



Communication

High Prevalence of Novel Beak and Feather Disease Virus in Sympatric Invasive Parakeets Introduced to Spain From Asia and South America

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Abstract: The psittacine beak and feather disease (Pbfd) is a globally widespread infectious bird disease that mainly affects species within the Order Psittaciformes (parrots and allies). The disease is caused by an avian circovirus (the beak and feather disease virus, BFDV), which is highly infectious and can lead to severe consequences in wild and captive populations during an outbreak. Both legal and illegal trading have spread the BFDV around the world, although little is known about its prevalence in invasive parrot populations. Here, we analyze the BFDV prevalence in sympatric invasive populations of rose-ringed (*Psittacula krameri*) and monk parakeets (*Myiopsitta monachus*) in Southern Spain. We PCR-screened 110 blood samples (55 individuals from each species) for BFDV and characterized the genotypes of five positives from each species. About 33% of rose-ringed parakeets and 37% of monk parakeets sampled were positive for BFDV, while neither species showed disease symptoms. The circovirus identified is a novel BFDV genotype common to both species, similar to the BFDV genotypes detected in several parrot species kept in captivity in Saudi Arabia, South Africa and China. Our data evidences the importance of an accurate evaluation of avian diseases in wild populations, since invasive parrots may be bringing BFDV without showing any visually detectable clinical sign. Further research on the BFDV prevalence and transmission (individual–individual, captive–wild and wild–captive) in different bird orders and countries is crucial to understand the dynamics of the viral infection and minimize its impact in captive and wild populations.

Keywords: circovirus; Pbfd; BFDV; rose-ringed parakeet; monk parakeet; invasive species

1. Introduction

Psittacine Beak and Feather Disease (Pbfd) is one of the most relevant infectious diseases affecting wild and captive parrot species [1,2]. This disease is caused by the Beak and Feather Disease Virus (BFDV), which is a highly infectious and mutable single-stranded DNA (ssDNA) circovirus [3]. The main Pbfd symptoms include feather lesions (loss of feathers, improper moulting, malformations and colour changes) and abnormal growth of the beak, although infected individuals can be asymptomatic [4]. Although information is scarce, the prevalence of BFDV is high in native parrots of Oceania, Africa and Asia [5], including wild populations of some threatened species [6–9]. To our knowledge, no surveillance of BFDV has been conducted on free-living parrots in South America, the other main stronghold for parrots. A screening conducted on captive individuals seized from illegal trade in Brazil showed evidence of BFDV at low prevalence in two native species [10]. The genomic

similarity of the isolates with reference strains from Asia and Oceania suggested an exotic origin of BFDV strains disseminated in captivity in South America [10,11].

BFDV has been detected in most captive parrot species around the world, showing a high genetic variability, with thousands of genotypes and novel ones described each year [5,12,13]. The international legal and illegal trade on captive birds [14,15] has induced a fast spread of BFDV worldwide, a threat that is enhanced in areas where parrots have established invasive populations [16]. Invasive parrot populations can be natural viral hosts, although their role in the genetic diversification of the virus and the spread into native populations of parrots and other avian orders has not yet been explored. However, it has been reported that invasive rose-ringed parakeets (*Psittacula krameri*) could have been involved in the outbreak of BFDV in the threatened population of Echo parakeets (*Psittacula eques*) on the island of Mauritius, with devastating effects due to its high mortality rate [2,9]. Moreover, the presence of BFDV has been recently reported in various non-psittacine species, which may both suffer the disease or act as reservoirs, with different prevalences depending on the order [17]. All these aspects increase the epidemiological complexity of the disease and its diagnosis [18], making urgent a comprehensive understanding of its drivers to prevent epidemic outbreaks impacting common but also threatened species.

The rose-ringed parakeet (*P. krameri*) and the monk parakeet (*Myiopsitta monachus*) were the first and fourth most traded parrots worldwide [14], and have established exotic populations in various countries around the world mostly as a consequence of accidental escapes of individuals kept as pets [19]. Recent studies have shown that the exotic populations of these two species mainly originated from individuals traded from India and an area between Argentina and Uruguay, respectively [20–22]. Governmental and environmental organizations have applied different measures to control these populations [23–25], highlighting that these invasive birds can be natural reservoirs of infectious bacteria, fungi and viruses of zoonotic concern. However, scientific evidence is scarce [26,27], and only available for rose-ringed parakeets introduced in some European countries [28,29] and on the island of Mauritius [9]. Given the BFDV mutagenic potential and unpredictable viral effects, its study in invasive populations is important to prevent dangerous outbreaks in novel hosts among wild species [9].

Here, we evaluate the prevalence of BFDV in sympatric populations of the invasive rose-ringed parakeet and monk parakeet in Spain and then proceed to its genetic characterization. We reviewed the information on BFDV *rep* gene isolated from captive rose-ringed parakeets worldwide and assessed whether specific BFDV variants can infect both parrot species despite their different geographic origin. Results are discussed in the context of the global spread of BFDV through wildlife trade and its potential impact on native species of the recipient communities.

2. Materials and Methods

From 2015 to 2018, parakeets of both species were captured with mist nets in Sevilla (Southern Spain), where they coexist in urban parks [30]. Individuals were banded, examined for lesions in the beak and plumage alterations, measured for several traits, banded and released. A sample of blood (ca. 0.05 mL) was collected from the brachial vein and stored in absolute ethanol for molecular analysis. A random sample of the parakeets (55 individuals from each species) was screened for the presence of BFDV ($n = 17, 16, 12, 10$ in 2015–2018 for *P. krameri*; $n = 25, 13, 17$ in 2016–2018 for *M. monachus*). The capture and extraction of blood samples from parakeets were approved by the Ethical Committee of EBD-CSIC (our national research institution), codes: 11_27-Tella and 12_48-Tella.

The DNA was isolated from blood using the Quick-DNA Miniprep Kit (Zymo Research) using an optimized protocol (i.e., samples were digested at 56 °C for 12 h in a solution containing 200 µL of genomic lysis buffer and 20 µL of 20-mg/mL proteinase K). The screening of BFDV was performed on all samples using two primer sets (5'-AACCCTACAGACGGCGAG-3' and 5'-GTCACAGTCCTCCTTGACC-3', [31]; 5'-TTAACAACCCTACAGACGGCGA-3' and 5'-GGCGGAGCATCTCGCAATAAG-3', [32]) that amplify a partial sequence of the replication-associated protein (*rep*) gene. PCR was performed in a reaction mixture of 20 µL containing 10 µL of 2x MyTaq HS Mix (Bioline), 250 nM of each primer

(final concentration) and ~20 ng of template DNA. The amplification protocol was composed of the following steps: 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s, 58 °C for 1 min, 72 °C for 30 s, and a final extension at 60 °C for 10 min. Negative controls and non-template controls were used in all PCR reactions to exclude contamination issues. Population-prevalence estimates and 95% CI were calculated with EpiTools [33] following Fogell et al. [16].

Samples were considered BFDV positive if at least one primer pair PCR had an accurate amplification of the expected size fragment. Five random positive samples of each species were bi-directionally sequenced by Sanger sequencing to assess genotype diversity. Sequences were visualized and edited using the software Geneious v.11.1.5. Then, our sequences were compared with data available in GenBank through a BLAST search [34]. The program BEAST v.2.6.0 [35] was used to construct a Maximum Clade Credibility (MCC) tree using the 100 sequences most related with the genotype characterized in this study. These sequences were obtained through a BLAST search and covered a total of 77 BFDV genotypes (see Supplementary database 1). The model of sequence evolution HKY + I + G4 was selected after careful estimation of this evolutionary parameter with jModelTest v.2.1.7 [36]. The proportion of invariable sites was set to 0.491 and the alpha shape parameter (α) was set to 0.643, using a normal distribution for the rate prior and letting the program to estimate the mutation rates. The strict clock and Yule model priors were used. The software run consisted of 200 million steps, with a sampling of the chains every 20,000 steps and a burn-in of 10%. The adequate convergence and mixing of the chains and sufficient effective sample sizes (ESS) were checked with Tracer v.1.7.1 [37]. The consensus tree was visualized and edited using the software FigTree v.1.4.4 [38]. The same procedures (changing only the model of evolution to HKY + G4 using $\alpha = 0.115$) were used to construct an MCC tree using only *P. krameri* *rep* gene sequences with a 100% coverage of the query sequence in the BLAST search.

3. Results

A similar prevalence of BFDV was found in both species (rose-ringed parakeet: 18 of 55, 32.7%, 95% CI: 21.8–45.9; monk parakeet: 20 of 55, 36.4%, 95% CI: 24.9–49.6; Fisher's exact test, $p = 0.84$). Individuals positive to BFDV were found in 2016 (50.0%, $n = 16$) and 2017 (83.3%, $n = 12$) but not in 2015 (0.0%, $n = 17$) and 2018 (0.0%, $n = 10$) for *P. krameri*. BFDV-positive samples were found in all sampling years for *M. monachus* (2016, 24.0%, $n = 25$; 2017, 38.5%, $n = 13$; 2018, 52.9%, $n = 17$). No individual showed visible signs of the disease.

The partial sequence of the *rep* gene isolated and characterized for rose-ringed parakeets and monk parakeets revealed a novel and unique BFDV genotype common to both species (GenBank accessions: MT303063 and MT303064). Our analysis indicates that the novel genotype differed by nine to 13 nucleotide substitutions with the most similar genotypes among all BFDV sequences described to date for different psittacine species (Figure 1). These closest variants were found in captive individuals of several parrot species (*P. krameri*, *Psittacus erithacus*, *Nymphicus hollandicus*, *Agapornis fischeri* and *Poicephalus gulielmi*) in Saudi Arabia, China and South Africa (Figure 1). Phylogenetic data suggest that genotypes isolated in Saudi Arabia and South Africa diverged from the novel circovirus genotype found in Spain (Figure 1). The phylogeny of the *rep* sequences isolated from *P. krameri* in various countries supports the proximity of the BFDV genotypes isolated from wild individuals in an invasive population in Spain with captive individuals in Saudi Arabia, which share an ancestor with the two genotypes also detected in captive individuals in Poland (Figure 2).

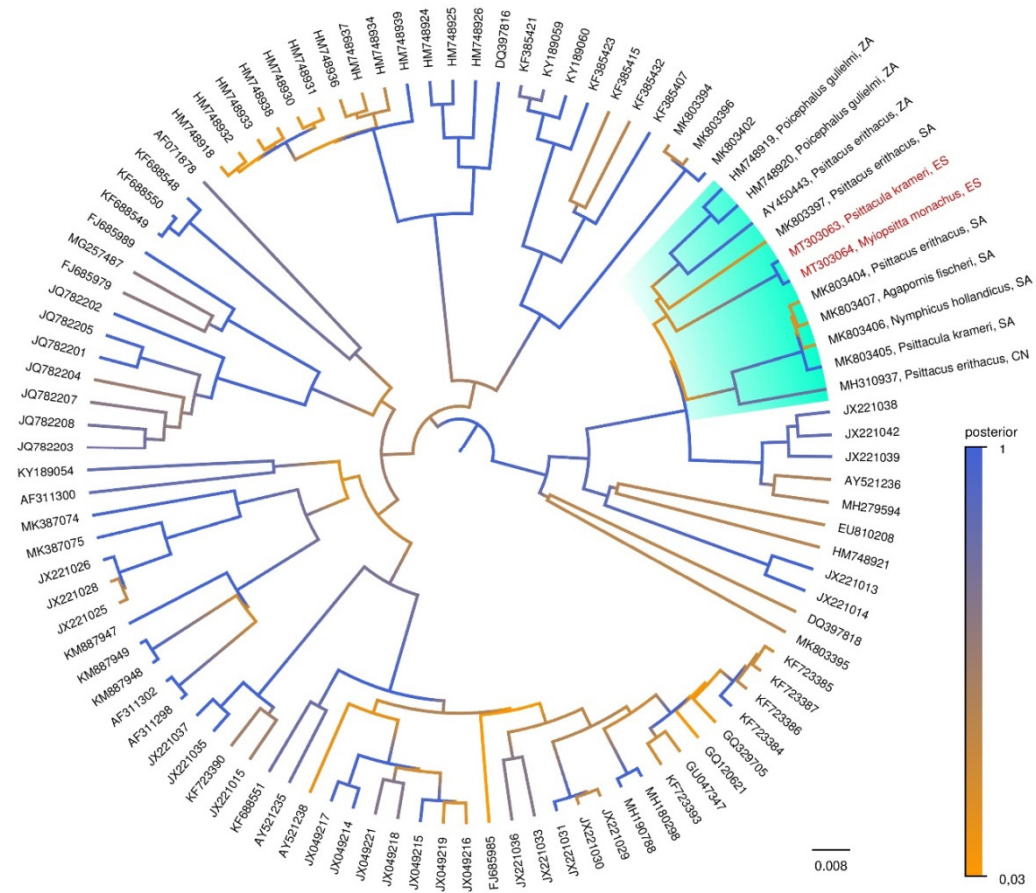


Figure 1. Maximum Clade Credibility tree for the beak and feather disease virus (BFDV) *rep* gene partial nucleotide sequences (603 bases). Posterior probabilities for the nodes are represented with a colour gradient scale. All partial *rep* sequences are identified by GenBank accession number. The clade that includes the novel circovirus genotype is highlighted and also shows species and country codes (ISO 3166-1 alpha-2). The detailed list of host species, country of virus isolation and genotypes are presented as supplementary database 1.

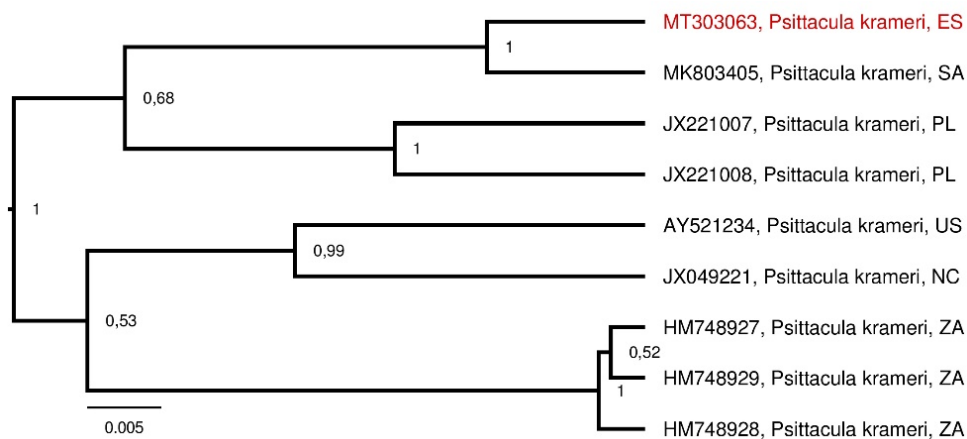


Figure 2. Maximum clade credibility tree inferred using partial nucleotide sequences of the BFDV *rep* gene isolated from rose-ringed parakeets (*Psittacula krameri*). Posterior probabilities are indicated for each node. All partial *rep* sequences are defined by GenBank accession number, species and country codes (ISO 3166-1 alpha-2). The novel circovirus genotype of Spain (ES) is highlighted.

The partial BFDV sequences isolated from rose-ringed parakeets in Spain and Saudi Arabia differ in 9 (1.5%) nucleotides and 1 (0.5%) amino acid (Table 1). However, the pattern of nucleotide and amino acid variations between isolates from different countries and continents is extreme, ranging from nine (1.5%) to 49 (8.1%) nucleotides and from zero to 16 (8.0%) amino acids (Table 1).

Table 1. Number of nucleotide (below diagonal) and amino acid (above diagonal) variations between partial sequences of the BFDV *rep* gene isolated from rose-ringed parakeets (*Psittacula krameri*). The percentage of variation considering the sequence length (603 nucleotides and 201 amino acids) is noted in parentheses.

	MT303063 (ES)	MK803405 (SA)	JX221007 (PL)	JX221008 (PL)	AY521234 (US)	JX049221 (NC)	HM748927 (ZA)	HM748929 (ZA)	HM748928 (ZA)
MT303063 (ES)	-	1 (0.5)	11 (5.4)	4 (1.9)	11 (5.4)	7 (3.5)	7 (3.5)	7 (3.5)	8 (4.0)
MK803405 (SA)	9 (1.5)	-	10 (5.0)	3 (1.5)	10 (5.0)	6 (3.0)	8 (4.0)	8 (4.0)	9 (4.5)
JX221007 (PL)	39 (6.5)	33 (5.5)	-	9 (4.5)	16 (8.0)	12 (6.0)	14 (7.0)	14 (7.0)	15 (7.5)
JX221008 (PL)	31 (5.1)	25 (4.1)	20 (3.3)	-	9 (4.5)	5 (2.5)	7 (3.5)	7 (3.5)	8 (4.0)
AY521234 (US)	33 (5.5)	27 (4.4)	46 (7.6)	36 (6.0)	-	7 (3.5)	9 (4.5)	9 (4.5)	10 (5.0)
JX049221 (NC)	28 (4.6)	22 (3.6)	44 (7.3)	32 (5.3)	22 (3.6)	-	6 (3.0)	6 (3.0)	7 (3.5)
HM748927 (ZA)	31 (5.1)	31 (5.1)	47 (7.8)	40 (6.6)	34 (5.6)	33 (5.5)	-	0 (0.0)	1 (0.5)
HM748929 (ZA)	32 (5.3)	31 (5.1)	47 (7.8)	40 (6.6)	33 (5.5)	34 (5.6)	1 (0.2)	-	1 (0.5)
HM748928 (ZA)	33 (5.5)	33 (5.5)	49 (8.1)	42 (7.0)	36 (6.0)	33 (5.5)	2 (0.3)	3 (0.5)	-

4. Discussion

Our study revealed a high prevalence of BFDV in the two sympatric invasive populations of rose-ringed parakeets and monk parakeets sampled in Southern Spain. No individual showed visible signs of the disease, which suggests that most of them were asymptomatic carriers or that ill (i.e., symptomatic) individuals die soon because of the disease [4,8,9] or are rapidly eliminated from the wild by natural selection [39]. The high prevalence of BFDV in the rose-ringed parakeet contrasts with the very low values in blood reported for other invasive populations of this species in Europe, Asia and Africa (from 0.0% in Germany [29] to 16.1% in Mauritius [16]). These values were lower than those reported in native populations in Asia (100% in Bangladesh and 71.4% in Pakistan [16]). It is worth mentioning that prevalence detected in our study area are similar to those found in native species from Australia and New Zealand, where the BFDV is endemic [40–42]. Differences in prevalence can be real among populations, although they can also arise due to the tissue analysed and to the selection of primers [16]. In our case, following previous studies showing high variability in the amplification specificity and sensitivity between different primers sets [43], we used two different sets of primers to search for BFDV. Positive samples for the virus only amplified with the primers reported by Ritchie et al. [32], likely due to variations in viral copy numbers or mutations in the primer binding sites in some BFDV genotypes. However, several studies only focused on one primer set for BFDV screening [10,12,17], and thus may have underestimated the actual prevalence in these populations. Further research using different molecular markers is thus needed to increase the robustness of the BFDV diagnosis test.

The evidence of BFDV infections in monk parakeets is restricted to a presumed case reporting beak lesions in a Greek invasive population, although no genetic, microbiological or other type of validation was provided [44]. No further information is available in its native or invasive range, so this is the first estimate of prevalence of BFDV in this species in the wild.

Genotype variations are frequent in circovirus and there are thousands of BFDV genotypes described and available through genome browsers [5,12,13]. This variability may explain species-specific susceptibility and infection impact, which deserves further research on the potential infectiousness to native species in the invasive range of these parakeet species. The novel genotype characterized in this study and its closest viral variants can colonize psittacids from all continents. This has important implications for the spread of this and other viral variants on native parrots interacting with invasive ones [9] or with individuals escaped from captivity, especially in the Neotropics where many parrot species are of conservation concern [45].

Phylogenetic data suggest that genotypes isolated in Saudi Arabia and South Africa diverged from the novel circovirus genotype found in Spain, which share an ancestor with the two genotypes also detected in captive individuals in Poland. These countries imported and exported thousands of captive-bred and wild parrots (and other birds) from and towards Spain and other European countries in recent decades according to CITES Trade Database [46]. This result highlights the potential role played by the international bird trade in the spread of wildlife infectious diseases and the emergence of zoonosis [8,47,48]. Specifically, the capacity of the same circovirus variant to colonize different parrot species, one a native of Asia and the other from South America, in one area of their European invasive range emphasizes the complex and concerning outcomes of trade-driven biological invasions on the global circulations of pathogens.

Invasive parrot populations with high prevalence of BFDV are not only of concern in areas of coexistence with other parrot species. There is evidence that BFDV can be transmitted from native parrots to several avian species of different orders [17], with some individuals showing the typical beak and plumage alterations of this disease [43,49]. A previous study showed BFDV symptoms and the presence of the causative virus in captive Gouldian finches (*Chloebia gouldiae*), which were suggested to be infected by invasive monk parakeets in Italy [50]. However, no test of the occurrence of the virus in the parakeets was conducted. Thus, the high prevalence of BFDV in the invasive parakeet populations sampled in southern Spain should be carefully considered, as it may have important consequences for the conservation of native birds, particularly those sharing habitats or nests with these invasive species and those preying on them both in urban and rural habitats [30,51,52]. These concerns increase due to the fast spread rates of these two parakeet species in Spain [19,53].

5. Conclusions

We show a high prevalence of a novel BFDV in free-ranging, sympatric invasive populations of two parakeets native to different continents in Southern Spain. Although previous studies have demonstrated the presence of BFDV in rose-ringed parakeets, this is the first genetic evidence of BFDV in monk parakeets. Both legal and illegal trade can contribute considerably to the dissemination of the virus in non-endemic regions, which can have important impacts not yet considered on native birds [54]. Thus, surveillance of invasive populations should be mandatory taking into account the high mutation rate of the virus and the possible cross-transmission to native species. We strongly encourage a strict control or total ban on the international bird trade to avoid the spread of this and other pathogens potentially threatening wildlife and public health.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1424-2818/12/5/192/s1>, Database 1: detailed list of GenBank accessions, host species, country of virus isolation and genotypes of the BFDV *rep* gene.

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