignment was then used to reconstruct a neighbour-joining tree (Saitou & Nei, 1987) using the MEGA5 default settings, except for gap deletions, where the pairwise deletion option was selected. Bootstrapping was performed on 2000 pseudoreplicates.

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