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# Evidence of an emerging Lorikeet-associated beak and feather disease virus

**Shubhagata Das,[1,2] Subir Sarker,[1,2] Ali Ghorashi, [1,2] Jade Forward, [2,3] David Phalen,[4] Shane R. Raidal[1,2]**

1. School of Animal and Veterinary Sciences, Charles Sturt University, Boorooma St, Wagga Wagga, NSW, 2678, Australia
2. Graham Centre for Agricultural Innovation, Pine Gully Road, Wagga Wagga NSW,, 2650, Australia
3. School of Biomedical, Charles Sturt University, Boorooma St, Wagga Wagga, NSW, 2678, Australia
4. Faculty of Veterinary Science, Wildlife Health and Conservation Clinic The University of Sydney, 425 Werombi Road, Camden NSW, 2570, Australia.

## Abstract

Beak and feather disease virus (BFDV) is well known for its wide host range, high genetic diversity and the capacity of flexible host switching or cross species transmission. Until now, phylogenetic analysis of BFDV genomes suggested host generalism and very shallow host based divergence with its origins in Australia. Budgerigars (*Melopsittacus undulatus*) host the most basal BFDV genotypes suggesting that the global spread of this infection may have occurred in the mid-19th century associated with the international trade budgerigars as pet birds. A broader hypothesis would be that the parrot subfamily Lorinae, to which the budgerigar, lorikeets and fig parrots belong, could be the Gondwanan antediluvian source of BFDV. Accordingly we analysed BFDV circulating in Australian budgerigars and lorikeets in order to investigate their potential role as distributors of BFDV across Australasia as well as to ascertain the likely threats they pose to vulnerable and endangered psittacine bird species. Phylogenetic and population genetic analyses of full length BFDV sequence data from wild lorikeets (n=34) and captive budgerigars (n=5) were compared with all available published full length BFDV genomes. Strong support for host tribe specific clustering was detected among lorikeet genotypes while budgerigar genotypes were basal and found in a wide variety of genetic clades with a diverse host-species mosaic. Within Australia, the BFDV genome in wild lorikeets occurred within a distinct subpopulation structure, genetically segregated from those infecting other psittacine hosts with no evidence of detectable gene flow and inter-population host switch events. The phylogenetic evidence indicates that Australian lorikeets maintain a deeply host adapted BFDV lineage introduced relatively recently from a foreign origin. The existence of genetically interlinked host based minor subpopulations demonstrates that BFDV genotypes evolved independently within each host with frequent episodes of cross species transmissions in the past.

## Introduction

Beak and feather disease virus (BFDV), one of the smallest and simplest viruses belonging to the genus Circovirus in the family Circoviridae, is the causative agent of Psittacine Beak and Feather Disease (PBFD). It has been recognized as a key threat for the endangered psittacine birds in Australia and has spread globally, now affecting a wide range of psittacine species both in wild and captive populations (Bassami et al., 2001; Raidal et al., 1993a; Ritchie et al., 1990). All endemic parrots, lorikeets and cockatoos are considered susceptible to this infection and evidence shows that it has been circulating naturally in wild Australian birds for more than 120 years (Ashby, 1907; McOrist et al., 1984; Powell, 1903). The evolutionary signature of BFDV identifies Australia as the most recent common ancestor of the extant BFDV lineages which co-evolved with the psittacine host in post-Gondwanan period (Raidal et al., 2015; Varsani et al., 2010). The dispersal of wild-caught Australian parrot species such as the budgerigar (*Melopsittacus undulatus*) since the early 1840's has most likely resulted in the global spread of PBFD as it now affects a wide range of psittacine species both in wild and captive populations worldwide (Bassami et al., 2001; Ha et al., 2007; Raidal et al., 1993a). A recent study based on *Rep* gene segment of BFDV from all over the world identified budgerigar genotypes as the basal clade of the phylogenetic tree which may represent that they are the most robust or deeply host-adapted host of BFDV. The budgerigar belongs to the Loriinae subfamily which also includes

the lorikeets, lories and fig parrots. Whilst the number of whole BFDV genomes from Australian lorikeets has been small they have been demonstrated in a wide variety of genetic clades as they were very distinct and genotypically divergent (Bassami et al., 2001) and suspected as the "true Australasian variant" (Heath et al., 2004). A BFDV genome from a wild caught Moluccan red lory from Indonesia (Sarker et al., 2013) and BFDV genomes from Deplanche's rainbow lorikeets in New Caledonia were closely related genetically despite wide geographical distances (Julian et al., 2012). Thus, psittacine hosts in the subfamily Loriinae may act as a super distributor of BFDV across Australasia and pose significant threats to endangered psittacine species.

Genome-wide patterns of sequence variation within and between closely related viral genomes can be used to efficiently infer the fine-scale genetic structures of virus populations (Prasanna et al., 2010). Determining genetic population structures which considers admixture or gene flows between different strata provides valuable insight in a variety of situations such as the establishment of sensible species/subspecies/strain classification criteria, the detection of geographical or biological barriers to gene flow, and the identification of demographic, epidemiological or evolutionary processes responsible for virus differentiation (Pritchard et al., 2000; Rosenberg et al., 2002). More importantly, a detailed knowledge of virus population stratification can provide important insights into how virus genetic diversity generated through mutation and recombination is shaped into discernible taxonomic groupings; a process that involves natural selection and genetic drift in the context of epidemiological fluctuations in virus population sizes and the spatial movement of viruses across land-masses (Barton and Clark, 1990; Slatkin, 1994). The deeper understanding of virus epidemiology and evolutionary history that can potentially be provided by studies of virus population structure is also directly applicable to the formulation of strategies for controlling the dissemination of viral diseases (Garcia-Arenal et al., 2001).

In the present study, we inferred the genetic population structure of BFDV alongside Bayesian phylogenetic reconstruction to understand the evolutionary process in with greater resolution. We sequenced and analysed new full length BFDV genome from Australian budgerigars and lorikeets to identify corresponding viral lineages and to ascertain the possible threats they might impose to endangered species. Our aim was to test the hypothesis whether Loriinae is the super distributor for BDFV in Australasia or not.

## Materials and Methods

### *Sampling and sequencing of full length BFDV genome*

Genomic DNA samples were obtained from PBFD infected budgerigar and lorikeets from different regions of Australia (NSW, QLD, WA) through samples collected and or submitted to the Wildlife Health and Conservation Clinic (WHCC), The University of Sydney or the Veterinary Diagnostic Laboratory (VDL) of Charles Sturt University. A total of 39 full length BFDV genomes (5 budgerigar and 34 lorikeets) were amplified, cloned and sequenced following standard protocol (Sarker et al., 2014b; Ypelaar et al., 1999). In addition to this, publicly available full length BFDV genome (n=303) sequences from NIH GenBank were also compiled together for conducting sequence based bioinformatics analysis. The individual sequences were then annotated according to the accession number, geographic origin, host species, taxonomic tribe name and sampling year.

### *Phylogenetic Analysis*

Full length BFDV sequence data were aligned with MAFFT (Katoh et al., 2002) and jModelTest 2.1.3 favored a general-time-reversible model with gamma distribution rate variation and a proportion of invariable sites (GTR+I+G4) for the BFDV phylogeny (Darriba et al., 2012). Maximum-likelihood (ML) phylogenetic tree from all available full length (n=342) sequences were estimated using the program PhyML v3.1 (Guindon and Gascuel, 2003). Branch support was evaluated by bootstrap analysis based on 1000 pseudo replicates. To reduce the computational burden for further analysis and easiness of result viewing a sub set (n=155) of sequences was selected representing all genotypic clades, geographical regions, host species and subsequently aligned in Geneious using same protocol described above. The Bayesian phylogenetic trees were inferred from the sequence subset using Beast v1.8.1 (Drummond et al., 2012). In Beast, two independent Monte Carlo-Markov chains (MCMC) were implemented for 500 million generation each with trees sampled every 50000 generations. Bayesian skyline coalescent demographic prior was chosen because it allows temporal changes in population size (Drummond et al., 2005). Each analysis was checked to ensure that a reasonable effective sample size (ESS>200) had been reached for all parameters. Tracer version v1.5 was used to derive parameters and Tree Annotator v1.8.1 was used to obtain the tree with the highest clade credibility and posterior probabilities for each node (Drummond et al., 2012), as well as FigTree v1.4 was used to generate the consensus tree (Andrew, 2009).

### *Genetic population structure analysis*

The BDFV population structure was investigated using the program STRUCTURE v 2.3.4. The full length sequences were converted to STRUCTURE compatible files using the program XMFA2Struct software (http://www.xavierdidelot.xtreemhost.com/index.htm). To estimate the number of population structure (the K parameter), the BFDVs dataset was analyzed allowing the value of

K to vary from 1 to 12 with an initial burn-in of 10,000 iterations followed by 50,000 iterations. Five independent runs were carried out for each of K value (equating to 60 runs in total). As advised in the STRUCTURE user's manual, we set most of the parameters to their default values (Pritchard and Wen, 2004) and used admixture model with the option of correlated allele frequencies between populations (Falush et al., 2003; Martin et al., 2005). Structure harvester was used to detect the optimum number of population structure (Earl and vonHoldt, 2012) by inferring the appropriate DK (highest change of likelihood function). After obtaining the optimum K value at least 30 independent run in STRUCTURE was performed to obtain the final result. Genetic stratification was visualized using CLUMPAK (Kopelman et al., 2015) using suitable distract setting and default color parameters. For validating the inferred genetic subpopulation structure and to determine the gene flow between subpopulations Dna SP v5(Librado and Rozas, 2009) and Arlequin 3.1(Excoffier et al., 2005) was used.

***Recombination analyses***

Evidence of recombination in global BFDV population was screened using the RDP(Martin and Rybicki, 2000), GENECONV (Padidam et al., 1999), Bootscan (Martin et al., 2005), MaxChi (Smith, 1992) , Chimaera (Posada and Crandall, 2001), Siscan (Gibbs et al., 2000) and 3Seq (Boni et al., 2007) methods contained in the RDP4 program (Martin et al., 2010). The non-tree like evolutionary relationship among Australian BFDV genomes in the presence of extensive recombination was assessed by neighbor net algorithm implemented in SplitsTree4 (Huson and Bryant, 2006).

***Spatial biogeographic reconstruction***

Evidence of virus movements between sampling locations was identified under a Bayesian stochastic search variable selection (BSSVS) process (Lemey et al., 2009). The Bayes factors were calculated with the computer software SPREAD (Bielejec et al., 2011) applying a BF cut-off of 3.0 (where a BF > 100 was considered as representing decisive support for one or more virus movements between the sampling locations in question, a BF>3.0 was taken to represent reasonable support for such movements and a BF <3.0 was considered to be a poor support for any direct movements between the locations).

***Analysis of Selection Pressure***

To discover evidence of positive selection sites in the protein coding genes of BFDV from lorikeets (where ω, the selection parameter which corresponds to the ratio of the non-synonymous and synonymous substitution rates, is greater than 1), a number of methods were used. A Fast Unconstrained Bayesian Approximation (FUBAR) was used, which allows sites experiencing positive and purifying selection (Murrell et al., 2013), where the number of grid points was 400. The data were further analyzed in the programs FEL and SLAC (Kosakovsky Pond and Frost, 2005). In addition, mixed effects model of evolution (MEME) was used to screen for episodic positive selection (Murrell et al., 2012). Finally to determine whether in any given time in any branch diversifying selection influenced the evolution of BFDV in Australian lorikeets, branch-site model (BSR) (Kosakovsky Pond et al., 2011) implemented to detecting episodic diversifying selection. All these analysis was performed on the Datamonkey web server (http://www.datamonkey.org/) to detect positive selection (initially data were screened for recombination).

## Results

***Genome Sequences***

A total of 39 new full length BFDV genome sequences from Budgerigars (n=5) and different species of Lorikeets (n=35) from various geographical locations of Australia were obtained in this study and deposited in GenBank (Accession numbers: KM887916–KM887951; KM978921-23). In addition to this, publicly available full length BFDV genome (n=303) sequences from NIH GenBank were also compiled together for conducting sequence based molecular analysis. The individual sequences then annotated according to the accession number, geographic origin, host species, taxonomic tribe name and sampling year.

***Phylogeny and population structure of BFDV in global context***

The Bayesian phylogenetic tree of newly isolated 39 full length BFDV genome sequences from budgerigar and lorikeets along with another 116 selected BFDV genome sequences exhibited strong tribe specific clustering for all species under tribe Loriini forming a monophyletic clade (Fig.1). However, this clade was subdivided into distinct sub-clades monophyletic by sampling location (Australia and New Caledonia). A single isolate from Moluccan red lory (KF673337) obtained from Indonesia formed a separate branch positioned between Australian and New Caledonian Loriini sub-clades sharing 100% posterior probability supports with both. Overall, all BFDV genome in the Loriini clade were related with BFDV genome from Thailand (GenBank accession no. GU015018, GU015021) sharing >72% clade identity. On the other hand, regardless of geographical origin BFDV genomes infecting budgerigars (tribe Melopsittacini) from different part of the world (Japan, China, Poland, South Africa and Australia) distributed at the basal clade of the phylogenic tree (Fig.1). Interestingly, BFDV genome from a crimson rosella (JX221043, tribe-Platycercini), a pacific parrotlet (JX221024, tribe-Arinae) and a rose-ringed parakeet (JX221010, tribe-Psittaculini) from Poland also integrated

within this clade which demonstrates a host generalist pattern of infectivity. Instances of spill over infection or cross species transmission was also observed in budgerigars as BFDV isolates from Poland (GenBank ac. no. JX221005, JX221012 and JX221014) found with distant lineages.

Analysis of gross population structure implemented by STRUCTURE software supported presence of nine genetic subpopulations in global BFDV population. The admixture model used in our STRUCTURE analysis automatically assigned individual BFDV whole genome sequences to particular subpopulations based on their relative membership scores with respect to each of these subpopulations. These relative membership scores got encoded as colored bars in the subpopulation structure maps generated by CLUMPAK and thus represent the membership probability of individual genome to particular subpopulation/s (Fig.1). These color coded bar plots when arranged alongside Bayesian MCC tree allowed us to simultaneously locate the topographic position of individual BFDV genome as well as to visualize their corresponding subpopulation membership, pattern of genetic admixture and gene flow between the subpopulations.

BFDV isolates from Australian lorikeets were all flagged with dark green colored plots which represent their genetic similarity. On the contrary, New Caledonian coconut lorikeets were designated with predominant orange color; completely separating them from that of Australian lorikeets. This color variation reveals that the BFDV genotypes circulating in Australian and New Caledonian lorikeets belong to two separate genetic subpopulations. However, the Moluccan red lory (GenBank ac. KF673337) isolate from Indonesia shared predominant color proportion from both New Caledonian and Australian lorikeets. BFDV isolates from Australian budgerigars (KM887947-51) at the basal clade of the phylogenetic tree were illustrated with sky blue color representing a subpopulation sharing spatially distant isolates from Australia, Japan, China and Poland (Fig 1). Interestingly, isolates from diverse hosts like crimson rosella (JX221043), pacific parrotlet (JX221024) and rose-ringed parakeet (JX221010) also demonstrated membership with the same subpopulation. This shows the global distribution of this particular genotype and host generalist pattern of infectivity. However, frequent admixture of genetic material with other subpopulations was also observed as one captive budgerigar from South Africa (GQ165757) and two from Japan (AB277747 and AB277750) showed considerable admixture of different colors indicating that a substantial number of polymorphisms of these genomes are characteristics of other genotypes.

***Genetic segregation in Australian BFDV population***

When only Australian (n=117) BFDV isolates were assessed for genetic population structure, the STRUCTURE software assumed existence of two major and seven minor subpopulations (structure harvester demonstrated highest change of likelihood function (DK) at k=2 followed by K=7). At K=2 the major subpopulation MSP-L comprised all isolates from lorikeets whereas subpopulation MSP-R represented rest of the sequences. This result essentially demonstrates that BDFV genotypes infecting Australian lorikeets are genetically segregated from other genotypes circulating in this region. However, many individual sequences within MSP-R subpopulation exhibited variable degree of admixture. Besides, the structure harvester showed another clear peak at k=7 demonstrating the potential of having minor subpopulations. Therefore, a second layer of population stratification with K=7 was performed and BFDV bar plots were arranged according to their host taxonomic tribes. Nonetheless, the segregation of lorikeet isolates remained constant as MSP-L while MSP-R divided into minor subpopulations (Melopsittacini, Platycercini, Cacatuini_1, Cacatuini_2, Cacatuini_3, Calyptorhynchinae and Pezoporini). Tribe Nymphicinae, Agapornithinae and Polytelini had less than four isolates and therefore compiled under 'others' and kept out from further analysis. The existence of these genetically distinct subpopulations within Australian BFDV population was validated using AMOVA which supported high overall Fst (Pairwise measures of population differentiation) statistics (Fst of 0.73; p=<0.001). The major subpopulation comprising lorikeet isolates (MSP-L) showed 41.15% diversity with rest of the Australian isolates infecting other hosts (MSP-R) and also with host dependent minor subpopulations of MSP-R (54.63% of the diversity). This fixed genetic differences between major and moor subpopulations collectively attributed for the overall high Fst value.

***Pattern of gene flow among Australian BFDV subpopulations***

From the STRUCTURE plots (Fig.2) it was evident that within Australia the BFDV genotypes circulating in lorikeets (MSP-L) do not contain or share nucleotide polymorphisms that are apparently characteristics of those infecting other host species (MSP-R). Additionally, when Fst statistics were calculated to determine the pattern of gene flow between different subpopulations, it demonstrated high (0.57 to 0.79) Fst statistics between Loriini and other subpopulations. This confirms that the BFDV lineage currently circulating in lorikeets is neither recipient nor donor for genetic material to and from other contemporary lineages of Australia. In contrast, the distribution of BFDV genomes in minor subpopulations under MSP-R was found to be homogenous with low degrees of gene flow and genetic admixture among them (Fig.2). For example, the BFDV genome infecting budgerigars formed independent subpopulation (demarcated as bright red bar plots) under tribe Melopsittacini but contributed genetic material to members of other minor subpopulations like Platycercini, Cacatuini_1, Ca-

lyptorhynchinae and Pezoporini. This suggests that the BFDV lineage circulating in budgerigars have potentially contributed substantial amounts of genetic material to other lineages with which they were co circulating and integrated genetic polymorphisms through recombination. However, tribe Calyptorhynchinae demonstrated that they got infection from BFDV genotypes infecting both Cacatuini_2 and Platycercini subpopulations. Similar events happened in 'Pezoporini' as at least two isolates; KF188691 and KF688551 possessed polymorphisms from distant subpopulations like Cacatuini_2 and Platycercini respectively. This intermixing of genetic material between different host groups demonstrates frequent cross transmission and generalized likelihood of infectivity under subpopulation MSP-R. The Fst statistics also reflected this phenomenon as Fst values dropped between highly admixed minor subpopulations (Fst=0.13 between Calyptorhynchinae and Cacatuini_1; 0.261 between Calyptorhynchinae and Platycercini) where cross transmissions were detected by STRUCTURE. Surprisingly, despite evidence of certain degree of genetic admixture, Fst values between many subpopulations were quite high which suggests that BFDV lineages evolved independently in those hosts.

***Contribution in BFDV recombination patterns***

Several detection methods implemented by RDP 4 software identified remarkably extensive number of recombinant genomes resulting from a large number of recombination events in the global BFDV population. Twenty one (A-U) recombinant events were statistically significant with at least three detection methods which influenced a total of 238 BDFV genome sequences worldwide. Most of the BFDV genomes infecting Australian lorikeets shared a single recombinant event with an ~880 bp recombinant fragment from an unknown donor genome (minor parents) overlapping intergenic and *Rep* gene regions where Australian budgerigars (tribe Melopsittacini) comprised the non-recombinant part (major parent). Except this, no other inter-lineage or extra population recombination events were detected in Australian lorikeet isolates. However, frequent intra-population recombination (n=11 affecting 29 isolates) was observed among different isolates. This result suggests a founder effect like spatial expansion of this particular lineage after a single introduction from an unknown source which is buffered by frequent within population recombination. Australian budgerigars (tribe Melopsittacini) on the other hand, exhibited an ancestral relationship with other budgerigar sequences all over the world and participated in several recombination events with Cacatuini and Platycercini tribes. This represents the frequent exposure of this host species with distant BFDV lineage from variety of psittacine birds.

Neighbor-Net analysis was also done to infer the evolutionary relationship among Australian BDFV genomes and to detect evidence of recombination. The BFDV genome sequences became distinctly separated into two networks; one comprising all isolates infecting lorikeets and another incorporates all other sequences (Fig. 2). This split of genome sequences eventually reflects the population subdivision inferred by STRUCTURE analysis and supports the existence of MSP-L and MSP-R. However, clear evidence of non-tree like evolution was revealed in both network indicated by reticular circles. This result demonstrates that the BDFV lineage circulating in Australian lorikeets evolving independently without inter-lineage/inter-population recombination.

***Spatial diffusion dynamics of BFDV in lorikeets***

Full genome sequence data supported two unidirectional spatial movement pathways of BFDV infection in lorikeets (Fig. 3). One pathway supports introduction of infection into New Caledonian coconut lorikeets from Thailand sometime near 2006 while another pathway demonstrated unidirectional diffusion of BFDV into Indonesia from Australia near 2004. However, the source of BFDV infection in Australian lorikeets remain obscure in absence of statistically (base factor) supported linkage by the full length genome. However, the *Rep* gene demonstrated sufficient statistical linkage between Australia and Thailand genotypes suggesting that sometime around 1972 the BFDV genome circulating in Australian lorikeets obtained its partial/entire *Rep* gene segment from an unknown host near Thailand.

***Selection Pressure analysis***

All four codon based analysis (SLAC, FEL, FUBAR and MEME) failed to demonstrate any consistent evidence of possible selection operating on any of the protein coding genes (*Rep* and *Cap*) of the BFDV genomes circulating in Australian lorikeets. However, higher proportion of sites was detectably evolved under negative selection than under positive selection (data not shown). For example, SLAC (the most conservative of fours tests applied) identified at least 2 sites evolving under negative selection pressure in *Rep* gene compared to 0 sites under positive selection. Similarly, in *Cap* gene no sites found selected positively/negatively by SLAC but few negatively selected sites were identified by FEL method. Nevertheless, episodic diversifying selection was evident by MEME in both genes. Branch site REL (BSR) analysis was conducted to identify episodic diversifying selection which also demonstrated overwhelming domination of purifying selection in almost every site in every branch of phylogenetic tree constructed from both *Rep* and *Cap* coding genes. This indicates that the observable polymorphisms in this population are transient and unique to individual sequences and that the population expansion has occurred relatively recently.

## Discussion

A focus of this research was to investigate the evolutionary pathways of BFDV in Australian budgerigars and lorikeets and to identify whether they impose potential threats to endangered host species. Strong intra-host divergences for tribe Loriini as Lories and lorikeets from different geographical location (Australia, New Caledonia and Indonesia) clustered together in a large monophyletic clade sharing common ancestry. However, BFDV genomes circulating in both Australian and New Caledonian lorikeets demonstrated a distinct population structure representing spatial divergence and founder-effect-like evolution acting on these host populations. Current understanding of circovirus evolution indicates that BFDV has its origins in its current hosts (psittacine birds) and has co-evolved with them since a post-Gondwanan period (Raidal et al., 2015). Phylogenetic evidence of diverse BFDV lineages circulating among Australian psittacine species in absence of deeper and definitely non-Australian-origin clades supports the post-Gondwanan origin of BFDV in Australia where the virus now exists as a pathogenic host-generalist capable of flexible host-switching amongst the psittacid avifauna (Sarker et al., 2014a). In this context, the host specific structure of BFDV among two geographically distant lorikeet populations (~3200 km) clearly demonstrate allopatric divergence following single introduction event from a single ancestral lineage.

Sequence based genetic structure analysis of Australian BFDV genomes demonstrated clear genetic segregation between BFDV in lorikeets and other host species where lorikeet isolates formed a distinct subpopulation (Fig.2) that does not share any polymorphism with genotypes circulating in other Australian psittacine hosts. In other words they represent a completely separate ancestry than all other BFDV genomes. This scenario should be reviewed with the evolutionary history of Psittaciformes birds in this region. Lories and lorikeets have evolved as specialized nectarivorous birds and are therefore physically distinguishable from their parrot counterparts (Sindel, 1987). While primary lineages of parrots and cockatoos most likely evolved in the supercontinent Gondwanaland during the Cretaceous (Cracraft, 1973; Rich, 1975; Smith, 1975) extant lorikeet genera, in contrast, are less differentiated and probably radiated more recently, springing from the Australo-Papuan platycercine parrots (Holyoak, 1973). Out of twelve different genera distributed throughout the southwest pacific region, Indonesia, New Guinea and Australia, three (*Trichoglossus*, *Psitteuteles* and *Glossopsitta*) represent Australian lorikeets. They have identical allelic constitutions (Christidis et al., 1991) and therefore can be considered as derivatives of a single lineage which entered Australia only recently from New Guinea. The speciation most likely happened after Miocene-Oligocene times (20-30 million years ago) when the New Guinean mainland was cut off from the Australian continental plate by the flooding of the Aure trough (Doutch, 1972; Pieters, 1982). Probably, during this period the ancestral BFDV lineage in lorikeets was separated from the main Australian lineage and became vicariant. Therefore, the distant BFDV subpopulation (MSP-L) in lorikeets might represent the reentrance of the primordial lineage that has been segregated from the mainland species for a very long time period.

As represented in Fig. 1 the overall BFDV genetic cluster infecting tribe Loriini from Australasia showed phylogenetic relationship with captive neotropical parrots (Red-shouldered macaw and Red-and-green macaw; GenBank accession. GU015018 and GU015021 respectively) collected from Thailand. It is conventionally accepted that the common ancestor of the parrot subfamily Arinae and Psittacinae lived in Antarctica and they became separated from the Australasian lineages when Antarctica began to split from Australia (Schweizer et al., 2010; Tavares et al., 2006). After that they colonized the neotropics and Africa giving rise to the Arinae and Psittacinae in the late Eocene or early Oligocene around 35 million years ago (Schweizer et al., 2011) before speciation of lorikeets. Therefore, it is possible that these hosts (Tribe Arini in our case), because of their separate habitat, retained some alleles of the primitive BFDV and did not undergo extensive evolutionary changes as appears to have happened across the Australian continent in parrots and cockatoos. The STRUCTURE plots (Fig.1) of these two genomes (GU015018 and GU015021) demonstrated that they share some polymorphic sites (coloured brown) with Australian lorikeet population, New Caledonian Deplanche's Lorikeet population (coloured yellow) and both colour in Indonesian Red lory (KF673337).

Other than a distant lorikeet subpopulation, the population structure analysis also revealed the existence of host dependent minor subpopulations within the Australian BFDV population (Fig.2). However, BFDV genomes within these minor subpopulations shared common polymorphic sites (as depicted by STRUCTURE plots) and frequent genetic admixture. This represents different co-circulating BFDV lineages which are continuously evolving through recombination. Not surprisingly, the budgerigar subpopulation shared genetic material into different BFDV lineage which suggests the pivotal role of budgerigar as a host for dispersal of BFDV infection into different host population. Cross species transmission and spill over events between hosts as a common attribute of BFDV infection (Peters et al., 2014) was also reflected in the BFDV genetic population structure as such instances reduced the Fst values indicating increased gene flow between corresponding subpopulations. Despite all these genetic admixture and host switch events AMOVA analysis supported existence of both major and minor subpopulations with high probability (<0.000) which stands for host based divergence of BFDV in Australian psittacine birds. However, this could be true in lorikeet population as they do not share any polymorphism and

participated in recombination or host switch events with any other host population. In fact, BFDV genomes infecting lorikeets created a separate sphere of intra-lineage recombination network apart from the larger intertwined network where all other host species underwent intra-host and inter-host recombination (Fig.3).

Several studies demonstrated that recombination has played an imperative role in evolutionary biology of BFDV (Julian et al., 2013; Sarker et al., 2014a) where close vicinity of reservoir host species like in aviaries in captivity and nesting hollows in wild intensified the trend of recombination. It is the multidirectional diffusion of BFDV lineages (Harkins et al., 2014) through pet bird trade and frequent inter-lineage recombination which has shaped the current face of global BFDV diversity. In this study, a 880 bp recombinant fragment (spaning from 5' end intergenic region to partial *Rep* gene) was found in BDFV genomes circulating in Australian lorikeets of which ancestry was unknown or unsampled. This could be due to lack of sampling from the population sequestering the primitive BFDV lineage as discussed before. Sampling in the lorikeet populations of south western pacific islands particularly from New Guinea and Torres straits is required to prove this. However, a predominant intra-lineage recombination pattern exists among the BFDV genomes infecting Australian lorikeets (Fig.4) which suggests very infrequent (if not zero) introduction of diverse BFDV lineages within these host species at least in the recent past. This could be achieved by complete isolation of hosts or transmission inability by the distant viral lineage. It is important to note here that, all lorikeet isolates in this study were wild caught birds sampled over a reasonably long period of time (2000 to 2013) and from different geographical location (Queensland, Victoria, New South Wales and Western Australia).

Lorikeets are highly mobile nomadic parrots that travel frequently a long distance in search of food resources (nectar and pollen) and abundantly found in suburban backyards and agricultural lands. Therefore, it is quite likely that they often come in close proximity of captive psittacine birds in aviaries and acquire BFDV infection from distant lineage. In Australia at least 48 bird species compete for breeding facilities in trees hollows for nesting and like many other psittacine birds rainbow lorikeets (*Trichoglossus haematodus*) are well known for occupying hollows used by other psittacine species. Like other circoviruses the BFDV virion is resilient and capable of remain structural viability in the environment of nest hollows for long periods (Raidal et al., 1993b). Oral or cloacal transmission and environmental persistence of BFDV, as well as the predisposition of young birds to become infected (Pass and Perry, 1984), suggests a potentially significant role of shared nest hollows in facilitating spillover, allowing abundant parrot species to act as reservoirs for BFDV infection. Such a situation has already been documented in Mauritius, where BFDV transmission occurs between invasive and abundant rose-ringed parakeets (*Psittacula krameri*) and the endemic, endangered Echo Parakeet (*Psittacula echo*) (Kundu et al., 2012). Taking all these into account, the absence of distant BFDV genotypes in Australian lorikeet population or even lack of gene flow between current BFDV lineage and rest of Australian BFDV population is quite surprising.

One explanation to this situation might lie in the physiology of lorikeets as they differ from parrots due to the morphological specialization of their alimentary tract to efficiently collect and digest nectar and pollen, including a modified gizzard musculature and a brush-tip tongue allowing them to harvest nectar rapidly (Collar, 1998; Richardson and Wooller, 1990). Their diet and anatomy may represent a key factor which might account for selective BFDV strain susceptibility. Such factors also might influence the clinical disease progression in lorikeets as they often do not present a classical syndrome as occurs in other hosts. Lorikeets might possess intrinsic barriers in which to resist transmission of distant BFDV genotypes. However, transmission studies are required to confirm this hypothesis.

Vertical transmission might be another force driving behind the formation of host specific population structure in lorikeets. But the role of vertical transmission in avian circovirus epidemiology is debatable (Duchatel et al., 2006; Rahaus et al., 2008) and when considered in broader terms of disease ecology, indicate BFDV to be a resource generalist. Flexible host switching is most likely facilitated by horizontal transmission and in the Australian context at least, this is most likely to occur in tree nest hollows where there is strong competition between Psittaciformes and other birds for reproductive opportunities (Heinsohn et al., 2003; Legge et al., 2004; Saunders et al., 1982)

The reconstructed spatial diffusion dynamics based on full length BFDV genomes failed to demonstrate any statistically supported migration pathways of BFDV lineage into Australian lorikeets (Fig.5) from any distant geographical location in near past. This result is comparable with that of recombination analysis demonstrating lack of sampling from its original source. However, unidirectional diffusion from Australian lorikeets into Moluccan red lory of Seram and from captive psittacine birds of Thailand into New Caledonian Deplanche's Lorikeet population were evident (Fig.5). BFDV genomes in lorikeets from New Caledonia theoretically should have a similar evolutionary history as Australian lorikeets. So a biogeographic linkage between captive psittacine birds of Thailand and New Caledonian Deplanche's rainbow lorikeets is surprising. There can be two possible explanation for this. Firstly, BFDV genomes were shown in birds (New Caledonian Deplanche's rainbow lorikeets) kept in captivity, an environment that could facilitate the spread of BFDV from a variety of host species with diverse BFDV

lineages. Recombination analysis demonstrating the introduction of genetic material from diverse host species and from various geographical locations supports this claim. Secondly, Thai BFDV genomes from diverse hosts (including Arini) retain polymorphisms that are characteristics of a primitive lorikeet lineage. Despite sharing an average ~91% nucleotide identity with the Australian lorikeet lineage no spatial diffusion pathway was supported statistically between New Caledonian lorikeets and Australian lorikeets which demonstrates that both lorikeet BFDV populations shared a common ancestry but evolved separately.

The present study demonstrated that the BFDV genomes currently circulating in Australian lorikeets are highly structured for host specific divergence and segregated from the host generalist BFDV lineages circulating in other psittacine species of Australia. Circumferential evidence suggests that they are the member of the BFDV lineage which most probably evolved outside of the Australian mainland and reemerged relatively recently and thereafter following demographic expansion in these nomadic birds. The predominant purifying selection pressure among the polymorphic sites throughout this lineage also represents the recent predisposition and rapid population expansion events. Nevertheless, phylogenetic and population genetics analysis did not support lorikeets as the distributor of BFDV to endangered species like orange bellied parrots. The physiology and behavioral biology of lorikeets might have played some role in such genetic segregation of lorikeet associated BFDV which should be tested by conducting transmission studies.

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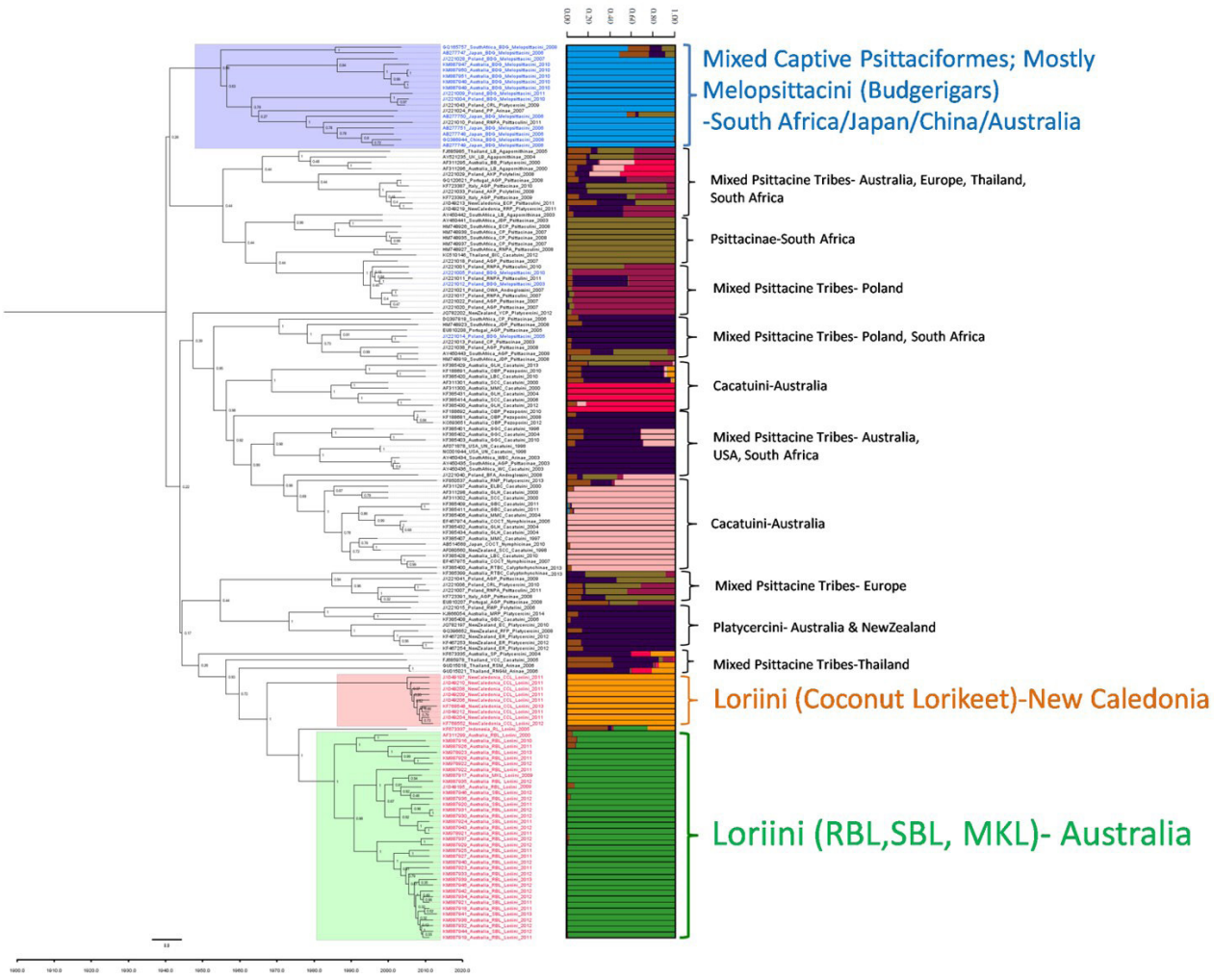


**Figure 1.** Left; Bayesian phylogenetic inference of evolutionary relationship among BFDV full genome sequences from Australian budgerigar and lorikeets in global context. Maximum clade credibility tree automatically rooted using a relaxed molecular clock model in Beast v1.7.5. Labels at branch tips refer to GenBank accession number with country name, species (abbreviation), taxonomic tribe of host and year of sampling. Clade posterior probability values are shown at tree nodes. Background shading highlights birds under same taxonomic tribe or geographical location. Red text highlights BFDV genomes from tribe 'loriini' (lorikeet species) and blue text denotes 'Melopsittacini' (budgerigars). Right; Genetic population structure of BFDV of Australian budgerigar and lorikeets in global context inferred by STRUCTUR v2.3.4. Individual genome showed by a horizontal bar plot with color coded according to the membership probability to a particular genetic subpopulation [STRUCTURE inferred nine genetic subpopulation (k=9) in global BFDV population, each represents a particular color]. Multiple colors within individual bars are indicative of admixture. Note distinct pattern of subpopulation clustering in BFDV created by Australian and New Caledonian lorikeets apparently without significant admixture while budgerigar genomes from different part of the world cluster together with significant admixture with distant subpopulations.

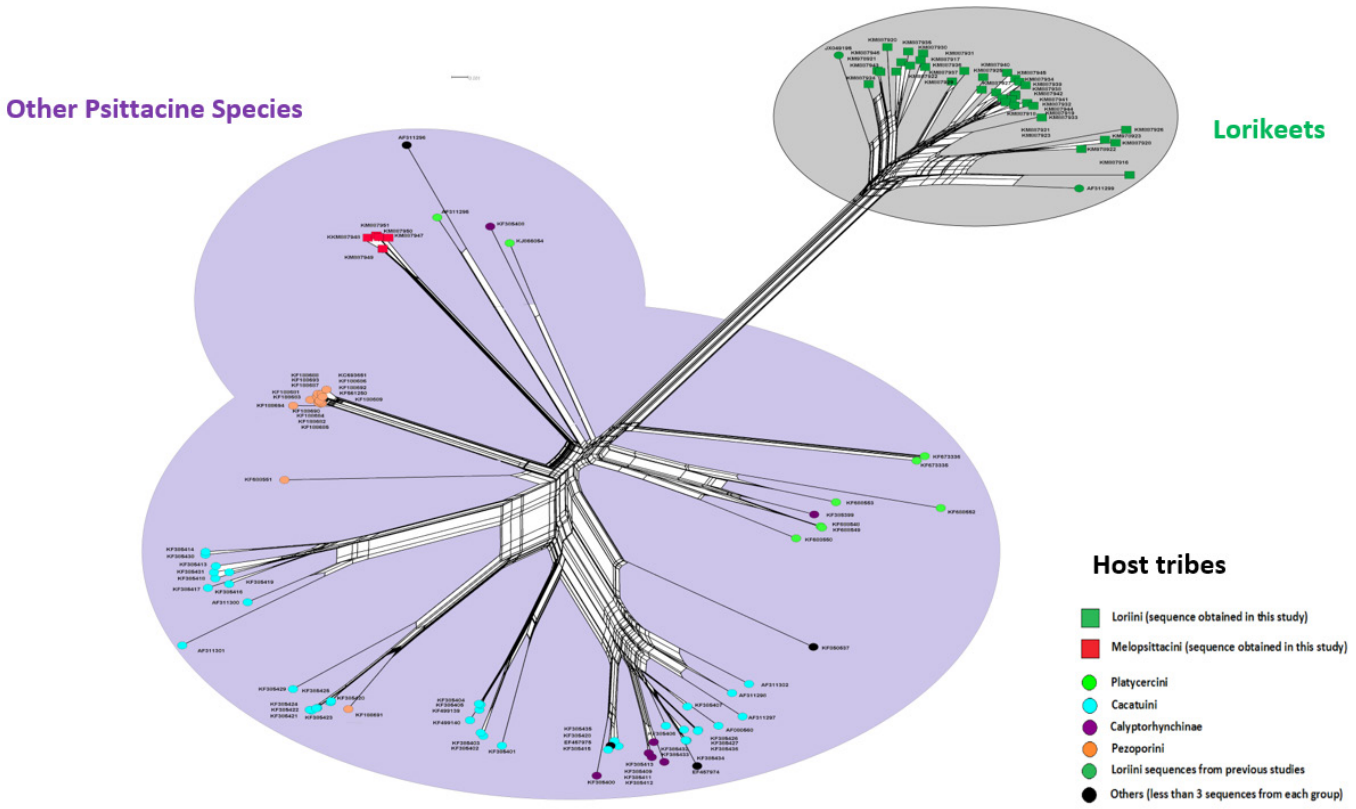


**Figure 2.** Neighbor-Net network analysis of Australian BFDV population. Result demonstrates phylogenetic evidence of separate recombination networks between BFDV genomes in lorikeets and other psittacine species. In both cases, formation of a reticular networks rather than a single bifurcated tree is suggestive of non-tree like evolution or recombination. The BFDV genomes were labeled with corresponding GenBank accession number and color coded according to the host's taxonomic tribe.

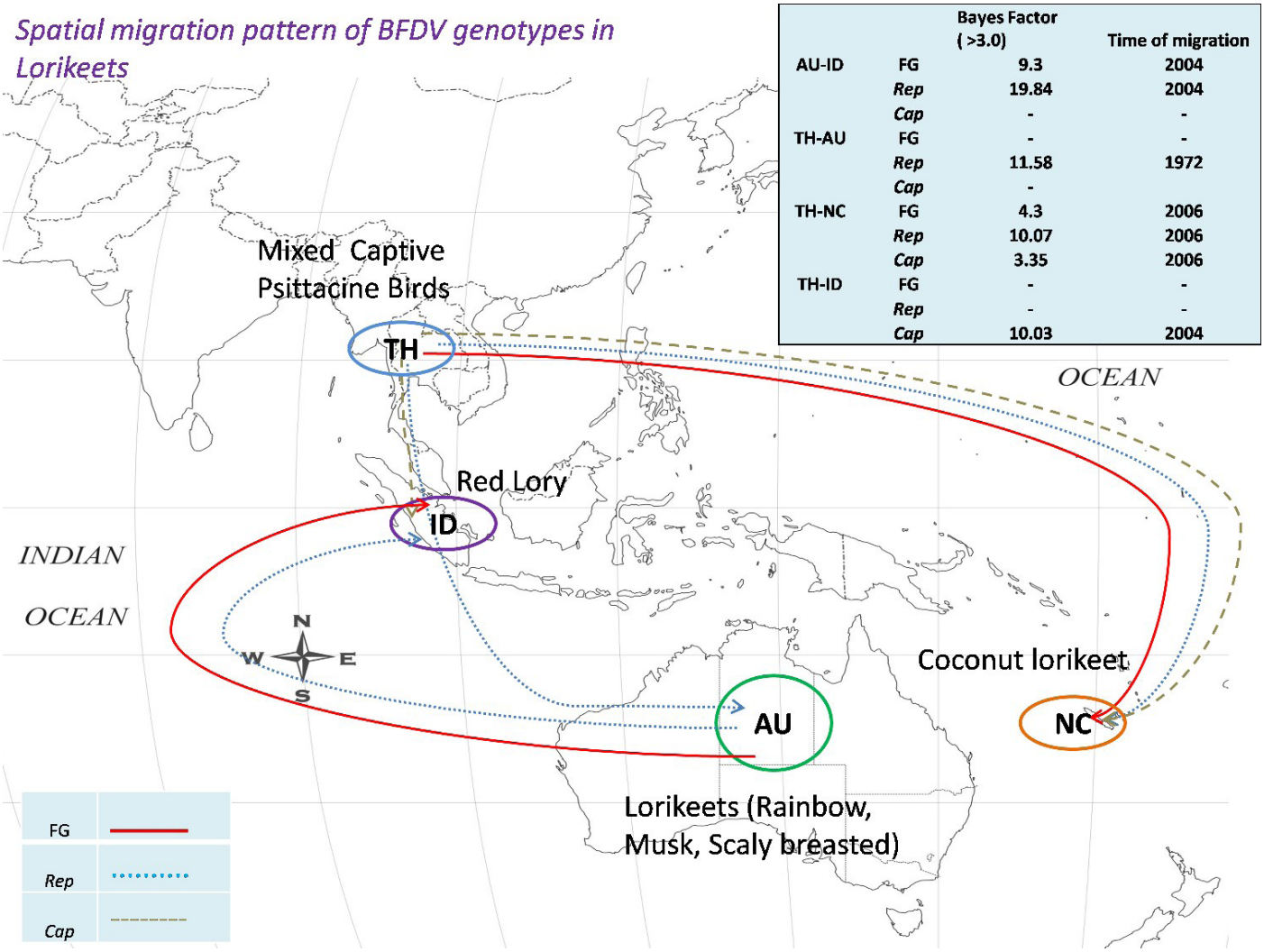


**Figure 3.** A graphical summary of the well-supported (BF>3.0) epidemiological linkage viral migration patterns inferred from the phylogeographic analysis of BFDV genomes in lorikeets. Countr1y names were represented as AU (Australia), ID (Indonesia), NC (New Caledonia) and TH (Thailand).