GENETIC ANALYSIS OF BEAK AND FEATHER DISEASE VIRUS ISOLATED FROM CAPTIVE PSITTACINE BIRDS IN THAILAND

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Abstract. Beak and feather disease virus (BFDV) is a causative agent of psittacine beak and feather disease. Genome sequences of BFDVs isolated from Thailand have not hitherto been reported. The whole genomes of 17 BFDV isolates, obtained from 12 psittacine genera, were amplified and subjected to direct sequencing revealing a length ranging from 1,990 to 2,015 nucleotides. The predicted open reading frames (ORFs) in the viral genome varied from four to six. Only ORF1, ORF2, and ORF5 were found in all isolates. Deduced amino acid sequences of BFDV ORF2 were used to construct a phylogenetic tree. The phylogram grouped BFDV into ten clusters, which showed either host species relationship or regional restriction. The Thai isolates, were grouped into three clusters, cluster I, II, and V. Cluster I and II showed restricted geographical region to Thailand, and cluster II also showed a close relationship with BFDV isolated from Australia. Cluster V demonstrated neither restricted region nor species specificity of birds. In this cluster, there was an insertion of 16 nucleotides at non coding region of all BFDV isolates. The genetic information obtained from this study can be used to help understand BFDV diversity and evolution in Thailand.

Keywords: beak and feather disease virus, genetic analysis, Thailand

INTRODUCTION

Psittacine beak and feather disease (PBFD), caused by beak and feather disease virus (BFDV), affects more than 60 different psittacine bird species, such as cockatoos, parrots, and lovebirds (Varsani *et al*, 2010). The clinical signs are very obvious with abnormal of feather and beak. However, some infected birds show subclinical signs which are difficult to diagnose (Fungwitaya *et al*, 2009). Thus, there is a requirement for laboratory diagnosis to confirm the disease. BFDV belongs to Circoviridae family (Ritchie *et al*, 1989). It is a non-enveloped virus with isometric symmetry and a particle diameter of 14-16 nm. The viral genome is an ambisense, circular, single-stranded DNA of approximately 1,992-2,018 nucleotides (nts) (Ritchie *et al*, 1989;

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Niagro *et al*, 1998; Bassami *et al*, 2001). Analysis of the replicative form of the virus predicts between three and seven open reading frames (ORFs), three of which are in virion strand and the rest in the replicative strand. The major ORFs of BFDV are ORF1 and ORF2. ORF1 encodes the putative replication associated protein, which is involved in the rolling circle replication, and ORF2 encodes the viral capsid protein (Bassami *et al*, 2001). There have many reports attempting to clarify the relationship of BFDV isolated from various host species and geographic regions. Some reports suggested that phylogenetic analysis of ORF1 and ORF2 of BFDVs indicates either geographical or host species specificity relationship (Bassami *et al*, 2001; de Kloet and de Kloet, 2004). Ritchie *et al* (2003) suggested that host species specificity occurs in BFDV isolated from New Zealand. Sequence analysis of BFDV isolated from Germany was able to separate BFDV causing acute infection from BFDV causing feather disorder suggesting the possible existence of BFDV genotypes (Raue *et al*, 2004).

Although, BFDV infected birds in Thailand have been detected (Banlunara *et al*, 2002) but nucleotide analysis and phylogenetic study of these viruses have not hitherto been reported. This study analyzed the nucleotide sequences of 17 BFDVs isolated from captive psittacine birds and the first report documenting the whole genome analysis of BFDVs in Thailand.

MATERIALS AND METHODS

Sample collection

Feathers and dried blood spot samples were collected from various psittacine birds (12 genera) during 2005-2006. Seventeen samples, BFDV positive by PCR, were analyzed. Details of the psittacine species and GenBank accession numbers are summarized in Table 1.

DNA isolation

DNA isolation from dry blood spots was performed as previously described (Sambrook *et al*, 1989). In brief, a few pieces of dry blood spot were transferred into lysis buffer solution (0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, 5% SDS), 0.6 mg/ml Proteinase K was added, and the sample was incubated at 56ºC for 4 hours. Subsequently, DNA was extracted using phenol: chloroform: isoamylalcohol (25:24:1 v/v/v) and precipitated with absolute ethanol and 3M sodium acetate. DNA was resuspended in Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and used as template for PCR. For feather bulb samples, DNA was isolated by rapid alkaline lysis method (Stoll *et al*, 1993). In brief, small pieces of feather bulbs were dissolved into 20 l of 0.2 M NaOH and incubated at 75ºC for 20 minutes. The solution was neutralized by adding 180 l of 0.04 M Tris-HCl, pH 7.5, and the supernatant used as template for PCR.

PCR amplification

Full length genome was constructed from two half-genome using 2 primer sets designed in this study. The first set, primers BFDV241f (5′ CTATGCCATCGTTGGACGG 3′) and BFDV1975r (5′ GCACCTCTAACTGCGCATGCG 3′) were used to amplify the up-stream half-genome. The second set, primers BFDV431f (5′ CTGGGCATTGTGGCGAGAG 3′) and BFDV908r (5′ GCTTCGGGTCACAGTCCTCCTTG 3′) were used to construct the remaining half-genome. PCR was performed in a thermocycler machine (PTC-200, MJ Research, USA) using expand high fidelity PCR system (Roche, Mannheim, Germany) according

to the manufacturer's instruction. The amplification was initiated at 94ºC for 2 minutes and followed by 35 cycles of 94ºC for 30 seconds, 52ºC for 30 seconds and 72ºC for 3 minutes. The DNA extension was continued at 72ºC for 5 minutes. Subsequently, PCR products were purified using gel extraction kit (Qiagen, Hilden, Germany).

Phylogenetic tree construction

Direct sequencing of the PCR fragments was determined using BigDye® terminator v3.1 cycle sequencing kit (Applied Biosystem, Carlsbad, CA) according to an instruction from the supplier. The sequencing reactions were performed by 25 cycles of 94ºC for 30 seconds, 50ºC for 15 seconds, and 60ºC for 4 minutes. Thereafter, the reactions were precipitated with absolute ethanol and 3M sodium acetate and subjected to Bio Basic (Amherst, NY) to read the sequences. Each fragment was assembled by BioEdit program (version 7.0.5). The complete nucleotide sequences of all isolates were converted to predict amino acid sequences of the major ORFs (BioEdit program) and were aligned with published capsid protein sequences using ClustalX program (version 1.8). Rooted phylogenetic tree construction was performed by dnadist, neighbor joining, and seqboot program implemented in PHYLIP package (PHYLIP 3.65). Chicken anemia virus (DQ991394), Columbid circovirus (GQ844278), and Goose circovirus (AY633653) were used as an out-group. Bootstrap values were calculated using the seqboot with bootstrap value of 1,000 replicates.

RESULTS

Nucleotide sequence analysis of Thai BFDV isolates

The whole nucleotide sequence similarity among Thai BFDV isolates varied from 91-100% identity. The genome sizes of BFDVs ranged from 1,990 to 2,015 nts. Most of BFDV genomes (12 of 17 isolates) have genome size of 1,990 nts. Nucleotide sequence analysis found insertions and deletions. For instance, the sequence of FJ685985 showed an insertion of 16 nts, GACCACTTGAAAAAAT, at a non coding region (nucleotide position 1,201 to 1,218; data not shown). The predicted sizes of each ORF were shown in Table 2. All isolates contained ORF1, ORF2 and ORF5. ORF1, ORF4 and ORF5 were located on the virion strand. ORF2, ORF3, ORF6 and ORF7 were located on the replicative strand. A potential stem-loop structure, containing the origin of rolling circle replication or nonanucleotide motif (TAGTATTAC), located between ORF1 and ORF2 of the virus genome, was conserved in all isolates. Downstream of stem loop was a second hairpin structure, which may serve as a binding site for the *rep* gene production. The size of ORF1 of all isolates was 867 nts, consisting of ATG as start codon. ORF1 of almost all isolates terminated at TGA, except for FJ685985 that was TAG. The size of ORF2 of all isolates was 732 nts and the stop codon was TAA. Twelve isolates including GU15012, GU15013, GU15014, GU15015, GU15016, GU15017, GU15018, GU15019, GU15020, GU15021, GU15022, and GU15023, had CCT (proline) as the start codon of ORF2, whereas the rest (FJ685978, FJ685979, FJ685980, FJ685985, and FJ685989) was TCT (serine). For ORF5, most of all isolates has a size of 708 nts except FJ685979, FJ685985 and FJ685989 with size of 309, 468, and 303, respectively. Isolate FJ685978 and FJ685980 have the size of ORF5 of 504 nts. The start codon of ORF5 was ATG in all isolates and the stop codon was TGA except for FJ685979 and FJ685989 that was

Table 1
BFDVs examined in the study.

GenBank accession no.	Host species		Country
	Scientific name	Common name	
GU015012	*Psittacus erithacus*	Grey parrot	Thailand
GU015013	*Psittacus erithacus*	Grey parrot	Thailand
GU015014	*Psittacula eupatria*	Alexandrine parakeet	Thailand
GU015015	*Psittacula eupatria*	Alexandrine parakeet	Thailand
GU015016	*Psittacula eupatria*	Alexandrine parakeet	Thailand
FJ685978	*Cacatua sulphurea*	Lesser Sulphur-crested cockatoo	Thailand
FJ685979	*Cacatua sulphurea*	Lesser Sulphur-crested cockatoo	Thailand
FJ685980	*Ara ararauna*	Blue Gold macaw	Thailand
GU015017	*Ara severa*	Chestnut-fronted macaw	Thailand
GU015018	*Ara nobilis*	Red-shouldered macaw	Thailand
GU015019	*Eclectus roratus*	Eclectus parrot	Thailand
GU015020	*Eclectus roratus*	Eclectus parrot	Thailand
FJ685985	*Agapornis* spp	Lovebird	Thailand
GU015021	*Ara chloropterus*	Green-winged macaw	Thailand
GU015022	*Probosciger aterrimus goliath*	Black cockatoo	Thailand
GU015023	*Ara ambigua*	Buffon's macaw	Thailand
FJ685989	*Cacatua moluccensis*	Salmon-crested cockatoo	Thailand

Table 2
Predicted ORFs of BFDVs obtained from the study.

GenBank accession no.	Genome length (nts)	Total ORFs	ORF1	ORF2	ORF3	ORF4	ORF5	ORF6	ORF7
GU015012	1,990	6	867	732	-	336	708	300	381
GU015013	1,990	6	867	732	-	336	708	300	381
GU015014	1,990	6	867	732	-	336	708	300	381
GU015015	1,990	6	867	732	-	336	708	300	381
GU015016	1,990	6	867	732	-	336	708	300	381
FJ685978	1,995	6	867	732	492	336	504	300	-
FJ685979	1,993	6	867	732	540	372	309	-	324
FJ685980	1,995	6	867	732	492	336	504	300	-
GU015017	1,990	6	867	732	-	336	708	300	381
GU015018	1,990	6	867	732	-	336	708	300	381
GU015019	1,990	6	867	732	-	336	708	300	381
GU015020	1,990	6	867	732	-	336	708	300	381
FJ685985	2,015	4	867	732	495	-	468	-	-
GU015021	1,990	6	867	732	-	336	708	300	381
GU015022	1,990	6	867	732	-	336	708	300	381
GU015023	1,990	6	867	732	-	336	708	300	381
FJ685989	1,994	6	867	732	492	372	303	-	306

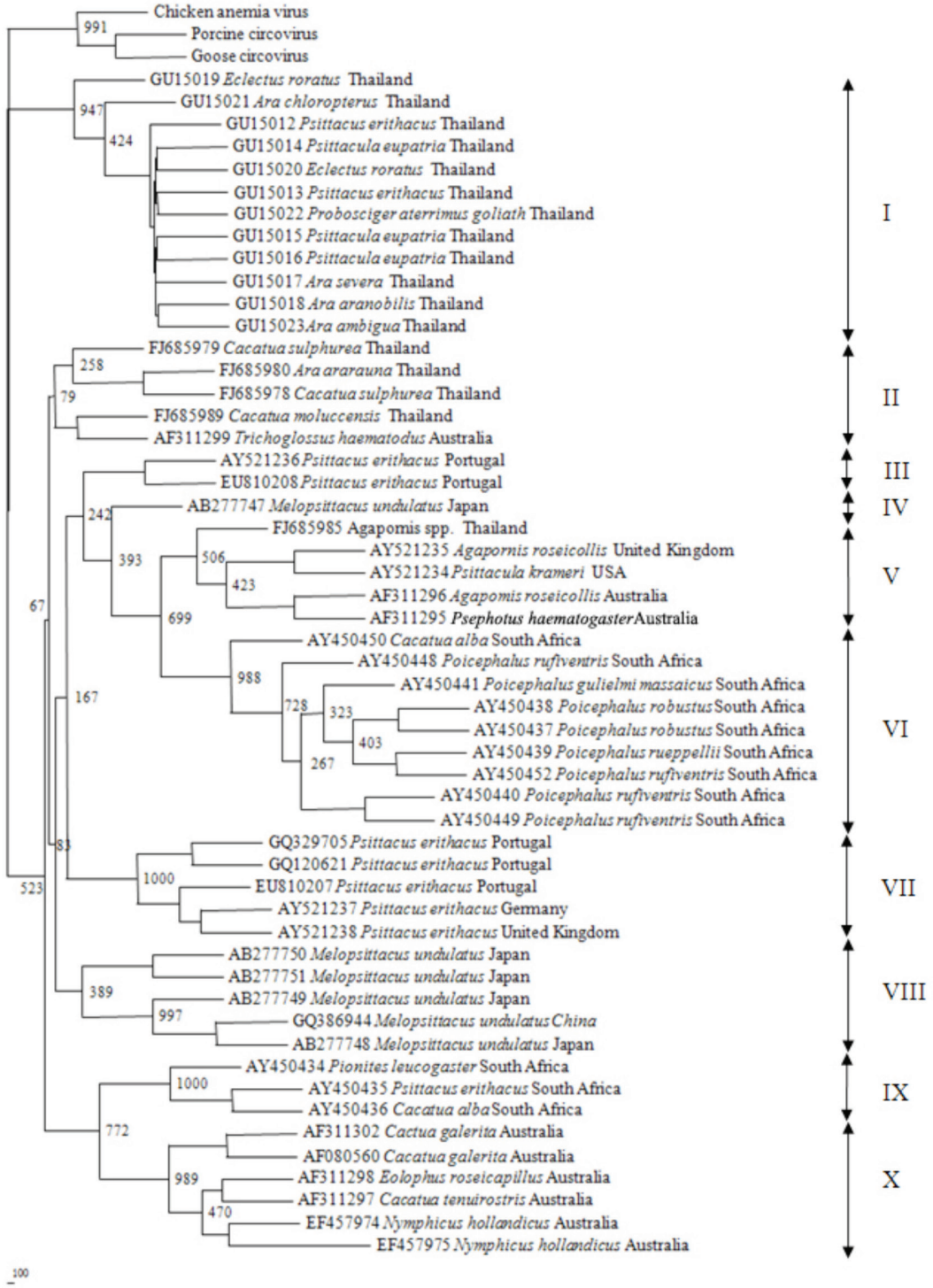

Fig 1–Phylogenetic tree generated from deduced amino acid sequences of BFDV ORF2. The number at the node indicates the bootstrap values. Genetic clusters are shown in Roman numerals.

TAA. Only 5 isolates, FJ685978, FJ685979, FJ685980, FJ685985, and FJ685989, contained ORF3. Initiation of translation of ORF3 of FJ685978, FJ685980, and FJ685989 isolates was ATG and terminates at TAG. For FJ685979 and FJ685985 isolates, the initiation and terminated codon were ATG and TAA, respectively. All isolates contained ORF4, except FJ685985 and used ATG as initiation of translation. Unlike most isolates which have the size of ORF4 of 336 nts, isolate FJ58979 and FJ685989 had 372 nts in size of ORF4. Fourteen isolates showed ORF6 and 3 isolates (FJ685979, FJ685985, and FJ685989) were lacking ORF6. The start and stop codon of ORF6 was CTG and TAG, respectively. Fourteen of 17 isolates contained OFR7, with start and stop codon of ATG and TAA, respectively, except ORF7 stop codon of FJ685989 was TGA.

Phylogenetic analysis of ORF2

To protect errors of sequence analysis, this report used deduced amino acid sequences to construct phylogenetic tree. The deduced amino acid sequences of ORF2 of 17 Thai BFDVs investigated in this study together with the deduced amino acid sequences of ORF2 of 43 sequences from different geographic regions previously published in GenBank were used to construct the phylogenetic tree (Fig 1). From the phylogram, the BFDV isolates were grouped into 10 genetic clusters. Seven of 10 clusters showed restricted geographic region including cluster I, II, III, IV, VI, IX, and X. Four of 10 clusters were related to the host of isolated BFDV (cluster III, IV, VII, and VIII). Only cluster III and IV showed both restricted regional geography and species specificity of birds. Cluster V showed neither restricted regional geography nor host species specificity. Thai BFDV isolates were restricted to three clusters: cluster I, II, and V.

DISCUSSION

From the entire sequence analysis, we found 1-3 nts insertion or/and deletion in some sequences. FJ68598 isolated from *Agapornis* spp showed an insertion of 16 nts which was also found in AY521234, AY521235, AF311295, and AF311296. This insertion sequence may not be involved in virus survival as it appeared in non-coding region. Based on amino acid sequences of ORF2, these isolates were grouped in the cluster V. Up to six ORFs were detected in Thai isolates, but only 3 ORFs, ORF1, ORF2, and ORF5 were able to be found in all 17 isolates. The start codon of all isolates was ATG except in ORF2 and ORF6. Bassami *et al* (1998) was reported ATG at position 1,029 as start codon of ORF6. However, at this position, we could not find ATG codon in virus sequences analyzed in this study but found ATT codon. Downstream of position 1,029, the alternative initiation codon CTG could be detected at position 1,065. The deduced amino acid sequence of the ORF6 investigated in this study was 75-82% identical to AF080560 previously identified (Bassami *et al*, 1998). For ORF2, the initiation codon ATG was undetected in all Thai isolates. Five isolates had an alternative initiation codon of ORF2 of TCT, which is similar to a previous report (Niagro *et al*, 1998). However, 12 isolates have no alternative initiation codon. These isolates have amino acid codon CCT located downstream of the KOZAK element (CCCGCCGCC), a consensus sequence in the 5′ non-coding sequence, believed to be involved in the initiation of translation in eukaryote (Kozak, 1987). This codon also has been reported as initiation codon in ORF2 of an aphid-infecting virus, *Rhopalosiphum padi* virus (Domier *et al*, 2000). However, this possibility should be confirmed by

sequencing mRNA of the BFDV capsid protein.

Several reports showed low incidence of an intra-individual sequence variation (Bassami *et al*, 2001; de Kloet and de Kloet, 2004). However, to protect errors of sequence analysis, this report used deduced amino acid sequences to construct phylogenetic tree. The predicted amino acid sequence of ORF2 can group BFDV into ten clusters. Cluster III, IV, VII, and VIII showed psittacine species relationship as previously reported (Ritchie *et al*, 2003). Cluster I, II, III, IV, VI, IX, and X revealed restricted regional geography. Cluster VI and IX isolated from southern Africa, had similar grouping as previously reported (Heath *et al*, 2004). The Thai BFDVs were grouped into clusters I, II, and V. Cluster I and II predominately consisted of Thailand isolates. However, in cluster II, the viruses isolated from Thailand were closely related to viruses isolated from Australia. These virus isolates may come from exotic birds imported from Australia.

In summary, this study showed that BFDVs may circulate and be restricted to only Thailand. However, movement of birds across countries may distribute the disease into new areas so that BFDV screening is important to protect the export and import markets of birds. Virus distribution from one site to other site may also result in genetic recombination that could generate new genotypes.

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