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Phylogenetic Analysis of Pigeon Paramyxoviruses Type-1 Identified in Mourning Collared-doves (*Streptopelia decipiens*) in Namibia, Africa

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ABSTRACT: We generated the complete sequence of the fusion (*F*) protein gene from six pigeon paramyxoviruses type 1 (PPMV-1) isolated from Mourning Collared-doves (*Streptopelia decipiens*) in Namibia, Africa between 2016 and 2017. All of the isolates had an *F* gene cleavage site motif of ^{112}RRQKRF^{117} characteristic of virulent viruses. A phylogenetic analysis using the full *F* gene sequence revealed that the viruses belonged to genotype VIa and were epidemiologically related to PPMV-1s from Asia, Europe, and North America.

Key words: Mourning Collared-doves, Namibia, phylogenetic analysis, pigeon paramyxovirus type-1, *Streptopelia decipiens*, subgenotype VIa.

Pigeon paramyxovirus type 1 (PPMV-1) is a nonsegmented, negative, single-stranded RNA virus that is a member of the family *Paramyxoviridae*, genus *Avulavirus* (Pestka et al. 2014). An antigenic and host variant of avian paramyovirus type 1 (APMV-1), also known as Newcastle disease virus, is one of the most important infectious diseases of poultry globally (Alexander 2000). Pigeon paramyxoviruses type 1 occurs in a number of wild bird species, including feral doves, kestrels, falcons, pheasants, robins, swans, woodpeckers, and African hornbills (Monne et al. 2006; Abolnik et al. 2008; Obanda et al. 2016; Wang et al. 2017). A global panzoonotic of PPMV-1 in 1983 (Alexander and Parsons 1984; Aldous et al. 2004) started among racing and show pigeons through cross-species transmission from Galliformes to Columbiformes (Chong et al. 2013). Transmission of PPMV-1 to poultry populations continues, making the surveillance and monitoring of PPMV-1 circulation an essential part of a country's prevention program against Newcastle disease (ND; Pestka et al. 2014).

A number of classification systems have been proposed for APMV-1 based on the phylogenetic analysis of partial or complete nucleotide sequences of the fusion (*F*) gene (Aldous et al. 2003; Czeglédi et al. 2006; Diel et al. 2012). There are two recognized classes, I and II, along with several genetic groups (lineages or genotypes; Aldous et al. 2003). In the most recent classification system, Class I viruses contain a single genotype, whereas Class II viruses can be divided into eighteen genotypes (Snoeck et al. 2009; Diel et al. 2012; Snoeck et al. 2013). The PPMV-1 belongs to genotype VI and can be further subdivided into nine subgenotypes, namely VIa, VIb, VIc, VIe, VIf, VIg, VIh, VIi, and VIj (Guo et al. 2013; Pchelkina et al. 2013; Wang et al. 2015; Ganar et al. 2017).

There is limited information available on the circulation of PPMV-1 in Africa. The virus has only been described and genetically characterized in Ethiopia (Damena et al. 2016), Kenya (Obanda et al. 2016), Nigeria (Snoeck et al. 2013), and South Africa (Abolnik et al. 2008).

Between July and November 2016, Namibia reported twelve separate outbreaks of ND in the north of the country along the Angolan border (Molini et al. 2017). As part of the control and prevention program implemented by the Namibian government, samples were collected and submitted to the Central Veterinary Laboratory in Windhoek. These samples included five dead (four feral and one captive) Mourning Collared-doves (*Streptopelia decipiens*) found in the same urban localities as the ND outbreaks. The feral birds were found dead or dying in the gardens of private houses. Residents reported the deaths of between 20–30 doves during the week prior

TABLE 1. GenBank accession numbers for the *F* gene sequences, dates of collection and locations of six pigeon paramyxoviruses type 1 isolated from Mourning Collared-doves (*Streptopelia decipiens*) in Namibia between 2016 and 2017.

GenBank no.	Location	GPS	Date	Bird status
MF681712	Grootfontein	19°34′S, 18°7′E	29 July 2016	Feral
MF681713	Windhoek	22°34′12″S, 17°5′1″E	16 November 2016	Feral
MF681714	Grootfontein	19°34′S, 18°7′E	18 November 2016	Captive
MF681715	Okahandja	21°59′S, 16°55′E	05 December 2016	Feral
MF681716	Windhoek	22°34′12″S, 17°5′1″E	12 December 2016	Feral
MF681711	Okahandja	21°59′S, 16°55′E	27 July 2017	Feral

to the samples being collected. The captive dove was one of 10 birds (from a flock of 60 birds) that died over a period of 30 d. No significant mortalities of other avian species were reported during the same period. The doves had shown signs of illness prior to death that included weakness, greenish-watery diarrhea, twisting of the neck, and respiratory distress (i.e., gasping and coughing). In addition to the samples collected in 2016, a PPMV-1 sample collected in July 2017 from another feral Mourning Collared-dove was included in the study (Table 1).

Samples were processed as previously described (Molini et al. 2017). Briefly, RNA was extracted from the spleen of the birds using the Maxwell®16 LEV SimplyRNA Tissue Purification Kit (Promega, Madison, Wisconsin, USA). The matrix gene of Newcastle disease virus was detected by One-Step RT-PCR using the commercial genesig® Advanced Kit for ND (Primerdesign Ltd, Chandler's Ford, UK). For further characterization of the viruses, the full *F* gene (1,662 base pairs [bp]) was amplified using the One-Step RT-PCR kit (Qiagen, Hilden, Germany) and the following four primer pairs: (Pair 1) Nam-PPMV1F (5′-CCATCGCCAAGTACAATCCC-3′) and Nam-PPMV1R (5′-CCTCCTTCCTCCTGATGTGG-3′), (Pair 2) Nam-PPMV2F (5′-CCCAAGGACAAAGAGGCATG-3′) and Nam-PPMV2R (5′-AAGTAATCCATGTTGCCGCC-3′), (Pair 3) Nam-PPMV3F (5′-GTTCGGGCCACAAATCACTT-3′) and Nam-PPMV3R (5′-TGCATCAAACTCCCCACTGA-3′), (Pair 4) Nam-PPMV4F (5′-CAAAGGCTCAGTCATTGCCA-3′) and Nam-PPMV4R (5′-CGACCGTTC TACCCGTGTAT-3′).

The following thermal profile was used for each primer pair: reverse transcription at 50 C for 30 min, initial denaturation at 94 C for 15 min and then 35 cycles of denaturation at 95 C for 30 s, annealing at 59.5 C for 1 min and elongation at 68 C for 1 min, followed by a final elongation at 68 C for 7 min. The expected amplified fragments of 530 bp, 539 bp, 667 bp, and 636 bp for primer pairs 1, 2, 3, and 4 respectively, were visualized on 1.5% to 2% agarose gels.

Positive RT-PCR amplicons were purified using a QIAquick PCR purification Kit (Qiagen) and were sent to LGC Genomics (Berlin, Germany) for sequencing. The Staden Package (Bonfield et al. 1995) was used to assemble the generated sequences. All sequences generated were deposited in GenBank under accession numbers MF681711 to MF681716 (Table 1). Multiple sequence alignment was performed using MUSCLE (Robert 2004) with default settings, incorporating all the sequences generated here combined with a selection of representative sequences available in GenBank (Figs. 1, 2). A phylogenetic tree was estimated using the maximum likelihood (ML) method available in MEGA 6 (Tamura et al. 2013), employing the Kimura two-parameter model of nucleotide substitution and 500 bootstrap replications. All of the isolates had an *F* gene cleavage site motif of ^{112}RRQKRF^{117} characteristic of virulent viruses, although in vivo pathogenicity tests are needed for confirmation

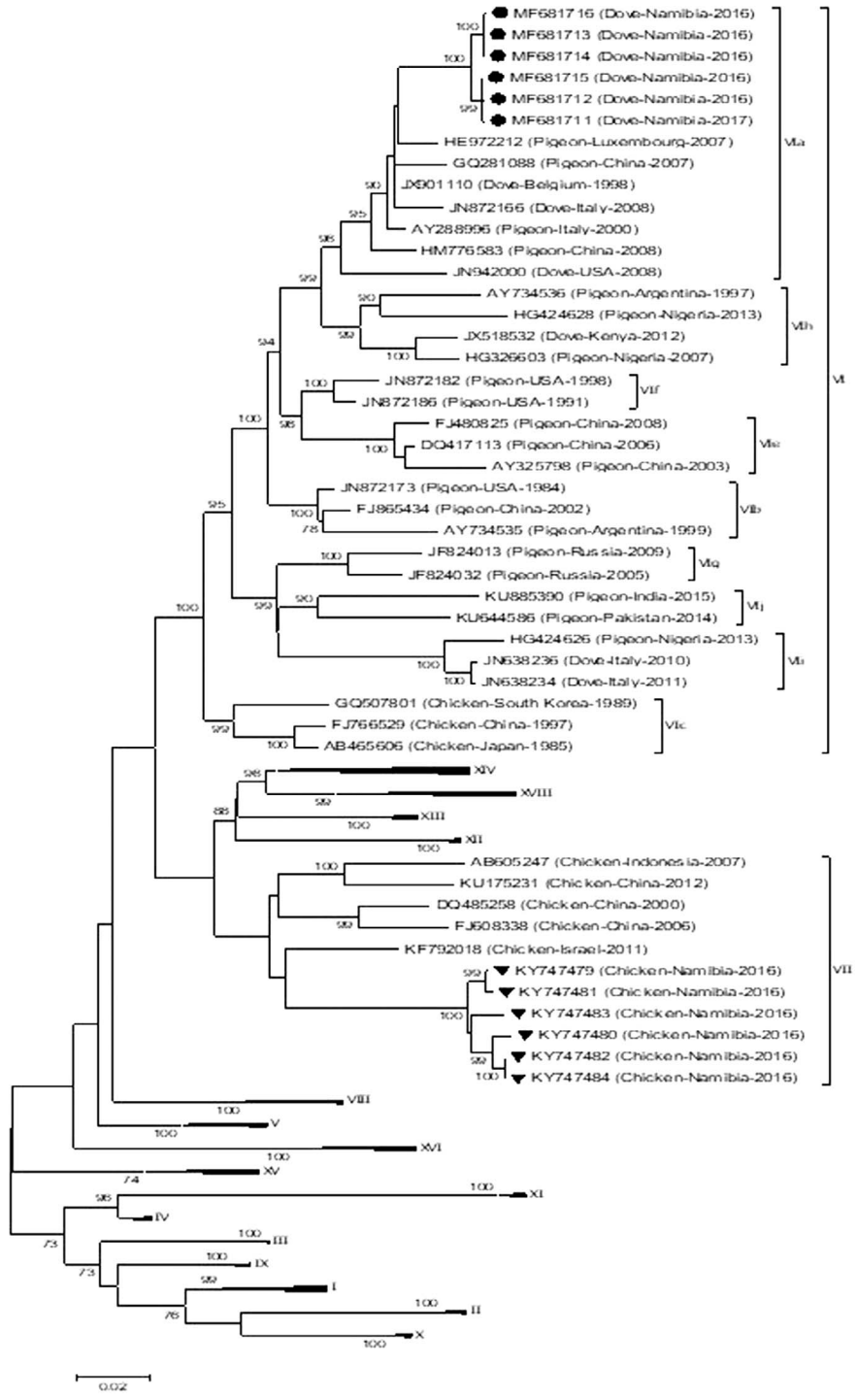

FIGURE 1. Maximum likelihood analysis using the MEGA6 software (Tamura et al. 2013) of the full *F* gene nucleotide sequence (1,662 base pairs) from six pigeon paramyxoviruses type 1 positive samples from Namibia (filled circles). The Kimura two-parameter model of nucleotide substitution was utilized. The numbers indicate the bootstrap values calculated from 500 bootstrap replicates. The different genotypes are numbered according to Diel et al. (2012). The recently characterized Newcastle disease viruses from Namibia (Molini et al. 2017) are also highlighted (filled triangles).

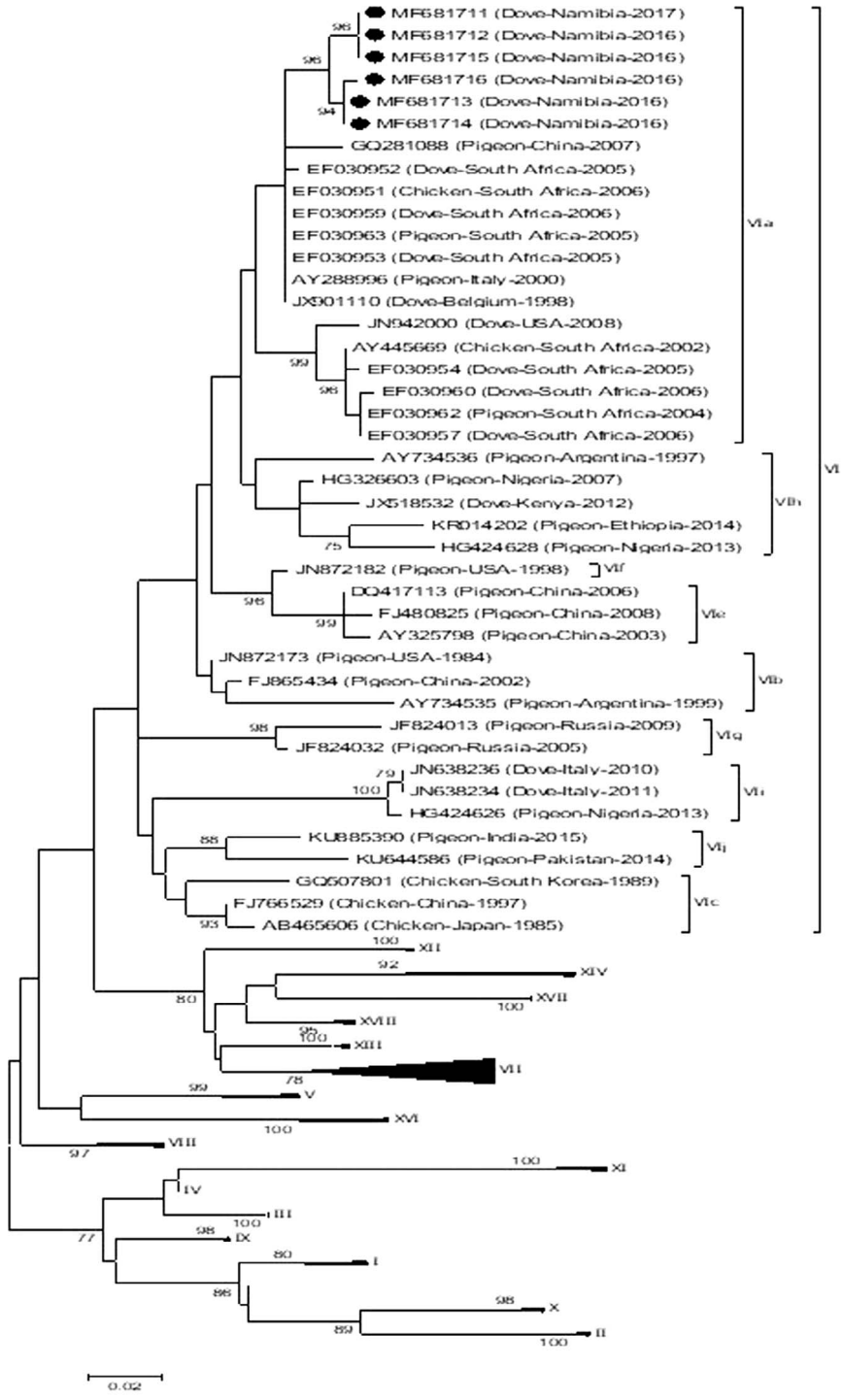

FIGURE 2. Maximum likelihood analysis using the MEGA6 software (Tamura et al. 2013) of a partial (259 base pairs) *F* gene nucleotide sequence from six pigeon paramyxoviruses type 1 positive samples from Namibia (filled circles). The Kimura two-parameter model of nucleotide substitution was utilized. The numbers indicate the bootstrap values calculated from 500 bootstrap replicates. The different genotypes are numbered according to Diel et al. (2012).

The ML analysis (Fig. 1) showed that the analyzed sequences grouped with genotype VI viruses. The six PPMV-1s from Namibia clustered together with VIa viruses from Asia, North America, and Europe. They did not cluster with viruses (both of the subgenotype VIh) from Nigeria or Kenya, the only African countries that have full *F* gene sequences available for analysis. In addition, the Namibian PPMV-1s were not epidemiologically related to the Newcastle disease viruses identified as the cause of poultry outbreaks in Namibia in 2016 (Fig. 1).

The PPMV-1s isolated from doves, pigeons, and chickens in South Africa in 2001 to 2006 (Abolnik et al. 2008) and in Ethiopia in 2012 to 2014 (Damena et al. 2016) could not be compared phylogenetically to the PPMV-1s from our study using the criteria defined by Diel et al. (2012) because only a partial *F* gene sequence is available for these earlier isolates in GenBank. To determine their relationships with other African PPMV-1, a second phylogenetic tree was estimated using ML and a 259 bp *F* gene segment from the same viruses used in the first phylogenetic tree but allowing for the inclusion of the earlier isolates from South Africa and Namibia (Abolnik et al. 2008; Damena et al. 2016). The tree generated using these shorter sequences was similar to the one using the full gene sequence (Fig. 2) but, in addition, it revealed that the Namibian and South African PPMV-1s both clustered in subgenotype VIa. Thus, the Namibian and South African viruses were epidemiologically related to one another and to PPMV-1s from Asia, Europe, and North America, confirming the global spread of subgenotype VIa PPMV-1s. The PPMV-1 strains continue to threaten wild avian species in the southern African region (Abolnik et al. 2008). This, and the fact that two of the South African PPMV-1s (EF030951 and AY445669; Fig. 2) that were epidemiologically related to the Namibian isolates were isolated from chickens, justifies the continued surveillance and monitoring of PPMV-1 in Namibia.

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