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NOVEL PARVOVIRUS FROM THE WORM LIZARD *TROGONOPHIS WIEGMANNI* – FIRST VIRUS EVER DETECTED IN AMPHISBAENIAN HOSTS

Judit J. PÉNZES[1*] and Mária BENKŐ[2]

Institute for Veterinary Medical Research, Centre for Agricultural Research, Hungarian Academy of Sciences, P.O. Box 18, H-1581 Budapest, Hungary

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To explore the diversity of some DNA viruses in reptiles, a continuous screening is going on, in our laboratory, by PCR using different consensus primers designed for the detection of the most conserved genome regions of adeno-, herpes- and parvoviruses. The test material consists essentially of dead specimens collected randomly from private pet owners, local pet shops, or at occasional exotic pet fairs. Here we report the partial sequence of a putative novel parvovirus obtained from a dead checkerboard worm lizard *(Trogonophis wiegmanni)* that had been wild-caught in its native habitat. An in-house-developed PCR with consensus primers targeting the gene of the parvoviral capsid protein was used. Other PCRs, intended to detect certain large DNA viruses, remained negative. The sequence of the PCR product indicated the presence of a hitherto unknown parvovirus in the internal organs of the checkerboard worm lizard. In phylogeny reconstruction, the novel sequence clustered with the members of the *Dependovirus* genus of the *Parvoririnae* subfamily, closest to the branch of snake adeno-associated virus. Since we could not demonstrate the presence of a potential helper virus, the putative amphisbaenian parvovirus supposedly can replicate autonomously. This is the first virus infection ever detected in any members of the suborder Amphisbaenia, and only the third parvoviral sequence obtained from any reptilian host.

Key words: Amphisbaenia, autonomous replication, *Dependovirus*, PCR, reptile, Trogonophidae, worm lizard

The genomic study of viruses occurring in lower vertebrates is a relatively new but rapidly progressing field that has been fuelled by the robust development and widespread use of sensitive molecular detection methods recently. Distinct viral infections have been described in representatives of some species of almost every larger reptilian taxon. Among the few exceptions with no virus de-

[*]Corresponding author; E-mail: penzes.judit@agrar.mta.hu; Phone: 0036 (1) 467-4083; Fax: 0036 (1) 467-4076

tection record are the order Rhynchocephalia and the group of Amphisbaenia (Essbauer and Ahne, 2001; Marschang, 2011).

The suborder Amphisbaenia is a less-known group of fossorial squamate reptiles completely adapted to their hidden burrowing lifestyle (Kearney, 2003). Their position in reptile taxonomy is controversial in respect of morphology and phylogenetic issues. Analyses based on morphology suggest a common origin with other limbless squamate animals such as serpents and dibamids (Conrad, 2008). On the other hand, according to molecular data, Amphisbaenia seems to be a monophyletic clade clustering with Old World terrestrial lizards, i.e. lacertids (Townsend et al., 2004; Wiens et al., 2012). The checkerboard worm lizard *(Trogonophis wiegmanni)* lives along the North Western Mediterranean coast of Africa (including Morocco, Algeria and Tunisia), buried in the earth in sandy-soiled habitats (Civantos et al., 2003). Less is known about the way of living and social life of these reptiles. Nonetheless, checkerboard worm lizards appear to have seasonal mating and parental care; this latter being a rather uncommon behaviour exhibited by members of the order Squamata (Martin et al., 2011). They are ovoviviparous, giving birth to two to five youngsters (Miras et al., 2009). According to the classification of the International Union for the Conservation of Nature (IUCN), the checkerboard worm lizard is listed as Least Concern in view of its wide distribution and tolerance of a broad range of habitats (Miras et al., 2009). This species is not included in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), i.e. the Washington Convention either.

As the popularity of reptiles as pets keeps increasing, our knowledge about their different diseases including viral infections becomes more and more comprehensive (Jacobson, 2007). Initially, the presence of several RNA and DNA viruses in different reptilian hosts was diagnosed essentially on the basis of light or electron microscopic histology findings, and successful virus isolation has been limited to a few occasions only. By histology, parvovirus-like particles were observed and described in a bearded dragon *(Pogona vitticeps)* with concurrent adenovirus (AdV) infection (Jacobson et al., 1996). A similar double infection by adenovirus and dependovirus in California mountain kingsnakes *(Lampropeltis zonata multicincta)* has been published more recently (Wozniak et al., 2000). A relatively early adenovirus isolation, from a captive corn snake *(Pantherophis guttatus)*, was reported from Germany (Juhasz and Ahne, 1993). Other AdV isolates, obtained from a royal python *(Python regius)* and from a boa constrictor *(Boa constrictor)* have been found to contain parvoviruses, too (Ogawa et al., 1992). The complete genome sequence of the corn snake adenovirus isolate, named as snake adenovirus 1, has been published recently (Farkas et al., 2008). It seemed to be identical with the virus obtained from the python and the boa. The full sequence of the snake parvovirus, co-isolated with the python adenovirus was published almost a decade ago (Farkas et al., 2004), yet ever since only

one partial sequence from an additional putative reptilian parvovirus, detected by PCR in an Indonesian pit viper *(Parias hageni)*, has been described (Farkas and Gál, 2008).

Members of the *Parvoviridae* family are small, non-enveloped particles of icosahedral symmetry. Their linear, single-stranded DNA genome (of 4–6.3 kb) contains two major ORFs coding for the replication or non-structural (NS) and for the capsid (VP) proteins. Parvoviruses occur in numerous vertebrate and invertebrate hosts. Accordingly, the family is divided into two subfamilies *(Parvovirinae* and *Densovirinae)*, the former of which contains the parvoviruses of vertebrates. This subfamily comprises five genera at present. Based on phylogeny inference, the two snake parvoviruses had been proposed to belong to the *Dependovirus* genus, but they have not been approved yet as official species (Tijssen et al., 2011). The name of the genus reflects a deemed common feature of its members, i.e. their dependence on some helper viruses (usually adeno- or herpesviruses) for efficient replication. However, several parvoviruses, known to cause serious disease, namely the so-called Derzsy's disease in goose and Muscovy duck, are exceptions to this rule. These viruses are capable of autonomous replication in spite of their genome organisation and phylogenetic place showing them clearly to belong to the *Dependovirus* genus (Le Gall-Reculé and Jestin, 1994; Brown et al., 1995; Zádori et al., 1995). Nonetheless, parvoviruses in reptiles have been found with concomitant adeno- or herpesvirus infection in all cases reported previously (Heldstab and Bestetti, 1984; Ahne and Scheinert, 1989; Jacobson et al., 1996; Wozniak et al., 2000; Kim et al., 2002; Farkas and Gál, 2008).

As no viruses at all have been described from any amphisbaenian hosts to date, our aim was to screen this group of squamate reptiles for the presence of different DNA viruses. However, amphisbaenians are neither too popular nor frequent in Hungary as well as in Europe, thus we were lucky to detect a novel putative virus in the very first specimen examined.

Materials and methods

The carcass of an adult (approx. 10 cm long) female checkerboard worm lizard *(Trogonophis wiegmanni)* was obtained from a Hungarian licensed collector. The animal had been wild-caught somewhere in North Africa, then sold at a reptile fair. It died unexpectedly without showing alarming disease signs. The carcass was slightly emaciated. Signs of autolysis were present due to the delayed discovery of death. For DNA extraction, small (15–25 mg) pieces from the internal organs (kidney, liver, lungs and intestine) were pooled and homogenised. We did the DNA purification with the use of the DNeasy Blood and Tissue Kit (Qiagen®, Hilden, Germany) according to the protocol recommended for animal tissues with an overnight incubation at 37 °C.

We subjected the sample to PCR screening for adenovirus, herpesvirus and parvovirus DNA. For the detection of adenoviruses, we used a nested PCR with consensus primers targeting the most conserved part of the DNA-dependent DNA polymerase gene as described by Wellehan et al. (2004). Amplification of the herpesviral DNA-polymerase gene fragment was attempted by another nested consensus PCR published by VanDevanter et al. (1996). Moreover, we also performed a wider-range PCR described for the DNA polymerase gene of certain other large DNA viruses (Hanson et al., 2006). For parvovirus detection, we designed a consensus (degenerate) primer pair based on the conserved amino acid (aa) motives found in the capsid protein (VP) of dependoviruses sequenced to date. The nucleotide (nt) sequences were 5'-GGYGCCGAKGGAGTGGGYAAT KCCTC-3' for the forward and 5'-TCAAARTTRTTBCCBGTYCTYAGCAT-3' for the reverse primer (we used the nt ambiguity codes recommended by the IUPAC). The calculated size of the expected specific product was around 600 base pairs (bp). After an initial denaturing step at 92 °C for 5 min, the PCR program consisted of 45 cycles of denaturation at 92 °C for 30 sec, annealing at 46 °C for 60 sec and elongation at 72 °C for 60 sec. A final elongation step (at 72 °C for 5 min) was added at the end. We run the reactions in 50 µl volume with the use of AmpliTaq Gold® DNA Polymerase (Life Technologies Corporation®, Carlsbad, CA, USA) according to the manufacturer's recommendation.

The PCR product was purified and sequenced directly by the PCR primers on both strands. We performed the sequencing reactions by using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies Corporation®, Carlsbad, CA, USA), and sent them for electrophoresis on an ABI PRISM 3100 Genetic Analyzer (Life Technologies Corporation®, Carlsbad, CA, USA) by a commercial service. For identification and comparison of the newly obtained sequence with earlier published parvoviral sequences, we used the BLASTx and BLASTn tools at the NCBI website.

Phylogenetic calculations were carried out essentially by a scheme described in detail elsewhere (Doszpoly et al., 2013). We collected the protein sequences of the homologue region from 15 parvoviruses so that all the five presently approved genera of the subfamily *Parvovirinae* be represented. The corresponding sequence of the *Bombyx mori* densovirus from the *Iteravirus* genus of the *Densovirinae* subfamily was selected as outgroup. A multiple alignment from these sequences, retrieved from the GenBank, was created using the Clustal X2 algorithm (Larkin et al., 2007). Model selection was performed by the JModelTest 0.1 package. We made a maximum likelihood analysis (WAG model with 100 bootstrap runs applying TOPALi v2.5) and a Bayesian phylogeny inference (MrBayes, 4 runs for 1,000,000 generations, 100 for sample frequency, burn in: 40%). For visualising the phylogenetic trees, we used the FigTree v1.3.1.

Results

The eventual pathological alterations of the internal organs could not be assessed due to their progressed autolysis. All PCRs, aiming at the detection of adenoviruses, herpesviruses, or any other large DNA viruses, remained negative. The only positive reaction was obtained by the PCR targeting parvoviruses. The nt sequence determined from the VP gene of the putative novel parvovirus present in the sample of the checkerboard worm lizard consisted of 538 bp after removal of the primer sequences. The deduced aa sequence, used for the alignments, contained 179 residues. The G+C content (48.64%) was found to be balanced. We deposited the sequence in the GenBank database under accession number KF289390.

In the BLASTn application, the newly determined sequence showed the highest score (77% nt identity) with the corresponding gene fragment of the snake parvovirus 1 (Farkas et al., 2004). According to the BLASTx results, the snake and amphisbaenian parvoviruses shared 83% aa sequence identity. In the phylogeny reconstructions, the new putative amphisbaenian parvovirus appeared as a separate branch among the members of the *Dependovirus* genus within the *Parvovirinae* subfamily. The monophyletic status of the two reptilian parvoviruses was strongly supported by the high bootstrap (98) and posterior probability (0.99) values. Since there was no significant difference between the topology of the trees obtained by the different inference methods, only the maximum likelihood tree is presented (Fig. 1).

Discussion

The occurrence of members of a large variety of different virus families in reptiles has been described in the past 30 years, yet the number of reptilian viruses with partial or complete genome sequences is still rather limited (Jacobson, 2007; Marschang, 2011). This is especially true for parvoviruses, sequence data from which have been reported only from snakes to date. The snake adeno-associated virus, co-isolated with snake adenovirus 1 from a royal python (Ogawa et al., 1992) is still the only reptilian parvovirus with a fully-sequenced genome (Farkas et al., 2004). A paper reporting partial sequences from a second putative reptilian parvovirus, detected in a captive Indonesian pit viper, was published more recently (Farkas and Gál, 2008). These authors determined the sequence of an approximately 1300-bp PCR fragment, containing the carboxyl terminal part of the NS and the amino-terminal fragment of the VP gene. Unfortunately, this fragment does not overlap with the sequence determined by us, thus the direct comparison of our amphisbaenian parvovirus with the proposed snake adeno-associated virus 2 was impossible. Nonetheless, our results provide the first evidence for the presence of a parvovirus in a non-serpent squamate reptile.

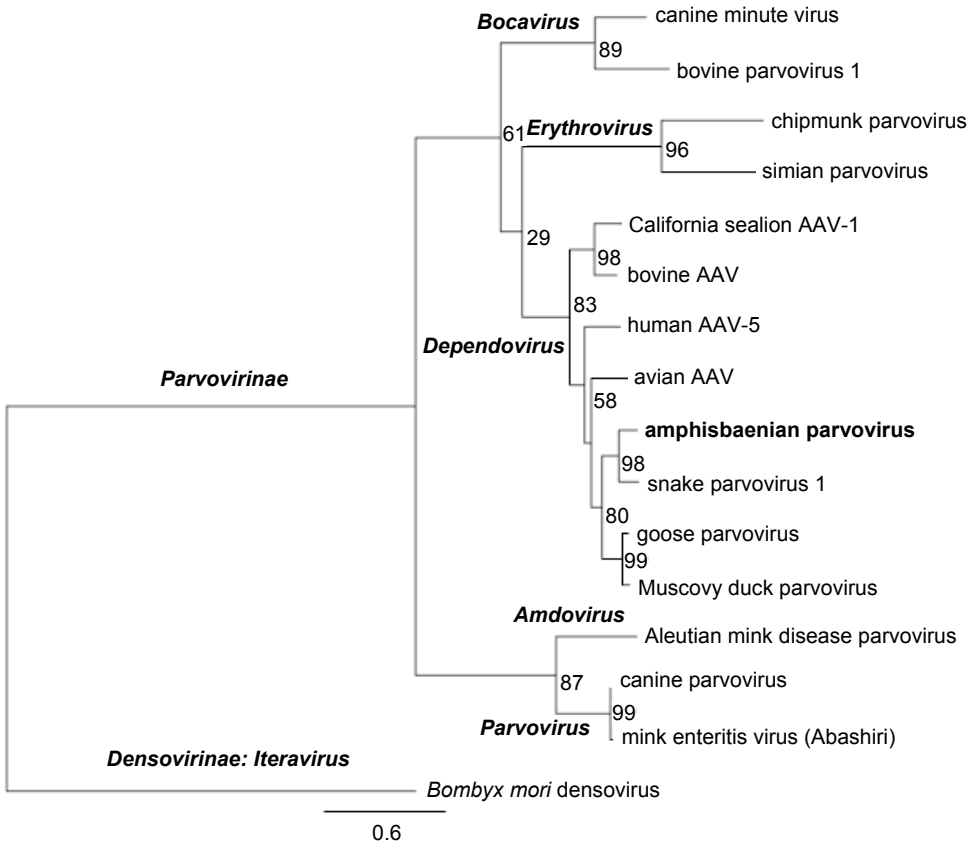

Fig. 1. Phylogenetic tree reconstruction based on maximum likelihood analysis (WAG model with 100 bootstrap runs) of a 179-amino acid-long fragment from the major capsid protein VP of parvoviruses. All accepted genera of subfamily *Parvovirinae* are represented. A member of the *Iteravirus* genus from the subfamily *Densovirinae* (infecting arthropods) was used for rooting the tree. The novel amphisbaenian parvovirus (highlighted in bold) clustered in the *Dependovirus* genus as the closest relative of snake parvovirus 1. AAV: adeno-associated virus

The phylogenetic analyses indicated the newly recognised amphisbaenian parvovirus to be unequivocally a member of the genus *Dependovirus*. In spite of the name referring to the dependence of most members on helper viruses, this genus also contains a couple of viruses, namely the goose and duck parvoviruses that are capable of replicating autonomously (Le Gall-Reculé and Jestin, 1994; Brown et al., 1995; Zádori et al., 1995). The other avian and mammalian parvoviruses, classified into this genus, are strictly dependent on helpers. Both snake parvoviruses have been found with concomitant adenovirus infection (Farkas et al., 2004; Jacobson, 2007; Farkas and Gál, 2008), and their ability for eventual autonomous replication has not been tested. The present paper contains the first description of a case when the occurrence of a parvovirus in a reptilian host

could be demonstrated without the detection of any helper (large DNA) virus. The negative result obtained with the wide-range PCR method excluded the presence of iridoviruses as well (Hanson et al., 2006). These findings suggest that autonomous replication of certain parvoviruses that are genetically closely related to members of the genus *Dependovirus* may occur in reptiles as well as in other diapsids. Further investigations including experimental work on virus replication in tissue culture are required to confirm this observation.

Although the gene fragment, amplified and analysed in this study, is slightly short for resolving intra-generic evolution of parvoviruses unambiguously or for studying the signs of co-evolution of dependoviruses with their vertebrate hosts, a strong monophyly of parvoviruses originating from the reptilian hosts could be observed (Fig. 1). For sound results, the analysis of longer gene fragments or preferably full genome sequences from additional viruses from more diapsid hosts would be needed.

Similarly, it would be essential to acquire information on the virulence and pathogenicity of the reptilian parvoviruses. The PCR system that we developed for this study seems to be suitable for routine screening of reptilian samples for parvoviruses.

As the evolution and physiology of the Amphisbaenia is still an intriguing issue for herpetology, getting to know their parasites, including viruses, can open a new perspective. The apparent social behaviour and parental care detected in this species could also affect the ecology and evolution of its pathogens as well. We plan to collect more samples from these fossorial reptiles and continue the exploration of viruses that may occur in such hosts.

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