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Adenoviruses in free-ranging Australian bearded dragons (*Pogona* spp.)

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ABSTRACT

Adenoviruses are a relatively common infection of reptiles globally and are most often reported in captive central bearded dragons (*Pogona vitticeps*). We report the first evidence of adenoviruses in bearded dragons in their native habitat in Australia. Oral-cloacal swabs and blood samples were collected from 48 free-ranging bearded dragons from four study populations: western bearded dragons (*P. minor minor*) from Western Australia (n = 4), central bearded dragons (*P. vitticeps*) from central Australia (n = 2) and western New South Wales (NSW) (n = 29), and coastal bearded dragons (*P. barbata*) from south-east Queensland (n = 13). Samples were tested for the presence of adenoviruses using a broadly reactive (pan-adenovirus) PCR and a PCR specific for agamid adenovirus-1. Agamid adenovirus-1 was detected in swabs from eight of the dragons from western NSW and one of the coastal bearded dragons. *Lizard atadenovirus A* was detected in one of the dragons from western NSW. Adenoviruses were not detected in any blood sample. All bearded dragons, except one, were apparently healthy and so finding these adenoviruses in these animals is consistent with bearded dragons being natural hosts for these viruses.

1. Introduction

Adenoviruses are non-enveloped viruses with linear, double-stranded DNA genomes. There are currently five genera in the family *Adenoviridae* (ICTV, 2018): *Atadenovirus*, which contains adenoviruses of ruminants, birds, snakes, lizards, testudines, and a marsupial; *Aviadenovirus*, which has so far only been described in birds; *Ichtadenovirus*, which contains a species of adenovirus described in a sturgeon; *Mastadenovirus*, which has been described in mammals and a lizard; and *Siadenovirus*, which has been found in frogs, birds and testudines.

In squamates (lizards and snakes), the atadenoviruses are the most commonly reported genus of adenovirus (Marschang, 2011, 2013). There is evidence for the co-evolution of adenoviruses with their hosts (Davison et al., 2003) and it seems likely that the atadenoviruses originated in squamates (Harrach, 2000; Farkas et al., 2002; Wellehan et al., 2004; Farkas et al., 2008; Pénzes et al., 2009). Currently, two species of atadenoviruses found in reptiles have been classified by the International Committee on Taxonomy of Viruses (ICTV, 2018): *Snake atadenovirus A*, sometimes referred to as snake adenovirus 1 (Farkas et al., 2002), and *Lizard atadenovirus A*, sometimes referred to as lizard adenovirus 2 (Bak et al., 2018) or helodermatid adenovirus 2 (Ball et al., 2014). The distinction between "snake" and "lizard" adenoviruses is an arbitrary one as lizards are paraphyletic without the inclusion of snakes (Wellehan, 2011). Moreover, *Lizard atadenovirus A* has been detected in at least one snake species before, a death adder (*Acanthophis antarcticus*) (Benge et al., 2019).

Reptiles infected with adenoviruses do not always show overt signs of disease (Kubiak, 2013). When they do, ill-thrift and sometimes gastrointestinal and neurological signs are described (Marschang, 2013). Hepatic necrosis, intranuclear inclusion bodies in the liver and gut, and secondary infections or infestations with coccidia and nematodes have been reported (Jacobson, 2007; Hyndman and Shilton, 2011; Doneley et al., 2014; Schilliger et al., 2016). Atadenovirus infections have been described in testudines (Garcia-Morante et al., 2016) and a number of snake and lizard species (Marschang et al., 2003; Farkas et al., 2008; Garner et al., 2008; Abbas et al., 2011; Schmidt et al., 2013), but the most commonly reported host of atadenoviruses are bearded dragons (Moormann et al., 2009; Hyndman and Shilton, 2011; Crossland et al., 2018), with many of the infections being a virus named agamid adenovirus-1 (Kübber-Heiss et al., 2006; Abbas et al., 2012; Kubiak, 2013; Doneley et al., 2014; Fredholm et al., 2015; Schilliger et al., 2016). This higher reporting frequency in bearded dragons is likely contributed to by their popularity as pets.

According to Cogger et al. (2014), there are seven species of bearded dragon: eastern (or coastal) bearded dragon (*Pogona barbata*), black-soil (or downs) bearded dragon (Rankin's dragon) (*P.*

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henrylawsoni), north-western (or Kimberley) bearded dragon (*P. microlepidota*), dwarf bearded dragons (*P. minor minima*, *P. m. minor* [western bearded dragon], and *P. m. mitchelli*), Nullabor bearded dragon (*P. nullabor*), and central (or inland) bearded dragon (*P. vitticeps*). All seven species are native only to Australia. Internationally, central bearded dragons are commonly kept as pets (Paré, 2006). The western bearded dragon is commonly kept in captivity in Western Australia and the coastal bearded dragon is kept by some reptile keepers in eastern Australia. The other species are seldom kept in captivity.

Adenoviruses are known to be present in captive Australian bearded dragons (Hyndman and Shilton, 2011; Doneley et al., 2014) and diagnostic testing for this virus suggests that the virus is relatively common in Australian captive central bearded dragons (Hyndman and Shilton, 2018). Testing free-ranging Australian bearded dragons for adenoviruses therefore provides an opportunity to gain further insight into the relationship between these viruses and these iconic species of reptile. In this study, we found adenoviruses in two Australian populations of free-ranging bearded dragons.

2. Materials and methods

Free-ranging bearded dragons were sampled opportunistically. Animals from four regions of Australia were sampled (Fig. 1) between the years 2014 and 2017: western bearded dragons (*Pogona minor minor*) from Perth in Western Australia (n = 4); central bearded dragons (*P. vitticeps*) from Alice Springs in central Australia (n = 2) and western New South Wales (NSW) (n = 29); and coastal bearded dragons (*P. barbata*) from south-east Queensland (n = 13). The animals from western NSW were sampled as part of a dedicated field study for this species. All other animals were presented to the authors (as registered veterinarians) by members of the public who were concerned for the health of the animal.

The use of animals from Queensland, Western Australia and central Australia that were used for this study was approved by the Animal Ethics Committee (AEC) of the University of Queensland (approval number 313/14). The use of animals from New South Wales was approved by the AEC of the New South Wales Department of Primary Industry (approval TRIM 17/947). Both of these animal ethics committees adhere to the Code of Practice for the Care and Use of Animals for Scientific Purposes (Australian Government, 2013).

The sex of each animal was determined by an assessment of hemipenal bulges (if present), the presence of everted hemipenes, and the size of the prefemoral pores. Each animal was classified as either juvenile or adult based on a subjective assessment of the snout to vent length relative to a healthy adult conspecific.

For sampling, a combined oral-cloacal swab was collected from each animal. Swab tips were broken off into tubes containing ~1–5 mL of sterile saline. Whole blood was collected from the ventral tail vein and anticoagulated with lithium heparin. The swab tips (in saline) and whole blood were frozen down immediately or were frozen down after being held at 4 °C for up to six hours after collection. All samples were submitted chilled (but not frozen), without further processing, to Murdoch University for testing.

DNA was extracted from swabs and whole blood using commercial kits. For swab samples, the Purelink® Viral RNA/DNA Mini Kit (cat. no. 12280050, Thermo Fisher) was used. The sample tubes containing the swab tips were vigorously vortexed (~30 s). A 200 µL aliquot was then added to carrier RNA, a lysis buffer and proteinase K as per the manufacturer's instructions. For whole blood, the QIAamp® *cador*® Pathogen Mini Kit (cat. no. 54104, Qiagen) was used. Two microlitres of whole blood was diluted to 200 µL using phosphate-buffered saline, and this was then added to proteinase K and lysis buffer as per the manufacturer's instructions. For the rest of the DNA extraction, the manufacturer's instructions were followed. DNA was eluted into 30 µL.

Extracted DNA was tested for the presence of adenoviruses using two PCRs: one that was designed to detect all known adenoviruses (Wellehan et al., 2004), and a second one that was designed to specifically detect agamid adenovirus-1 (Fredholm et al., 2015). For the nested PCR described by Wellehan et al. (2004), 10 µL of 2x master mix from the Platinum™ Green Hot Start PCR Master Mix (2X) (cat. no. 13001013, ThermoFisher) was added to 1 µM (final concentration) of primers AdenopolFouter and AdenopolRouter, and 1 µL of extracted DNA, with the final volume brought up to 20 µL using PCR-grade water. Cycling conditions were 94 °C x 2 min, 40 x (94 °C x 20 s, 50 °C x 45 s, 72 °C x 50 s). One microlitre of PCR product was then added to 1 µM (final concentration) of primers AdenopolFinner and AdenopolRinner and PCR master mix and water as above. The products of this second round of PCR amplification were then separated by agarose gel electrophoresis using standard techniques. Amplicons close to the predicted size (321 nucleotides) were excised from the gel, purified, added to forward and reverse primers, and then submitted for Sanger sequencing using standard techniques. After the primer sequences were removed, sequenced amplicons were used as search terms in BLASTN to look for homologous sequences in GenBank. Unique sequences were uploaded to GenBank under the accession numbers MK597918-MK597923 (Table 1).

For PCR testing for agamid adenovirus-1, the methods described by Fredholm et al. (2015) were followed.

3. Results

All dragons sampled in this study, except one, were apparently healthy. Only bearded dragon 28 (BD028) from the western New South Wales (NSW) population was obviously unwell; it had lethargy and bilateral exophthalmos, but was still in good body condition and no abnormalities could be detected following coelomic palpation, oral examination, or auscultation of the heart. No further diagnostic testing was performed. Several of the bearded dragons sampled from south-east Queensland had traumatic injuries (dog bites, vehicular trauma) but none were systemically unwell.

In total, 48 oral-cloacal swabs and 47 blood samples were collected from 48 free-ranging Australian bearded dragons. All samples were tested with the pan-adenovirus PCR and the agamid adenovirus-1 PCR (Table 1). Adenoviruses were not detected from the bearded dragons sampled from Western Australia or Alice Springs. In contrast, adenoviruses were detected in nine of the 29 central bearded dragons sampled from western NSW. Of these nine dragons, eight were positive using the PCR for agamid adenovirus-1. Of these eight animals, five were also positive for agamid adenovirus-1 using the pan-adenovirus PCR. The ninth PCR-positive animal from western NSW was infected with *Lizard atadenovirus A*. One of the 13 animals sampled from south-east Queensland was PCR-positive for agamid adenovirus-1 with both PCRs.

All blood samples were PCR-negative for adenovirus using both PCRs. DNA from a species of *Bacteroides* was identified in two swab samples: one from an animal that was PCR-negative for adenovirus, and the other from an animal that was positive using the PCR specific for agamid adenovirus-1.

The partial agamid adenovirus-1 polymerase gene sequences from animals BD003, BD004, BD018, BD025 and BD026 (all from the western NSW population) showed nucleotide identities of greater than 98.8% to each other and to eight sequences on GenBank all listed as agamid adenovirus-1 (e.g. GenBank accession number AY576678.1). These sequences were identical to each other when the predicted amino acid sequences were compared. By comparison, the sequence obtained from BD13 from the south-east Queensland population showed identities of 87.2% and 94.3% to the cognate region of the nucleotide and predicted amino acid sequences, respectively, of the same agamid adenovirus-1 (AY576678.1 [nucleotide], AAS89694.1 [amino acid]). The partial nucleotide sequence of the adenovirus from animal BD016 was 98% identical to the equivalent region of the genome of an adenovirus belonging to the *Lizard atadenovirus A* species (KY552649.1).

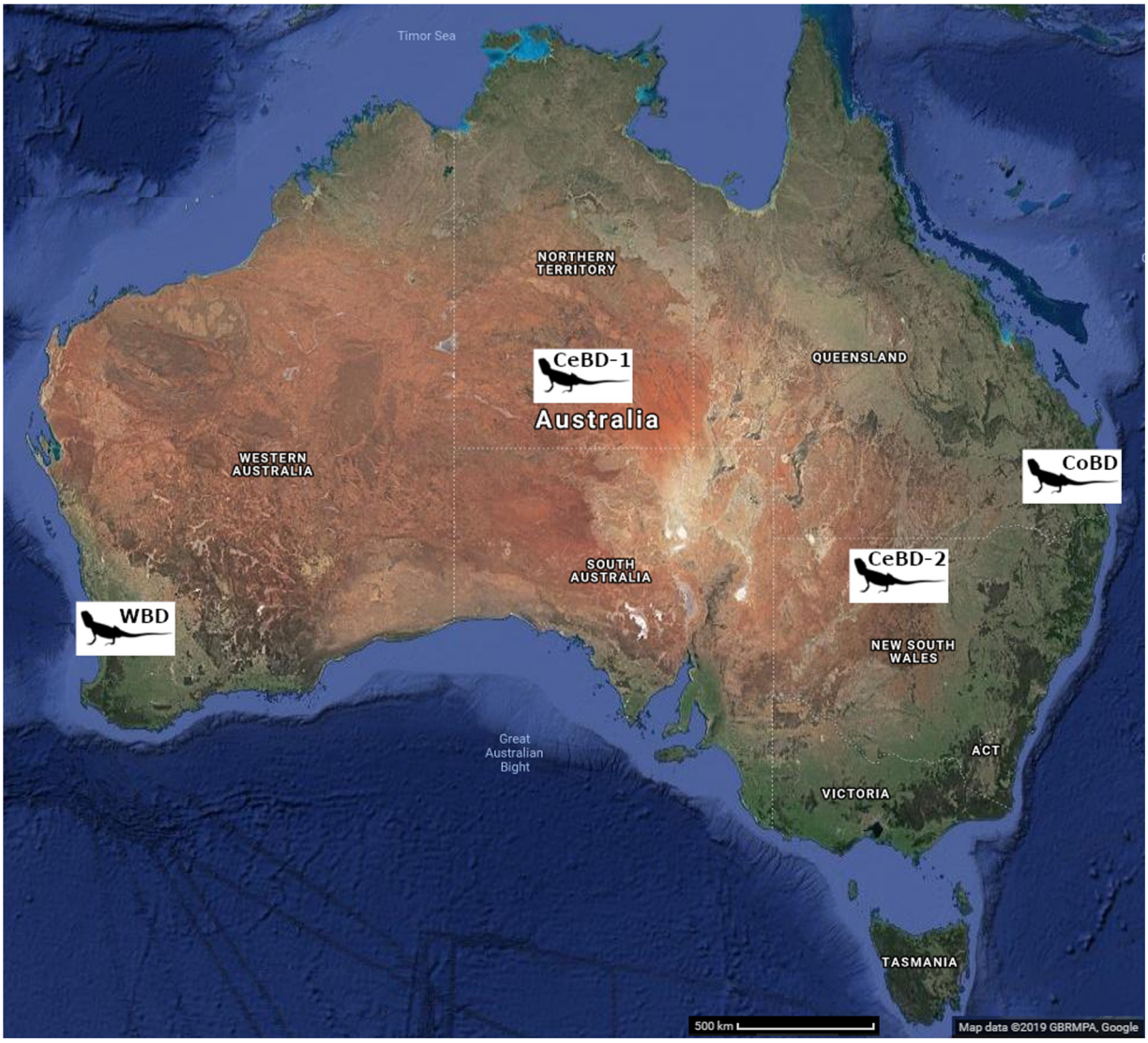

Fig. 1. Free-ranging bearded dragons from four locations in Australia were sampled for the presence of adenoviruses. Four western bearded dragons (*Pogona minor minor*) (WBD) were sampled from Perth. Two central bearded dragons (*P. vitticeps*) were sampled from Alice Springs in central Australia (CeBD-1). Twenty-nine central bearded dragons were sampled from near to Bourke in western New South Wales (CeBD-2). Thirteen coastal bearded dragons (*P. barbata*) were sampled from south-east Queensland, near to Gatton (CoBD).

4. Discussion

This is the first report of the detection of adenoviruses in free-ranging bearded dragons in their native Australian habitats. Given the reported co-evolution of atadenoviruses with squamates, this result is unsurprising. Other authors have also described the detection of adenoviruses by PCR in free-ranging reptiles. Adenoviruses have twice been detected in free-ranging eastern box turtles (*Terrapene carolina carolina*) from Illinois and Tennessee (Archer et al., 2017; Adamovicz et al., 2018). They have also been detected in lizards from Spain and islands north of Africa (Szirovicza et al., 2016) and Gila monsters (*Heloderma suspectum*) from USA (Benge et al., 2019). However, not all studies have detected adenoviruses in free-ranging reptiles (Schmidt et al., 2014; Aplasca et al., 2019). It is important to note that the studies where adenoviruses were detected nearly always involved more animals being tested than the studies where these viruses were not detected.

In our study, we were able to identify an infected free-ranging bearded dragon (*P. barbata*) from a peri-urban population in south-east Queensland. It is possible that this animal was an escaped captive individual that had become infected before its escape, or that it had acquired infection from an escaped captive individual, but this is unlikely because in this area, this species is not sold by pet stores and is rarely kept as pets. Additionally, we were able to identify another eight infected animals in a second study population near to Bourke in western New South Wales. The animals from this second study population were collected from an area of approximately 45,000 square kilometres, most of which was within the Shire of Bourke. This Shire had a population of 2834 people in 2016 (ABS, 2016) and is remote from urban human populations. Our results are therefore consistent with these adenoviruses being endemic in wild populations of Australian bearded dragons.

The role that these viruses played in the health of the dragons in our study is unknown. Only one of the dragons was obviously unwell but given the assessment of animals was limited to physical examination, and that animals were presented for assessment by concerned members of the public, it is possible that more than one animal was unwell. It is also possible that adenoviral infection contributed to misadventure in the animals presented with traumatic injuries. However, given that most of the animals we detected adenovirus in were collected from a

Table 1
PCR Results for the detection of adenoviruses in free-ranging Australian bearded dragons.

Animal number[a]	Sex and age	PCR result			
		oral-cloacal swab		blood	
		Pan-adenovirus (GenBank accession number)	Agamid adenovirus-1	Pan-adenovirus	Agamid adenovirus-1
Western bearded dragons from Perth, Western Australia					
Dragons 1-4	1 x AF, 1 x JM, 1 x JF, 1 x n.r.	negative	negative	negative	negative
Central bearded dragons from Alice Springs					
Dragons 1-2	n.r.	negative	negative	negative	negative
Central bearded dragons from western New South Wales					
BD001-BD002	2 x AF[b]	negative	negative	negative	negative
BD003	AF	**AgAdV-1** (MK597918)	**positive**	negative	negative
BD004	AF	**AgAdV-1** (MK597919)	**positive**	negative	negative
BD005-BD007	3 x AM	negative	negative	negative	negative
BD008	AM	negative[c]	negative	negative	negative
BD009-BD010	1 x AM, 1 x AF	negative	negative	negative	negative
BD011	AM	negative	**positive**	negative	negative
BD012-BD015	1 x AF[b], 3 x AM	negative	negative	negative	negative
BD016	AM	***Liz. atadeno. A*** (MK597922)	negative	negative	negative
BD017	AF	negative	negative	n.s.	n.s.
BD018	AM	**AgAdV-1** (MK597920)	**positive**	negative	negative
BD019	AF	negative	negative	negative	negative
BD020	AM	negative	**positive**	negative	negative
BD021	AM	negative[c]	**positive**	negative	negative
BD022-BD024	1 x AF[b], 1 x AF, 1 x AM	negative	negative	negative	negative
BD025	AM	**AgAdV-1** (MK597921)	**positive**	negative	negative
BD026	AF[b]	**AgAdV-1**[d]	**positive**	negative	negative
BD027-BD028	2 x AM	negative	negative	negative	negative
Coastal bearded dragons from south-east Queensland					
Dragons 1-12	7 x AM, 5 x AF	negative	negative	negative	negative
Dragon 13	1 x AF	**AgAdV-1** (MK597923)	**positive**	negative	negative

JM = juvenile male; AM = adult male; JF = juvenile female; AF = adult female. n.r. = not recorded. n.s. = not sampled. *Liz. atadeno. A* = *Lizard atadenovirus A*.
[a] All animals were tested individually.
[b] Gravid.
[c] *Bacteroides* spp. DNA was detected.
[d] Incomplete sequence that is identical to the 179 nucleotides in the cognate region of BD003, BD004 and BD025.

free-ranging population, and were apparently healthy, the most congruous explanation for our results is that the animals were asymptomatically infected.

The detection of agamid adenovirus-1 and *Lizard atadenovirus A* in these animals contributes information to the possible provenance of these viruses. It is tempting to assume that these two viruses originated in Australian bearded dragons, especially considering agamid adenovirus-1 is commonly detected in captive bearded dragons worldwide. Raising doubt to this is that *Lizard atadenovirus A*, sometimes referred to as helodermatid adenovirus 2, after the helodermatid hosts it has been detected in, Mexican beaded lizards (*Heloderma horridus*) and Gila monsters (*Heloderma suspectum*) (Wellehan et al., 2004; Papp et al., 2009; Benge et al., 2019), has been detected in free-ranging Gila monsters (Benge et al., 2019). This raises the intriguing possibility that these viruses may have co-evolved from an ancestor to the Toxicofera (a diverse group of squamates that both bearded dragons and helodermatids belong to).

We decided to screen each dragon using two different PCRs that were capable to detecting adenoviruses in bearded dragons. The PCR described by Fredholm et al. (2015) was designed to specifically detect agamid adenovirus-1, which is the most commonly reported adenovirus infection of bearded dragons (Kübber-Heiss et al., 2006; Abbas et al., 2012; Kubiak, 2013; Doneley et al., 2014; Fredholm et al., 2015; Schilliger et al., 2016). In the study by Fredholm et al. (2015), this PCR was shown to be able to detect this virus in more samples than the pan-adenovirus PCR described by Wellehan et al. (2004). This result is consistent with our results, where of the nine animals we identified to be infected with agamid adenovirus-1, three were PCR-positive using the PCR by Fredholm et al. (2015) but were PCR-negative using the PCR by Wellehan et al. (2004). No animal was PCR-positive for agamid adenovirus-1 using the PCR by Wellehan et al. (2004) but PCR-negative using the PCR by Fredholm et al. (2015). Despite using two PCRs for the detection of adenoviruses, it is still possible that there were animals in our study that were infected with adenoviruses that escaped detection. For example, if the amount of adenoviral DNA present in the blood of an infected animal, or being shed from that animal, was beneath the detection limit of our tests, it would be PCR-negative.

The utility of this approach to use two PCRs was also evident from the detection of *Lizard atadenovirus A* in one of our study animals. Given the PCR described by Fredholm et al. (2015) was designed to specifically detect agamid adenovirus-1, it was unsurprising that this PCR did not detect *Lizard atadenovirus A*. We therefore recommend that future studies surveying populations for microbial agents by PCR consider this dual approach where a broadly-reactive PCR, capable of detecting less common or novel genotypes, is used in parallel with a second PCR that can detect the most common genotype(s) with superior sensitivity.

We also tested two different sample types: oral-cloacal swabs and whole blood. We did not detect adenoviruses in any whole blood samples. In contrast to our results, adenovirus infections have been detected in the blood of other bearded dragons (Doneley et al., 2014). In the study by Doneley et al. (2014), both cloacal swabs and blood were tested for adenoviruses in a subset of their study population. From this population, adenoviruses were detected in 18 animals. Only the cloacal swabs were PCR-positive in 12 of these animals, both the cloacal swabs and blood were PCR-positive in five of these animals, and only the blood was PCR-positive in one animal. Although the higher proportion of positives in swab samples is consistent with our results, we did not detect adenoviruses in any blood sample. In the study by Doneley et al. (2014), juvenile bearded dragons with acute neurological disease were tested and so it is possible that testing the blood of acutely-

infected animals identifies more animals that are viraemic. Transient adenovirus viremia has been described in human children receiving bone marrow transplants (Walls et al., 2005). It is also possible that different methods of blood storage or testing also influenced the difference in the results seen between our study and the study by Doneley et al. (2014). However, in a separate study that provided results consistent with what we observed, adenoviruses were detected in cloacal (+/- oral) swabs, but not blood, from two adult central bearded dragons from Germany (Papp et al., 2009). Based on these findings, we therefore recommend that if resources are limited, swabs are prioritised over blood, especially for apparently healthy animals.

5. Conclusion

Our study has provided the first report of adenoviruses in free-ranging bearded dragons in their native Australian habitat. This provides a valuable insight into the evolutionary history of reptile adenoviruses. We also demonstrated that oral-cloacal swab samples are more useful than whole blood for detecting these infections and that using a broadly-reactive PCR in concert with a specific PCR for the detection of the most common genotype is a useful approach for molecular surveys of reptiles.

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Declarations of interest

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