Tracking Viral Evolution during a Disease Outbreak: the Rapid and Complete Selective Sweep of a Circovirus in the Endangered Echo Parakeet

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Circoviruses are among the smallest and simplest of all viruses, but they are relatively poorly characterized. Here, we intensively sampled two sympatric parrot populations from Mauritius over a period of 11 years and screened for the circovirus Beak and feather disease virus (BFDV). During the sampling period, a severe outbreak of psittacine beak and feather disease, which is caused by BFDV, occurred in Echo parakeets. Consequently, this data set presents an ideal system for studying the evolution of a pathogen in a natural population and to understand the adaptive changes that cause outbreaks. Unexpectedly, we discovered that the outbreak was most likely caused by changes in functionally important regions of the normally conserved replication-associated protein gene and not the immunogenic capsid. Moreover, these mutations were completely fixed in the Echo parakeet host population very shortly after the outbreak. Several capsid alleles were linked to the replication-associated protein outbreak allele, suggesting that whereas the key changes occurred in the latter, the scope of the outbreak and the selective sweep may have been influenced by positive selection in the capsid. We found evidence for viral transmission between the two host populations though evidence for the invasive species as the source of the outbreak was equivocal. Finally, the high evolutionary rate that we estimated shows how rapidly new variation can arise in BFDV and is consistent with recent results from other small single-stranded DNA viruses.

Managing the effects of virally mediated infectious disease in host populations requires an understanding of how these pathogens evolve. Rapid evolution and selection can enable a virus to escape immune recognition and to reinfect individuals that have been previously exposed. However, logistical difficulties in surveying wild populations may undermine our ability to understand evolution in pathogens that do not infect humans or domestic livestock. Endangered species, which are often monitored and regularly sampled over long periods of time, offer a resolution. Using a data set of samples collected over 11 years from two populations of parrot, the endangered Echo parakeet (Psittacula echo) and the rose-ringed parakeet (Psittacula krameri), and from both symptomatic and asymptomatic birds, we tracked the evolution of Beak and feather disease virus (BFDV) before, during, and after a significant disease outbreak.

BFDV (family Circoviridae) is one of the most common infections of parrots (57). It is a small and simple virus with a circular, ambisense ssDNA genome of around 2,000 nucleotides encoding two products of known function (52). ORF1 encodes the replication-associated protein Rep, which is responsible for viral replication (16) and has been shown to be highly conserved (23, 30, 34). ORF2 encodes a capsid protein responsible for encapsidation of the virus and its entry into the cell via cell surface receptors (37, 38, 52). Another protein and other ORFs have been putatively identified, but they are not consistently present (3, 4, 9, 51, 52). The virus has been shown to be environmentally stable (61) and may be transmitted both horizontally and vertically (18, 48, 63). Infection by BFDV leads to psittacine beak and feather disease (PBFD), which is characterized by immunosuppression, feather dystrophy, and, in some species, beak deformity (33, 41, 51, 52).

The Echo parakeet is an endangered parrot species endemic to the Indian Ocean island of Mauritius. By the early 1980s, this species had declined to such a degree (around 20 individuals) that it was regarded as the world’s rarest parrot (28). Intervention by a conservation program has resulted in a period of population growth over the last decade, with the number of birds currently estimated at around 500. Between 2005 and 2006 the recovery was interrupted by the presence of a pathogen; a large proportion of the population showed clinical signs consistent with PBFD. One suspected source of infection in the Echo parakeets is a population of feral rose-ringed parakeets. Since being founded in about 1886 (6), this population has grown rapidly to number over 10,000 birds and is widespread throughout Mauritius. In the context of infectious disease, invasive species represent an especially pernicious problem. Besides the potential for introducing novel pathogens and pathogen strains for which the endemic species may have no immunity, in such large and growing host populations natural
Two independent Monte Carlo-Markov chains were run for common ancestor (TMRCA) were inferred using the program Beast,ian phylogenetic trees, the evolutionary rate, and the time to most recent (32); no insertions or deletions were inferred from the alignments. Bayes-47 Echo parakeet and 31 rose-ringed parakeet BFDV isolates at ORF2.

RESULTS

Changes in BFDV in Echo parakeet host population. Analyses using microsatellite DNA, cytochrome b, and cytochrome c oxidase subunit I (COI) loci amplified from the host (data not shown) indicated no obvious evidence for cross-contamination between host individuals or species. All sequences derived from the present study have been submitted to GenBank under the following accession numbers: HQ641457 to HQ641563 and HQ662336 to HQ662409. To identify the mutations that may have elicted the 2005/2006 season (2005/06) PBFD outbreak, we constructed phylogenies using the Echo parakeet BFDV isolates. We found no significant evidence for recombination using any method and, consequently, the capsid and rep genes were concatenated to produce the dated phylogeny of Echo parakeet isolates shown in Fig. 1 (inferred using a relaxed lognormal molecular clock; the frequency histogram of the mean coefficient of variation [mean CoV = 1.09] does not sit against zero, indicating rate heterogeneity). Of the isolates sampled during the PBFD outbreak, the majority (37 of 42) group together within one large clade (highlighted by the gray box). All of the isolates collected after this
period are also found in this clade. In contrast to the exception of an isolate collected in 2004/05, all of the isolates that predated the outbreak were found outside of the highlighted clade. The clade support values indicate good evidence for this differentiation of BFDV isolates sampled prior to the PBFD outbreak from those sampled during and afterwards, although they also indicate that there is no clear pattern of genetic differentiation within the highlighted clade. Of the 10 mutations that appear to differentiate the highlighted clade from the remaining isolates eight occur in the replicase gene, two of which are nonsynonymous substitutions. The first is a mutation within a potential pyrophosphatase domain (38), giving rise to a histidine residue (from tyrosine) at codon 14. The second occurs at codon 34, which is in a rolling-circle replication motif (26), and was a substitution from a phenylalanine residue to a leucine (both changes have been highlighted in Fig. 1). The capsid gene had just two substitutions, both synonymous, that clearly differentiated the two phylogenetic groups. However, we observed eight polymorphic sites with nonsynonymous changes involving more than one isolate of which codons 88, 148, 167, and 234 appeared to be particularly variable (these are mapped onto Fig. 1). The pattern of changes indicates that prior to 2005 the “14Y34F” replicase allele and “88T148V167E176L234M” capsid alleles were present in the majority of Echo parakeet isolates. From early 2005 a new replicase allele, “14H34L,” appears in the population and represents almost all of the isolates sampled during and after the PBFD outbreak; after February 2006, the “14Y34F” replicase allele is no longer present. Multiple capsid alleles are evident both during and after the outbreak, including the “88T148V167E176L234M” allele that was present in the population before 2005.

Epidemic history. A Bayesian skyline plot depicting the inferred effective number of BFDV infections in the Echo parakeet population over time is shown in Fig. 2a (from the Beast analysis

FIG 1 Maximum clade credibility tree showing the inferred phylogenetic relationships between BFDV isolates collected from the Echo parakeet population (from Bayesian analysis of the capsid and replicase genes using Beast). The tips are labeled with the Echo parakeet breeding season from which the sample was collected. The tree was automatically rooted by using a relaxed clock model in Beast. Nodes with posterior probability of ≥0.9 are indicated with asterisks and with a double-dagger for P ≥ 0.7. The dark grey vertical bar highlights the 2005/06 PBFD outbreak season. Inferred nonsynonymous substitutions at codons in the replicase (in boldface) and capsid (in italics) are indicated at the appropriate lineages (where the majority of isolates in a clade possess a particular substitution then the ancestral node has been labeled). The clade highlighted by the gray box represents all of the isolates with the 14H and 34L replicase gene substitutions identified by the present study as closely corresponding to the PBFD outbreak. The estimated mean TMRCA for all of the isolates is 1959 (95% HPD 1920 and 1988).
used to infer the phylogenetic relationships in Fig. 1). The skyline plot, which gives an indication of change in viral diversity over time (13), indicates no evidence for any change from the estimated TMRCA (in 1959). The plot shows a period of exponential decline in viral diversity (highlighted as “A”) coinciding with the 2005/06 PBFD outbreak (highlighted by gray shading), followed by a subsequent increase (“B”). This decline continues until approximately the middle of the PBFD outbreak, after which there is a period of increase (labeled “B”). The skyline plots inferred for each gene separately show a similar pattern to that in Fig. 2a (data not shown). The estimated nucleotide diversity of BFDV in each Echo parakeet breeding season from 2004/05 is shown in Fig. 2b (alongside the prevalence); we did not calculate for previous seasons since there were too few isolates to obtain a reliable estimate. The pattern is consistent with that indicated by the skyline plot and shows a decline in genetic diversity during the outbreak (\( \pi = 0.0064 \), standard deviation \( SD = 0.0013 \)) and in season 2006/07 (\( \pi = 0.0019 \), SD = 0) compared to that estimated from 2004/05 (\( \pi = 0.012 \), SD = 0.005).

Transmission between the Echo parakeet and feral rose-ringed parakeet population. Figure 3 shows the phylogenetic relationships among BFDV isolates from both parrot populations in Mauritius (also inferred using the relaxed molecular clock since...
the frequency histogram for the coefficient of variation indicated evidence against the strict clock; for ease of visualization, we do not include all Echo parakeet isolates but instead retain 15 isolates representative of the polymorphisms identified in Fig. 1. The rose-ringed parakeet isolates, which in Fig. 3 are indicated by “(R),” form two clades: the majority are closely related to the Echo parakeet viral isolates carrying the 14Y34F rep allele, i.e., those that were identified from Fig. 1 to not correspond to the PBFD outbreak. This group includes rose-ringed parakeets sampled at the same time as the PBFD outbreak in the Echo parakeet population. The second group (highlighted by the gray box), comprising just three isolates, have the 14H34L rep allele that appears to closely correspond to the outbreak. This second group also shares some of the synonymous substitutions that correspond to the PBFD outbreak; one isolate shares three of six of the rep gene substitutions and two others each carry one of the capsid gene substitutions. The five sites in the capsid gene that we observed to be particularly polymorphic in the Echo parakeet isolates (codons 88, 148, 167, 176, and 234) appeared to be much less variable in the rose-ringed parakeet isolates. Only codons 148 and 167 showed nonsynonymous polymorphism.

**Positive selection in the BFDV genes.** Table 1 shows the positively selected sites inferred by the FEL, PAML, and SLAC methods. These analyses failed to find any significant evidence for positive selection in either the capsid gene of rose-ringed parakeet isolates or the rep gene of Echo parakeet isolates. Four of the five capsid gene polymorphisms highlighted in Fig. 1 have been identified as positively selected, but inconsistently across the three methods: codons 167 and 176 by PAML (significant evidence [1%] in favor of the selection model, M8, over M7 ΔL = 10.714, v = 2, where ΔL is the test statistic and v is the number of degrees of freedom), codon 148 by FEL and codon 234 by SLAC. The polymorphic sites in the replicase gene that we identified as most closely associated with the PBFD outbreak, 14 and 34, have not been identified by any method. The substitution counts determined by SLAC showed that except for sites 88, 167, and 176, there is no evidence for synonymous substitutions at any of the sites in Fig. 1. The phylogenetic pattern of these substitutions (Fig. 1)
suggests that tests of episodic selection may be more appropriate than the site-prediction methods in FEL, PAML, and SLAC. The results of the MEME analyses for episodic selection (Table 1) indicate that seven codons in the capsid (including sites 148, 167, 176, and 234) and one in the rep gene showed evidence of episodic selection in the evolutionary history of BFDV in the Echo parakeets. Codons 14 and 34 in the rep have not been identified.

Secondary structure in BFDV genes. Previous studies have highlighted the selection potential of synonymous substitutions in viruses with highly compact genomes primarily as a consequence of their effect on the formation of secondary structure. Indeed, analyses of the intergenic region between ORF1 and ORF2 in BFDV and the related porcine circovirus have identified a putative stem-loop structure that may be involved in replication (3, 59). Eight of the substitutions that closely correspond to the PBFD outbreak were synonymous, and we investigated their effects upon the predicted secondary structure (data not shown). Of the two observed in the capsid, only the substitution at codon 174 has an effect, resulting in the formation of a number of additional stem-loop structures. We observed the synonymous substitution at codon 121 in the rep gene to have a minimal and localized effect altering a predicted loop structure. Substitutions at codons 54 and 84 have even smaller effects, resulting in minor changes to the loop structures that these sites occur in. The remaining substitutions appear to have no effect whatsoever.

Evolutionary rate of BFDV genes. Across all of the clock models, the mean evolutionary rate estimates varied only slightly (data not shown), falling within a narrow range between $10^{-4}$ and $10^{-3}$ substitutions per site per year, which is high but consistent with rates estimated from other ssDNA and ssRNA viruses (15). The coefficient of variation histograms indicated that for the Echo parakeet isolates there is not sufficient evidence to reject the strict clock, but there is for the rose-ringed parakeet isolates. Table 2 shows the rate estimated from the strict clock for the Echo parakeet isolates and the relaxed clock (uncorrelated lognormal) for the rose-ringed isolates. The inferred rates do not differ substantially between the two genes or between the two host populations, although the 95% HPD intervals for the capsid gene analyses from rose-ringed isolates are very wide, suggesting that there may not be very much temporal structure. Given the phylogenetic evidence for allele-sharing between the two host populations, we also estimated the rate based on the combined data where there was sufficient evidence to reject the strict molecular clock for the rep gene but not for the capsid gene.

To assess the level of clock-like evolution in the data, we re-screened the root-to-tip genetic distances, inferred from neighboring trees, against sampling time by using Path-O-Gen. The results indicate that there is good evidence for temporal signal in the capsid gene but not in the replicase: (i) Echo parakeet isolate capsid gene, $r^2 = 0.625$, residual mean square (rms) $= 3.23 \times 10^{-6}$; and replicase gene, $r^2 = 0.122$, rms $= 1.55 \times 10^{-6}$, and (ii) rose-ringed parakeet isolate capsid gene, $r^2 = 0.461$, rms $= 4.32 \times 10^{-6}$, and replicase gene, $r^2 = 0.073$, rms $= 7.21 \times 10^{-7}$. Following Duffy and Holmes (14), to assess the temporal structure of the evolutionary rate, analyses were repeated for each data set, but with the sampling dates randomly shuffled among the tips. In both genes of the Echo parakeet isolates the actual estimated mean

| Table 1 | Positively selected sites in the BFDV genes of the Echo parakeet and feral rose-ringed parakeet isolates inferred by a number of methods$^a$ |
|---|---|---|---|---|
| Species | Gene | No. of sequences | PAML, model M8 ($\omega$, $P$) | FEL ($\omega$, $P$) | SLAC ($\omega$, $P$) | MEME ($P$) |
| Echo parakeet | capsid | 50 | 167 (3.8; 0.99), 176 (10.3; 0.99) | 126 ($\approx 0.03$), 148 ($\approx 0.05$) | 234 (7.28; 0.04) | 36 (0.09), 121 (0.005), 126 (0.001), 148 (0.02), 167 (0.07), 176 (0.07), 234 (0.03) |
| | rep | 73 | No sites | No sites | No sites | 8 (0.06) |
| Rose-ringed parakeet | capsid | 23 | No sites | No sites | No sites | 60 (0.03), 246 (0.01) |
| | rep | 34 | 227 (2.26; 0.95 [NS]) | 180 ($\approx 0.08$) | No sites | 159 (0.08), 180 (0.05) |

$^a$ The number of sequences in the analysis is specified. $\omega$ is the selection parameter, and $P$ the posterior probability of $\omega > 1$. NS, not significant. The PAML, FEL, SLAC, and MEME methods are described in the text.

| Table 2 | Mean evolutionary rate and the 95% HPD intervals estimated for the capsid and replicase genes of BFDV isolates from Echo and feral rose-ringed parakeets$^a$ |
|---|---|---|---|
| Host species | Gene | Clock model | Mean evolutionary rate (per site/yr) | 95% HPD interval |
| | | | Lower | Upper |
| Echo parakeet | capsid | Strict | $1.01 \times 10^{-3}$ | $5.63 \times 10^{-4}$, $1.44 \times 10^{-3}$ |
| | rep | Strict | $3.44 \times 10^{-4}$ | $1.39 \times 10^{-4}$, $5.67 \times 10^{-4}$ |
| Rose-ringed parakeet | capsid | Relaxed (ULN) | $5.50 \times 10^{-3}$ | $1.99 \times 10^{-6}$, $9.63 \times 10^{-3}$ |
| | rep | Relaxed (ULN) | $2.75 \times 10^{-3}$ | $7.31 \times 10^{-4}$, $4.44 \times 10^{-3}$ |
| Combined data set | capsid | Relaxed (ULN) | $1.08 \times 10^{-3}$ | $6.15 \times 10^{-4}$, $1.63 \times 10^{-3}$ |
| | rep | Relaxed (ULN) | $4.74 \times 10^{-4}$ | $1.65 \times 10^{-4}$, $8.31 \times 10^{-4}$ |

$^a$ Estimates obtained by combining the data from the two host populations are also shown. For each data set, we only included results from the significant clock model, i.e., the strict clock was rejected for the rose-ringed and combined datasets (in favor of the relaxed uncorrelated lognormal clock [ULN], which had a higher marginal likelihood than the relaxed uncorrelated exponential clock).
characterized by a rapid reduction in variability at linked loci (7, 36). Since the fixation of rep alleles occurred during an outbreak, we suggest that strong directional selection for adaptive changes was responsible rather than drift. Moreover, no significant declines have been observed in the Echo parakeet population since 1993 and analyses of the host genetic diversity using microsatellite DNA loci has not identified any significant evidence for a secondary bottleneck (49). Although changes in viral coat proteins are most commonly associated with disease outbreaks, mutations in viral replication genes have been shown to result in increased replication activity and virulence (20, 53). It is notable that the changes that we observed occur in potentially important regions (a pyrophosphatase domain and a rolling-circle replication motif). It is still possible that the rep gene changes that we observed are nonadaptive and have simply been “carried” through a sweep by genetic hitchhiking to other mutations. The regularity of our sampling suggests that it is unlikely that we missed any other key mutations, and the phylogenetic pattern of nonsynonymous changes in the capsid gene does not correspond well to the chronology of the outbreak. However, we did observe a number of synonymous substitutions in both BFDV genes that also correspond to the appearance of the PBFD outbreak. Previous studies in RNA viruses, particularly those carrying a positive-stranded genome, have indicated that synonymous substitutions may be under selection, often as a consequence of their impact upon secondary structure, which itself can affect translation and gene expression (39, 58, 8). Small ssDNA viruses may behave in a similar way, and our analyses indicate that two of the synonymous substitutions that we observed, one in the capsid gene and the other in the rep gene, may indeed affect the secondary structure. However, a recent experimental analysis of synonymous substitutions in viruses found that while they may have significant fitness effects in ssRNA viruses, the same may not be true for ssDNA viruses, where the effect appears to be considerably diminished (8). These observations, taken together, suggest that the two nonsynonymous substitutions in the rep gene at codons 14 and 34 remain the most plausible explanation for the PBFD outbreak in the Echo parakeets, although ultimately some degree of experimental validation to ascertain their functional relevance will be needed. Currently, it is difficult to conceive of how this may be achieved since our study system includes a critically endangered host species and a virus for which there is currently no cell culture system (24).

While adaptive changes in the rep gene appear to be the likeliest explanation for the PBFD outbreak, the presence of variation in the capsid may also have been important. The analyses showed the presence of multiple capsid alleles linked to the outbreak, and almost 10% of the gene showed some level of nonsynonymous diversity. Mutations in capsid proteins can affect a virus’ ability to avoid the immune system and cause infection. Consequently, it may be that while the outbreak was elicited by changes in the rep gene, variation in the capsid gene was an additional factor in the dissemination of the virus.

Our analyses indicated little consistent evidence for positive selection despite the presence of potentially adaptive mutations. Although current site prediction methods arguably represent the most rigorous tools for analyzing selection in DNA sequences (2), it has been suggested that the statistical methods that underpin them have some limitations (40). Specifically, many of these methods require considerable sequence divergence (1) and have difficulty in identifying rare mutational events that are adaptive (40). The phylogenetic analyses suggest that the nonsynonymous substitutions in the rep gene that we have identified as most closely
linked to the outbreak arose only once. These sites also had no other nonsynonymous variation, i.e., they are characterized by a pattern of selective neutrality or purifying selection punctuated by a single potentially adaptive event. The results thus seemingly corroborate previous studies suggesting that site prediction methods may be inappropriate for detecting rare adaptive events, including those that have gone through a major selective sweep. Given the role of the capsid, adaptive changes are expected to be comparatively frequent and thus more detectable. A previous study of BFDV did indeed identify evidence of positive selection in this gene, albeit using only a single method of analysis (23). However, even here our results were not unequivocal across three of the site-prediction methods that we used (FEL, PAML, and SLAC). The fourth method for detecting selection, MEME, attempts to identify sites that have periodically experienced positive selection over their evolution (45). The majority of the polymorphic sites that we documented in Fig. 1 were identified by this method, suggesting that the evolution of the capsid gene is characterized by episodic positive selection. This is consistent with a pattern of frequency-dependent selection, which is often observed in the dynamic between pathogens and the host immune system (11, 31, 42, 47).

Considering the proximity of the two parrot populations in Mauritius and the recent common ancestry of the host species (22), we expected to find good evidence for viral transmission. Indeed, the phylogenetic analyses indicated the presence of BFDV allele sharing between the two populations. Although the literature (28) and field reports indicate behavioral and ecological separation between the parrot populations in Mauritius, the environmental stability of BFDV (62) means that the virus still can be indirectly transmitted. The most recent common ancestor for all Echo parakeet isolates dates to around 1959 (or 1949 when the rose-ringed isolates are included in the analysis), which approximately coincides with a period when the rose-ringed parakeet started expanding into native forest, coming into direct competition with the Echo parakeet for nest sites (6). Interestingly, the oldest rose-ringed parakeet isolate was taken from a bird housed in aviaries that were central to the initial recovery of the Echo parakeets. Thus, the apparent transmission of BFDV actually may have occurred in some cases be attributable to human intervention. Although the evidence for viral transmission between the two parrot populations appears to be clear, the evidence for the rose-ringed parakeets being the source of the PBFD outbreak is more equivocal. We only identified three rose-ringed parakeet isolates that possessed the 14H34L rep allele, and all of them were collected at least one season after the outbreak. One possibility is that the mutations responsible for the outbreak originated in the Echo parakeet population with subsequent transmission to the rose-ringed population. Indeed, one of the three rose-ringed isolates was collected from an individual that most likely became infected after indirect contact with the endemic population (it was found in an Echo parakeet nest in the 2006/07 season). Nevertheless, given the considerable size and distribution of the invasive population, it undoubtedly remains the likeliest source of the PBFD outbreak in the Echo parakeets. It may be that our sampling of this population prior to 2005/06 was not sufficient to identify the 14H34L mutations.

Documented evolutionary rates for ssDNA viruses are very comparable to those of RNA viruses (15, 50, 55, 56). It has been suggested that ssDNA viruses may tolerate such high rates since their generally small genome size may be less likely to accumulate deleterious mutations (25). The only previous study to have estimated an evolutionary rate for the Circoviridae (27) suggested that this group of viruses have an evolutionary rate only about three times higher than that of the vertebrate host. Clearly, this rate is lower than that implied by our study and appears contrary to the relationship between viral genome size and evolutionary rate; circoviruses have the smallest genomes of all known DNA viruses. However, Holmes (25) points to a number of flaws in the Johne et al. study (27), including the limited number of host species and the presence of within host genetic diversity. A caveat to our own findings is the potential effect of the background mutation rate to inflate our estimates for the evolutionary rate. It is possible that some of the variation identified here may be from deleterious mutations that have yet to reach fixation. The randomization analyses of the rose-ringed parakeet isolates suggested that there is insufficient support for the evolutionary rate inferred from this data set. However, our analyses suggest that the rate estimated from the BFDV capsid gene in the Echo parakeet isolates is well supported. This result indicates that as with other ssDNA viruses, BFDV has a high evolutionary rate.

Concluding remarks. The growing impact of emerging infectious disease in humans, livestock, and endangered species has increased the importance of understanding the changes that can elicit an outbreak and how a pathogen subsequently evolves during such a disease. Although infectious disease in endangered populations represents a major problem for biodiversity, such events also present an excellent opportunity to resolve evolutionary patterns in pathogens. Populations such as the Echo parakeet, which have been sampled in a frequent and unbiased (in terms of disease) fashion, enable studies to access a level of detail not normally possible in a natural population.

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