Evidence of multiple introductions of beak and feather disease virus into the Pacific islands of Nouvelle-Calédonie (New Caledonia)

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Psittacine beak and feather disease (PBFD) is a viral disease affecting parrots. The causative pathogen of PBFD is beak and feather disease virus (BFDV), a circovirus in the family Circoviridae. BFDV is an icosahedral virus, 14–16 nm in diameter, with a circular ssDNA genome of approximately 2 kb (Ritchie et al., 1989). Like all circoviruses, the genome of BFDV is bidirectionally transcribed and has two major ORFs, one of which is located on the virion strand coding for the replication-associated protein (Rep), while the other codes for the capsid protein (CP) and is found on the complementary strand (Niagro et al., 1998). BFDV is highly contagious, and can be picked up from feather dust, faeces and crop secretions of infected birds (Ritchie et al., 1991), and can also be passed from infected mothers to their eggs (Rahaus et al., 2008). This ease of transmission, coupled with the general environmental stability of circoviruses (Todd, 2000), makes BFDV one of the most common viral diseases affecting psittacine birds (Ritchie et al., 2000). BFDV was first described in the 1970s in Australia (Pass & Perry, 1984) and now has a global distribution because of the international trade in exotic birds. It must be noted that there is no credible molecular evidence to date to indicate that BFDV originated in Australia.

Nouvelle-Calédonie (New Caledonia) is an external French territory that comprises a group of islands in the southwest Pacific Ocean. It has four parrot species, three of which are endemic and classed as threatened by the IUCN (IUCN Red List of Threatened Species, version 2011). The endemic genus Eunymphicus comprises two species of parakeet: the Horned Parakeet (Eunymphicus cornutus),
present on the mainland of Nouvelle-Calédonie, and the Ouvea Parakeet (Eunymphicus ouveaensis) found only on the island of Ouvea, part of the adjacent Loyalty Islands. The New Caledonian Parakeet (Cyanoramphus saisseti), once considered a subspecies of the New Zealand Redfronted Parakeet (Cyanoramphus novaezelandiae), is now recognized as a separate species in its own right, basal to other Cyanoramphus species. The Horned Parakeet and New Caledonian Parakeet are considered vulnerable, while Ouvea Parakeet, with its more restricted range, is considered endangered. The only local species not considered at risk is a subspecies of the Rainbow Lorikeet (Trichoglossus haematodus). Up to 20 subspecies of Rainbow Lorikeets are recognized, based on differences in colour, and they are widespread throughout northern and eastern Australia, as well as Indonesia and many Pacific Islands (Forshaw, 2010). Deplanche’s Rainbow Lorikeet (Trichoglossus haematodus deplanchii), however, is only found in Nouvelle-Calédonie. As is true throughout the world, Nouvelle-Calédonie also has a variety of introduced species of parrot, in zoos and other captive facilities. It is not, therefore, unreasonable to assume that BFDV might have been introduced along with these exotic birds. Although there have been no previous reports of BFDV in Nouvelle-Calédonie, the recent unexplained death of a Deplanche’s Rainbow Lorikeet, along with the discovery of others suffering from feather loss, raised the possibility of an outbreak of BFDV.

We sampled blood and/or blood feathers from 168 exotic captive parrots (all born and raised in New Caledonia) from 16 genera, and also sampled 79 birds from the four New Caledonian parrot species (Fig. 1). These were Cyanoramphus saisseti (n=14), E. cornutus (n=17), E. ouveaensis (n=8) and T. haematodus deplanchii (n=40). All of these parrots were captive, with the exception of 17 T. haematodus deplanchii that had been rescued from the wild after they were found with severe feather loss, and that were being nursed in captivity. Finally, a feather sample from a symptomatic and deceased nesting of Trichoglossus haematodus from Australia was also analysed. Blood samples were collected on filter paper by venipuncture of the medial metatarsal vein in the leg. Total DNA from samples (feather or blood) was extracted using Genomic DNA extraction kit for blood (Intron Biotechnology) and pre-screened for BFDV using primers targeting the ~605 bp region of the replication-associated protein gene (rep) (Ritchie et al., 2003).

Twenty-six of the 247 birds tested positive for BFDV including 18 birds from local New Caledonian species (Fig. 1). Of these, 17 were Deplanche’s Rainbow Lorikeet showing classical symptoms of BFDV infection, including failure to grow primary feathers and generalized feather loss (17/40, prevalence=42.5 %, 95 % confidence intervals (CI): 27–59.1 %). Of concern is the susceptibility to BFDV infection of the endemic New Caledonian Parakeet (C. saisseti), a species already considered vulnerable, with a single bird being found to be infected (1/14, prevalence=7.14 %, 95 % CI: 0.2–33.9 %). Exotic species in which infections were detected included six Eclectus Parrots (Eclectus roratus vosmaeri; 6/10, prevalence=60 %, 95 % CI: 26.2–87.8 %) and single specimens of the Red-rumped Parrot (Psophothus haematonotus; 1/1, prevalence=100 %, 95 % CI: 2.5–100 %) and the Ring-necked Parakeet (Psittacula krameri; 1/9, prevalence=11.1 %, 95 % CI: 0.3–48.2 %).

The viral genomes from BFDV-positive samples were enriched by rolling circle amplification using TempliPhi (GE Healthcare) as described previously (Massaro et al., 2012; Ortiz-Catedral et al., 2010; Piasecki et al., 2012; Varsani et al., 2010, 2011). These enriched viral targets were used as a template for a PCR-based full genome amplification using back-to-back primers (target in the conserved domain of rep: AV-BFDV-F 5’-CYTACYCT-KGGCATTGTGGC-3’, AV-BFDV-R 5’-TATHACRTBCBC-CYTCYTTACTGCA-3’) using Kapa HiFi HotStart DNA polymerase (Kapa Biosystems) and the following touchdown PCR protocol: 94 °C for 2 min, 10 cycles of 98 °C (20 s), 62 °C (20 s), 72 °C (2 min), 20 cycles of 98 °C (20 s), 52 °C (20 s), 72 °C (2 min) and a final extension of 72 °C for 2 min. The resulting ~2 kb amplicons were cloned into pJET1.2 vector (CloneJET PCR cloning kit; Fermentas) and sequenced by primer walking at Macrogen Inc.

The assembled BFDV genome sequences were aligned with all full genome BFDV sequences available in GenBank (Table S1, available in JGV Online) using CLUSTAL W (gap open penalty=10; gap extension penalty=5) with manual editing done using MEGA5 (Tamura et al., 2011). Within all the genomes we identified: (i) the circovirus nonanucleotide origin of replication sequence (TAGTATTAC); (ii) three conserved rolling circle replication motifs (FTLNN, GxxHLQGY and YxxK) in Rep; and (iii) nuclear localization motif (RRR x ARPY x RRRH x RR x R xx RRRR x FRRRRFST x RIYTLRL x RQ) on the N terminus of CP. Maximum-likelihood (ML) phylogenies were inferred using PHYL (Guindon & Gascuel, 2003) with 1000 non-parametric bootstrap replicates and model GTR+I+G4 as determined by RDP4 (Martin et al., 2010). Based on the system of classification proposed by Varsani et al. (2011), the 26 Nouvelle-Calédonie isolates can be split into two strains: BFDV-J1 (n=9) and BFDV-P1 (new strain; n=17). The additional isolate of BFDV from an Australian Rainbow Lorikeet represents a variant, BFDV-G2, which shares ~95 % pairwise identity to a previously characterized isolate from Rainbow Lorikeet (Fig. 2).

The 17 BFDV-P1 isolates were all from Deplanche’s Rainbow Lorikeet and were highly similar to each other, sharing >97 % genome wide pairwise identity, whereas the two Rainbow Lorikeet isolates from Australia share 95 % pairwise identity. However, pairwise comparison of the Deplanche’s Rainbow Lorikeet BFDV isolates with the Australian Rainbow Lorikeet isolates (BFDV-G1, -G2) reveals that they share ~90 % pairwise identity (Fig. 2).
Fig. 1. (a) Sampling collection details in Nouvelle-Caledonie. An additional sample from an Australian Rainbow Lorikeet (T. haematodus) was also analysed. (b) Locations and hosts of full genomes of BFDV isolated from around the world. (c) Illustration of recombination events detected in the Nouvelle-Caledonie isolates using RDP4. R, G, B, M, C, S and T indicate recombination detection by the RDP, GENCONV, BOOTSCAN, MAXCHI, CHIMERA, SISCAN and 3SEQ methods, respectively, with the $P$-value shown for the method indicated in bold.
In addition, all the Rainbow Lorikeet isolates (BFDV-P1, -G1 and -G2) are distinct from all other BFDV isolates, and ML phylogenetic analysis shows that they share a common ancestor. This demonstrates that the Nouvelle-Calédonie BFDV-P1 strain found in the Deplanche’s Rainbow Lorikeet most probably originated from a strain present in Australian counterparts. The remaining nine isolates infecting Eclectus Parrot (n=6), New Caledonian Parakeet (n=1), Red-rumped Parrot (n=1) and Ring-necked Parakeet (n=1) share >98% pairwise identity amongst themselves and they also share similar identity to BFDV-J1 isolated from an infected African Grey Parrot (Psittacus erithacus) in Portugal and ~95% identity to other African Grey Parrot BFDV-J isolates from Europe (Germany, UK and Portugal; Fig. 2).

ML phylogenetic trees of amino acids for Rep and CP were constructed using the LG model using PHYML with aLRT branch support (Anisimova & Gascuel, 2006). Branches with <60% support were collapsed using Mesquite (version 2.75). Pairwise comparison (p-distance with pairwise deletion of gaps) of the full BFDV genomes, Rep and CP residues were performed using MEGA5 (Tamura et al., 2011).

Rep of Deplanche’s Rainbow Lorikeet (BFDV-P1) isolates share >98.6% pairwise identity, and 91% to the two Rainbow Lorikeet isolates (BFDV-G1, -G2) from Australia whose Reps share 97.6% identity. All the Reps of BFDV-J1 isolates from Nouvelle-Calédonie share >99% pairwise identity amongst themselves and other BFDV-J1 isolates.
Fig. 3. ML relationships of Rep and CP of BFDV isolates.
from Europe and BFDV-K1 (GenBank accession no. FJ685985) from Thailand. ML phylogenetic analysis of Rep indicates that BFDV-K2 and -K3 (GenBank accession nos AF311295 and AF311296), both from Australia, and BFDV-K1 (FJ685985) from Thailand cluster with the BFDV-J1 Reps (Fig. 3). BFDV-P1 and BFDV-G1 and -G2 share ~91.3–92.7 % pairwise identity. The CP of BFDV-P1 share >96.3 % identity and 86.4–91.8 % to the two Rainbow Lorikeet isolates (BFDV-G21, -G11). Interestingly, the two Australian Rainbow Lorikeet isolates show divergence in CP sharing only 88.9 % pairwise identity (Fig. 3). On the other hand the CP of BFDV-J1 shares 95.1 % identity. The CP of BFDV-P1 and BFDV-G1 and -G2 share ~85–87 % identity. It is clearly evident from the ML phylogenetic analysis of Rep (Fig. 3) that the BFDV-J and -P strain clusters are maintained. However, there is variation in clustering in the CP and Rep ML phylogenetic trees, possibly as a result of recombination. For example, in the Rep ML phylogenetic tree, BFDV-D1 and -D2 are more closely related to BFDV-B3, -B4 and -B5 than BFDV-B1. Similarly the BFDV-K isolates cluster with BFDV-J isolates in the Rep ML phylogenetic tree, whereas in CP they cluster with BFDV-L isolates. This highlights the need for full genome analysis in order to classify BFDV variants and identify the source/reservoirs of these viral isolates.

The calculated ratios of normalized synonymous (dS) and non-synonymous (dN) substitution rates from codon alignments of the cp and rep genes using the slac method (Kosakovsky Pond & Frost, 2005; Kosakovsky Pond et al., 2006) implemented in the online server Datamonkey (http://www.datamonkey.org/; Delport et al., 2010) revealed that both rep (dN/dS=0.1896) and cp (dN/dS=0.3533) are evolving under purifying selection (dN/dS <1). Within the 249 codons of the cp gene we note 0.75 substitutions per site in contrast to the rep gene where within 291 codons it is 0.49 substitutions per site. It is clearly evident that the rep genes are evolving under a greater degree of negative selection compared with the cp genes. As demonstrated by the ML phylogenetic trees (Fig. 3), overall the rep gene is more conserved than the cp gene (Fig. 3), which may be attributed to species-specific immune evasion. As a result, it is essential to base genome/variant analyses on complete genomes and not individual genes and comparisons cannot be made between phylogenies solely based on either Rep or CP.

We found evidence of two events of recombination amongst the Nouvelle-Calédonie samples (Fig. 1) using RDP4 RDP (Martin & Rybicki, 2000), GENECONV (Padidam et al., 1999), BOOTSCAN (Martin et al., 2005), MAXCHI (Smith, 1992), CHIMERA (Posada & Crandall, 2001), SISCAN (Gibbs et al., 2000) and 3SEQ (Bonì et al., 2007). All the BFDV-J1 isolates are recombinants (the C-terminal portion of the cp gene and intergenic region) with a ~700 bp fragment from BFDV-K isolates from Australia. This clearly demonstrates that the BFDV-J1 isolates in Nouvelle-Calédonie have been introduced from Europe, possibly from the importation of infected parrots from European facilities. The second event involves a single isolate of Deplanche’s Rainbow Lorikeet BFDV-P1 [NC-2NC40F-2011], which has a ~70 bp recombinant region (within the rep gene) from an unsampled BFDV variant. Various studies (Heath et al., 2004; Varsani et al., 2011; Lefèvre et al., 2009) have demonstrated the recombinant nature of circoviruses and highlighted that the recombination hotspots are on the C-terminus of the cp gene and in the intergenic region. The recombination results in this study amongst the Deplanche’s Rainbow Lorikeet indicate the BFDV diversity within Nouvelle-Calédonie may be significantly greater than the diversity we have found in this study.

Our analysis clearly shows evidence of multiple introductions of the virus into Nouvelle-Calédonie. Most of the 27 new full BFDV genome sequences in this study, as is true for most of the known full genome sequences, were isolated from birds kept in captivity, an environment that can facilitate the spread of BFDV. Seventeen Deplanche’s Rainbow Lorikeet that were being nursed in captivity after being rescued from the wild and which were suffering from feather loss all tested positive for BFDV. The source of the BFDV infection in the Deplanche’s Rainbow Lorikeets is unclear; however, this strain does seem to be very specific to this particular subspecies of Rainbow Lorikeet.

Sampling of wild parrot populations throughout Nouvelle-Calédonie is necessary to determine the geographical and species distribution of BFDV since all three endemic species of Nouvelle-Calédonie are classed as threatened, with the Ouvea Parakeet found only in one location, and therefore extremely vulnerable to stochastic events such as a disease outbreak. While no Horned or Ouvea Parakeets were found to be positive in this study, the susceptibility of the genus to BFDV infection has previously been reported (Tomasek & Tukac, 2007). Data on population trends of the four species of parrots in Nouvelle-Calédonie, as well as baseline information on the distribution and prevalence of BFDV, is necessary to characterize the threat this virus may pose, and feed into conservation management decisions.

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References
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