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Meta-transcriptomic analysis of the virome and microbiome of the invasive Indian myna (*Acridotheres tristis*) in Australia

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ABSTRACT

Invasive species exert a serious impact on native fauna and flora and have become the target of eradication and management efforts worldwide. Invasive avian species can also be important pathogen reservoirs, although their viromes and microbiomes have rarely been studied. As one of the top 100 invasive pest species globally, the expansion of Indian mynas (*Acridotheres tristis*) into peri-urban and rural environments, in conjunction with increasing free-ranging avian agricultural practices, may increase the risk of microbial pathogens jumping species boundaries. Herein, we used a meta-transcriptomic approach to explore the microbes present in brain, liver and large intestine of 16 invasive Indian myna birds in Sydney, Australia. From this, we discovered seven novel viruses from the families *Adenoviridae, Caliciviridae, Flaviviridae, Parvoviridae* and *Picornaviridae*. Interestingly, each of the novel viruses identified shared less than 80% genomic similarity with their closest relatives from other avian species, indicative of a lack of detectable virus transmission between invasive mynas to native or domestic species. Of note, we also identified two coccidian protozoa, *Isospora superbusi* and *Isospora greineri*, from the liver and gut tissues of mynas. Overall, these data demonstrate that invasive mynas can harbor a diversity of viruses and other microorganisms such that ongoing pathogen surveillance in this species is warranted.

1. Introduction

Originally native to southeast Asia, the Indian (common) myna bird (*Acridotheres tristis*) was introduced into Australia to control insects in market gardens during the late 1800s following the expansion of agriculture and urban development. These animals are ominivorous, capable of exploiting diverse landscapes and are aggressive to other birds and small mammals, driving their rapid spread throughout Australia and abroad. As a consequence mynas are now classified as one of the top 100 invasive pest species globally [1]. Many invasive species are highly adaptable, competing for food, roosting and nesting resources with native Australian species [2]. Since their population is now widely distributed and rapidly expanding both in Australia and globally, the ecological significance of invasive mynas in Australia makes them attractive to study from the perspective of population dynamics, biology, aggressive behavior, and as potential vectors for known and novel infectious agents. For example, the Indian myna will spread the seeds of agricultural pest weeds and damage ripening fruits [3]. Invasive mynas have also shown a capacity to carry pathogens such as *Trichomonas gallinae* and Haemosporidian parasites (*Plasmodium* and related *Haemoproteus* spp.), [4] *Salmonella* sp. and mites [5]. Although a previous study detected avian siadenovirus [6] and low pathogenic avian influenza [7] in free-living mynas, little is known about the clinical and zoonotic risks of any viruses these animals may carry.

Invasive and native birds can function as reservoirs for a variety of important human and veterinary pathogens, such as members of the family *Flaviviridae* (positive-sense RNA viruses), including avian-associated flaviviruses that infect domestic animals and cause considerable socio-economic impacts on public health and the agricultural industries [8]. Notably, two related genera of the *Flaviviridae* - hepaciviruses and pegiviruses - have been detected in an increasingly wide array of species, including Australian wildlife, providing a new perspective on the diversity of this important group of viruses [9]. For example, pegiviruses have been identified in a variety of mammalian hosts, including human and non-human primates, bats, horses, rodents, cetaceans [10] and more recently geese [11] and felines [12]. Infections

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Table 1
Detailed information on the RNA-seq libraries generated in this study. The library ID, number of animals, host tissue, total trimmed reads and read abundance of each library are presented. The virus read abundance of each family and a host gene marker (RPS7) are presented as Reads per Million (RPM).

Library ID	Myna 1 L -VERT70	Myna 2 L -VERT79	Myna 1B -VERT80	Myna 2B -VERT69	Myna 1G -VERT71	Myna 2G - VERT78
No. of animals	8	8	8	8	8	8
Tissue	Liver	Liver	Brain	Brain	Gut	Gut
Total reads	62,477,255	89,923,130	103,780,074	70,380,196	64,848,217	93,735,914
Flaviviridae	44.00	49.82	0.87	1.25	0.30	34.22
Caliciviridae	0.19	0.31	0	0	0	0.54
Picornaviridae	0.64	44.99	0.019	0.057	0.21	2.44
Adenoviridae	0	0	0	104.39	0.40	10.42
Parvoviridae	14.8	0	0	0	0	4.99
RPS7 (host)	379.2	367	74.70	110.17	9.20	491.20

with human pegivirus (HPgV) are frequently persistent, but not associated with the development of identifiable disease. Although largely non-pathogenic, pegivirus infection in other animals has been associated with persistent viraemia, acute to chronic hepatitis and liver failure in horses, defined as Theiler's disease [13], as well as lymphocytic enterocolitis in common marmosets [14].

Avian caliciviruses (*Caliciviridae*; positive-sense RNA viruses) have been identified in a broad range of avian species showing either asymptomatic infection and/or presenting as diseases such as gastro-enteritis, poor feathering and infectious runting-stunting syndrome [15,16]. Similarly, avian picornaviruses (*Picornaviridae*; positive-sense RNA viruses) can cause a reduction of egg production, catarrhal enteritis, avian encephalomyelitis and hemorrhagic hepatitis in a range of waterfowl and poultry, and have been associated with avian keratin disorders (AKD) and beak deformity in wild birds [17]. Collectively, picornaviruses exhibit tremendous phylogenetic diversity and a wide host range, and some have the potential to contribute to economic loss and negative impacts on wildlife conservation.

Metagenomic sequencing has greatly increased our understanding of viral diversity and cryptic pathogens in wildlife [18]. For example, metagenomic studies of seemingly healthy avian species have revealed a diverse array of novel avian picornaviruses and caliciviruses from ducks, penguins, cranes and lorikeets [19–24] with no clear disease association. Importantly, characterizing the virome of highly invasive species through metagenomics can provide a better understanding of the potential ecological consequences and microbial interplay between introduced and native species in an urban bird community [25]. Herein, we used meta-transcriptomics to reveal the diversity of viruses and other microbes in apparently healthy mynas in Australia, with the specific aim of documenting any that may have transmitted to or from native bird species.

2. Methods

2.1. Sample collection

Sixteen Indian (common) mynas (*A. tristis*) were captured from a suburban environment in North Sydney, NSW, Australia in early 2019. The mynas used in this study were trapped and euthanised during routine community pest control. Birds were trapped in wire cages, transported less than 30 min, euthanised with carbon dioxide and immediately subject to gross post-mortem examination and sample collection. After necropsy, the sample tissues were preserved in RNA-later® and stored under −80 °C.

prior to RNA extraction. All methods were approved by Taronga Conservation Society Australia's Animal Ethics Committee (approval number 3b1218) in compliance with the Australian Code of Practice for the Use of Animals for Scientific Purposes.

2.2. RNA library construction and metatranscriptomic sequencing

RNA was extracted from samples of brain, liver and large intestine from the 16 birds using the RNeasy Plus mini kit (Qiagen, Hilden, Germany) then pooled into six libraries based on these tissue types. RNA concentration and integrity were determined using a NanoDrop spectrophotometer (ThermoFisher, MA, USA) and TapeStation (Agilent, CA, USA). RNA samples were pooled in equal proportions based on animal tissue type. Illumina RNA libraries were then prepared from the pooled samples following rRNA depletion using a RiboZero Gold kit (Epidemiology) at the Australian Genome Research Facility (AGRF), Melbourne, Australia. The rRNA depleted libraries were sequenced on an Illumina NovaSeq platform (paired 150-nt reads). Raw sequencing reads were quality trimmed with Trimmomatic [26] then *de novo* assembled using Megahit [27]. To identify potential virus-like transcripts, sequence reads were assembled into contigs and blasted against the NCBI non-redundant protein (nr) database with searches using Diamond blastx at an e-value cut-off $1E^{-5}$ [28]. All viral reads were then reassembled to obtain a complete virus genome using the Geneious Assembler program implemented in Geneious Prime 2021.1.1. Putative cleavage sites and motifs were identified through comparative genomic analysis.

2.3. Phylogenetic analysis of the novel viruses

A diverse set of nucleotide and amino acid sequences, including the RNA-dependent RNA polymerase (RdRp) that is conserved among RNA viruses, were downloaded from GenBank and employed as background reference sequences in phylogenetic analyses. Accordingly, the putative NS3 and NS5B proteins of pegiviruses (used for Pin virus), the hexon protein of adenoviruses (for myna adenoviruses), the polyprotein of picornaviruses (for myna hepatovirus), the nonstructural gene of the *Parvoviridae* (myna chaphamaparvovirus) and the polyprotein for caliciviruses (myna caliciviruses) determined here were aligned using the E-INS-i algorithm in MAFFT v7 [29], after which ambiguously aligned regions were removed using TrimAL employing the gappyout setting [30]. This resulted in a set of final sequence alignments that were subjected to phylogenetic analysis using the maximum likelihood (ML) method available within IQ-TREE 1.6.7 and employing the LG model of amino acid substitution with SPR branch-swapping [31]. Bootstrap resampling (1000 replications) was used to assess nodal support. The phylogenetic trees obtained were visualized using Figtree version 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) and midpoint rooted for purposes of clarify.

2.4. Virus and host gene abundance

To compare with the extent of virus gene expression of that of host genes, the abundance of the ribosomal protein S7 (RPS7) was evaluated through mapping trimmed raw reads to the RPS7 gene transcript of the common starling (*Sturnus vulgaris*), the closest relative of the Indian myna for which an appropriate sequence is available (GenBank accession number: XM_014883722.1). RPS7 has been reported to be a stable reference gene for quantitative gene expression studies in multiple avian species [32]. Putative viral reads were extracted to estimate viral abundance using RPM (reads per million) per virus genome and

Table 2
Abundance of bacterial and eukaryotic microbial organisms (protozoa and fungi). Abundance is measured using RPM (Read per Million). Color grey indicates RPM 0–10, yellow indicates RPM 10–1000, orange indicates RKM1000-100000 and red indicates RPM > 10,000.

Family/RPM	Myna1L-VERT70	Myna2L-VERT79	Myna1G-VERT71	Myna2G-VERT78
Archaea				
Halorubraceae	0	292.38	0	0
Natrialbaceae	0	85.75	0	0
Bacteria				
Acetobacteraceae	0	0	0	5.7
Alcaligenaceae	0	34.36	0	0
Brachyspiraceae	0	0	6.44	0
Campylobacteraceae	0	0	0	143.63
Clostridiaceae	0	0	14.95	10.29
Enterobacteriaceae	14.86	0	18.28	64.4
Enterococcaceae	0	0	0	16.67
Erwiniaceae	0	0	0	8.54
Helicobacteraceae	0	0	0	548.48
Lachnospiraceae	0	0	5.24	0
Mycoplasmataceae	0	0	1506.25	72.93
Pasteurellaceae	15.54	0	0	0
Peptostreptococcaceae	0	0	194.59	0
Streptococcaceae	4.41	0	0	0
Vibrionaceae	0	6.53	0	105.07
Fungi				
Glomeraceae	0	0	2.4	0
Malasseziaceae	0.97	0	14.93	0.52
Pleosporaceae	0	0	3.68	0
Sclerotiniaceae	0	0	1.07	0
Trichosphaeriaceae	0	0	0	5.19
Eukaryota				
Eimeriidae	864.52	2013.98	1191.73	22186.22
Geminigeraceae	0	0	2.62	0
Peronosporaceae	2.8	0	0	0
Plasmodiidae	3.14	89.71	7.73	4.85
Salpingoecidae	0	0	7.98	0
Sarcocystidae	0	0	3.63	0
Theileriidae	0	0	0	9.06
Trichomonadidae	0	0	0	10.88

mapping against the predicted viral contigs using BBmap [33].

2.5. Screening for potential bacterial, eukaryotic and protozoan pathogens

To determine the presence (i.e. active gene expression) of any known bacterial and eukaryotic pathogen, trimmed reads were taxonomically classified using CCMetagen [34]. We specifically searched for bacteria, eukaryotes and protozoa of likely pathogenic importance, or known to infect avian species. Accordingly, the results for each microbe were presented as the number of reads per million (RPM), excluding those hits that had <0.5 RPM and less than 90% sequence similarity to a known reference sequence.

3. Results

3.1. Overview of meta-transcriptomic virome data

In total, six rRNA-depleted RNA sequencing libraries were constructed from the brain, liver and large intestine of 16 mynas, resulting in an average of 96,766,596 (ranging from 70,380,196 to 129,823,626) high-quality trimmed reads in each library (Table 1). Overall, seven novel viruses from the families *Adenoviridae*, *Caliciviridae*, *Flaviviridae*, *Parvoviridae* and *Picornaviridae* were identified after Blast analyses to previously described viruses. The viral reads obtained were also used to estimate the abundance of each virus, with the host RPS7 gene used as reference for comparison (Table 1). Further details of each virus identified are described below. (See Table 2.)

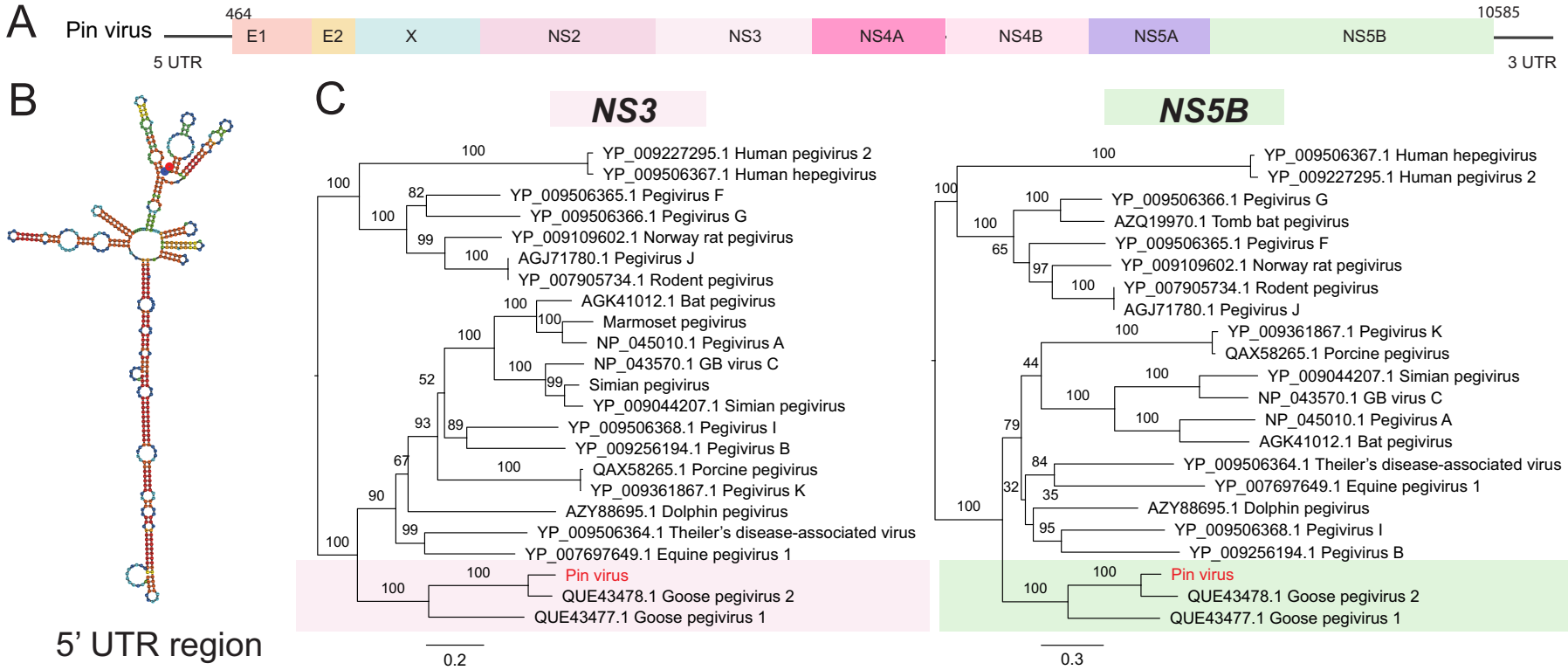

Fig. 1. Genome characterization and phylogenetic analysis of representative pegiviruses. (A) Genome structure of Pin virus. (B) Structure of the 5'UTR region of Pin virus predicted by RNAfold. (C) Phylogenetic trees of the NS3 gene (shaded pink) and NS5B genes including the RdRp (shaded green) of selected pegiviruses. Branches are scaled according to the number of amino acid substitutions per site and bootstrap support values are shown. Pin virus is shown in red font. Trees are midpoint-rooted for clarity only. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

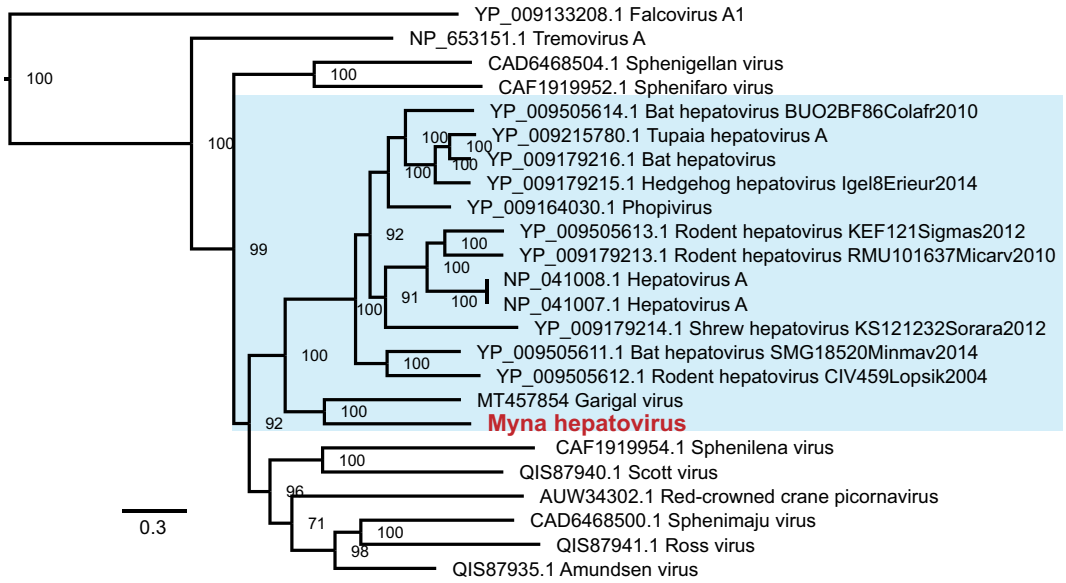

Fig. 2. Phylogenetic analysis of myna hepatovirus. Phylogenetic tree of the polyprotein, containing the RdRp region, of relevant picornaviruses focusing on the novel myna hepatovirus identified here (bold red font). The tree was midpoint rooted, corresponding to the division between mammalian and avian viruses. The blue box indicates members of the genus *Hepatovirus*. Bootstrap values >70% are shown for key nodes. The scale bar indicates the number of amino acid substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Discovery of a novel avian pegivirus

Abundant pegivirus-related transcripts were obtained from seemingly healthy mynas. Remapping the trimmed raw reads onto the putative pegivirus-like transcript revealed a nearly completed pegivirus genome (mean read depth coverage of 143 per nucleotide position). We tentatively termed this novel virus Pin virus (Passerine INvasive myna pegivirus) (Fig. 1). This virus was previously partially described by our group [9], and herein we provide a more complete investigation. Typical virion features of pegiviruses are a potentially infectious single-strand, positive-sense RNA, genome size ranging from 8 to 11 kb, encoding a polyprotein and possessing an IRES element. Pin virus comprises 11,053 nucleotides (nt) including a 5′ untranslated region (UTR) of 463 nt, with a single open reading frame encoding a polyprotein of 3373 amino acids and a 3′UTR of up to 468 nt. The stem-loop-like structure of the 5'UTR region of Pin virus was predicted using RNAfold (Fig. 1B). Comparisons with goose pegiviruses revealed that the polyprotein of Pin virus contained three structural (E1, E2, p7) and six nonstructural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins (Fig. 1A).

Two highly conserved regions - the NS3 (protease) and the NS5B (RdRp) - were used for phylogenetic analysis. This revealed a distinct avian clade of pegiviruses that comprises Pin virus and goose pegiviruses 1 and 2. Indeed, Pin virus exhibited 71.54% amino acid identity across the virus polyprotein with its closest relative - goose pegivirus 2 (NCBI accession number MW365447.1) isolated from swan geese in China [11] (Fig. 1C).

Through comparison with the goose pegiviruses we also identified conserved functional motifs that likely impact virus packaging and replication in the putative coding regions (Fig. S1). These included ATP-binding sites (GSGKS helicase motifs) at positions 1268 to 1272 and a nucleotidase domain (QRRGRXGR) at positions 1525 to 1532 in NS3. A D-X4-D motif at positions 2869–2974 and 3022–3027, a GX2TTX3N (GVLTTSSSN) motif at positions 3081–3038, and a highly conserved GDD motif (at positions 3115–3117) were also identified in the NS5B protein that contains the RdRp (Fig. S1).

3.3. Myna calicivirus and a novel myna hepatovirus

Additional myna-associated RNA viruses were identified from the families *Picornaviridae* and *Caliciviridae*. Specifically, an hepato-like virus (*Picornaviridae*) transcript was identified in the liver and gut RNA-seq libraries. The length of the nearly complete virus genome was 7928 nt (mean read depth of 149 reads/position), encoding a polyprotein of 2208 amino acid residues. A phylogenetic analysis of the polyprotein revealed that this novel picornavirus was most closely related to Garigal virus (NCBI accession number: MT457854.1), previously identified from a rainbow lorikeet hepatovirus in Australia [19], with which it shared 70% polyprotein amino acid similarity. Notably, Garigal virus was found in both diseased and non-diseased lorikeets (Fig. 2).

Two partial caliciviruses transcripts, a 945 nt and a 2444 nt fragment of the partial polyprotein containing RdRp, were obtained from the gut library (Myna-2G VERT78) (Table 1). Phylogenetic analysis of the polyprotein demonstrated that the two caliciviruses clustered with other avian caliciviruses, forming a distinct avian clade. BLASTx searches indicated that both fragments were most closely related to a sequence denoted as Caliciviridae sp. (NCBI accession number: QKN88791.1), identified from bird fecal metagenomic samples from China and exhibiting 52.96% and 58.49% amino acid sequence identity, respectively (Fig. 3).

3.4. Novel DNA viruses identified in mynas

Adenoviruses have been detected in a variety of vertebrate hosts from fish, reptiles, marsupials as well as avian species worldwide. Most avian-related adenoviruses belong to three genera: *Aviadenovirus*, *Siadenovirus* and *Atadenovirus* [35]. We identified several abundant transcripts associated with aviadenoviruses and siadenoviruses, which were reassembled into two partial genomes of adenoviruses and denoted here as myna aviadenovirus and myna siadenovirus. Hexon gene sequences were then used to infer phylogenetic relationships among adenoviruses. Accordingly, myna aviadenovirus exhibited 78.26% amino acid similarity with its closest documented relative, Southern Psittacara leucophthalmus aviadenovirus (NCBI accession number: MN153802.1), identified from the feces of white-eye parakeets in Brazil [36]. Similarly, myna siadenovirus, shared 78.69% amino acid similarity with its closest relative - Great tit adenovirus 1 (NCBI accession number: NC_043405.1) isolated from a diseased great tit (*Parus major*) in Hungary [37]

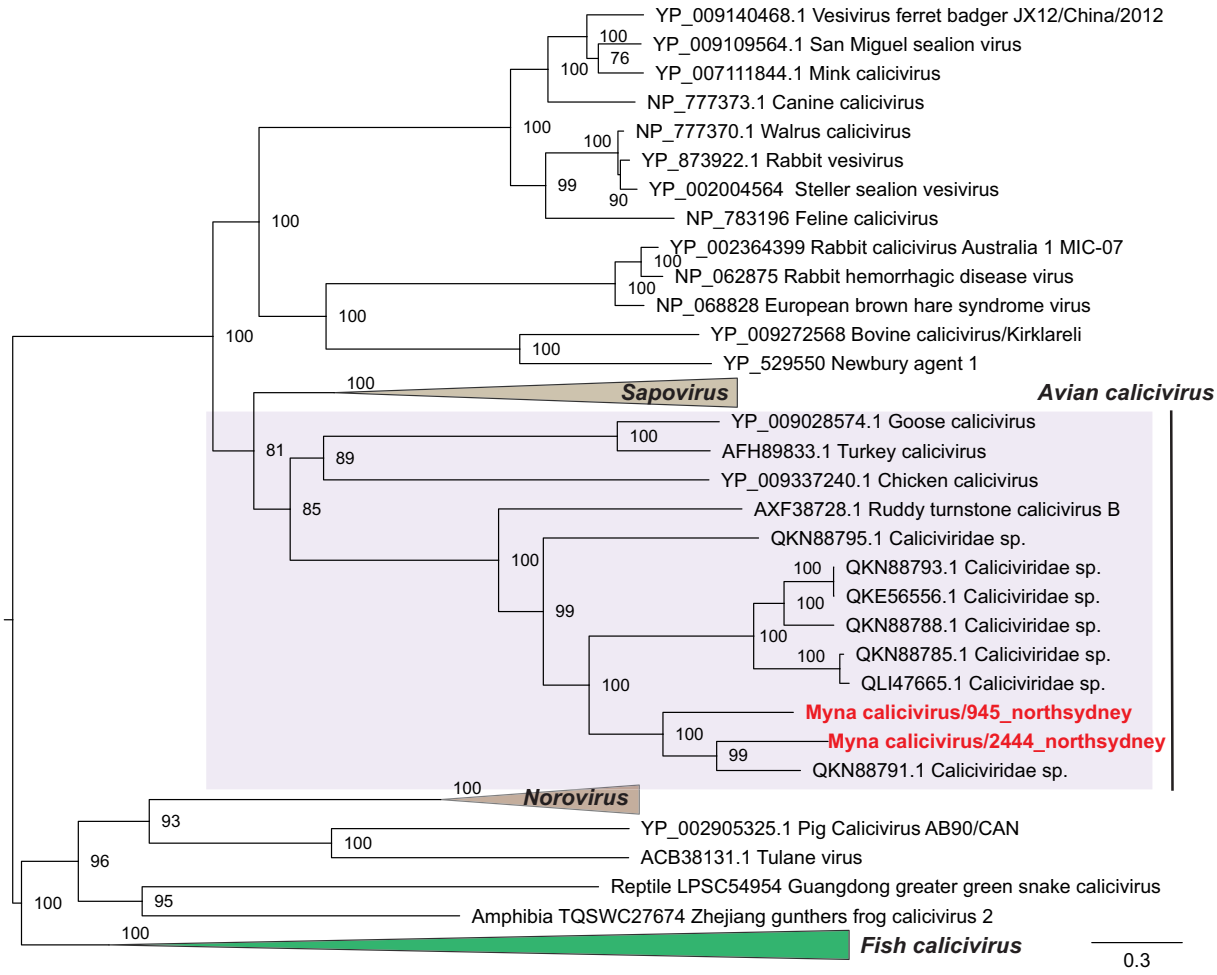

Fig. 3. Phylogenetic tree of the polyprotein, including the RdRp, of myna caliciviruses. The tree was midpoint rooted, corresponding to the division between mammalian calicivirus, sapovirus, norovirus, fish caliciviruses and avian viruses (shaded purple). Some monophyletic groups have been collapsed to improve visualization (shaded green). Bootstrap values >70% are shown for key nodes. The myna caliciviruses obtained here are shown in bold red font. The scale bar indicates the number of amino acid substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 4A).

To compare our myna associated adenoviruses to those identified previously in birds from New South Wales [6], nucleotide sequences of the DNA polymerase gene (~232 nt to accord with the data provided in [6]) were incorporated into a broader phylogenetic analysis. This revealed that myna aviadenovirus clustered with Aviadenovirus spp. isolates WHC1055 and WHC1073 (NCBI accession number: MN238650.1 and MN238651.1.), identified from the feces of the Eastern yellow robin (*Eopsaltria australis*) and Eastern shrike-tit *(Falcunculus frontatus*), although exhibiting only ~38% nucleotide similarity. Similarly, the myna siadenovirus shared 68% nt identity with the Siadenovirus sp. isolate WHC1110 (MN238652.1) detected in the Silvereye (*Zosterops lateralis*). Arguably of most note was that the myna siadenovirus only shared 65% similarity with a siadenovirus previously identified in the kidney of an invasive myna in Australia (accession MN238647.1), suggesting an understudied diversity of myna adenoviruses (Fig. 4B).

Finally, a myna chaphamaparvovirus (mchPV) was detected from an intestinal RNA-seq library (VERT79). The virus genome comprised 4334 nt with two distinct ORFs encoding the nonstructural protein (NS, 631 amino acids) and the hypothetical structural protein (VP, 531 amino acids). Phylogenetic analysis of the predicted amino acid sequence of the complete NS protein revealed that the mchPV fell within the avian Chaphamaparvovirus lineage, clustering with chicken chapparvovirus 2 and chestnut teal chaphamaparvovirus 1. (Fig. 5). The amino acid sequences of mchPV shared only 36.99% pairwise identity in NS gene (NCBI accession: AXL64655.1) and 31.14% in VP gene (NCBI accession: AXL64656.1), respectively, to chicken chapparvovirus 2. (See Fig. 6.)

3.5. Microbial profiling and the identification of Isospora spp.

Both gut libraries showed a greater diversity in the number of bacterial families compared to the liver. *The most abundant bacterial family of interest in the gut was the Mycoplasmataceae (RPM 1506.25). However, the patterns of bacterial composition of the two gut libraries were different, which may be related to animal diet, sample location and/or individual variation.* The most abundant fungi in the data set were members of the *Malasseziaceae*, observed at low abundance in the gut library (myna1G-VERT71). In the case of eukaryotes, sequence reads for the *Plasmodiidae* were found in all libraries but at relatively low abundance, ranging from 3 to 90 RPM (Fig. S2). This result is unsurprising given previous descriptions of malaria in Indian mynas in Australia [4].

Notably, the dominant eukaryote family identified were the *Eimeriidae,* ranging from 864 to 22,186 (RPM) across all liver and gut libraries. The *Eimeriidae* is a family of protozoans of the order *Coccidia* that includes the *Eimeria* and *Isospora* genera that are of medical and veterinary importance. The transcripts associated with *Eimeriidae* were retrieved and reassembled into one complete mitochondrial genome of

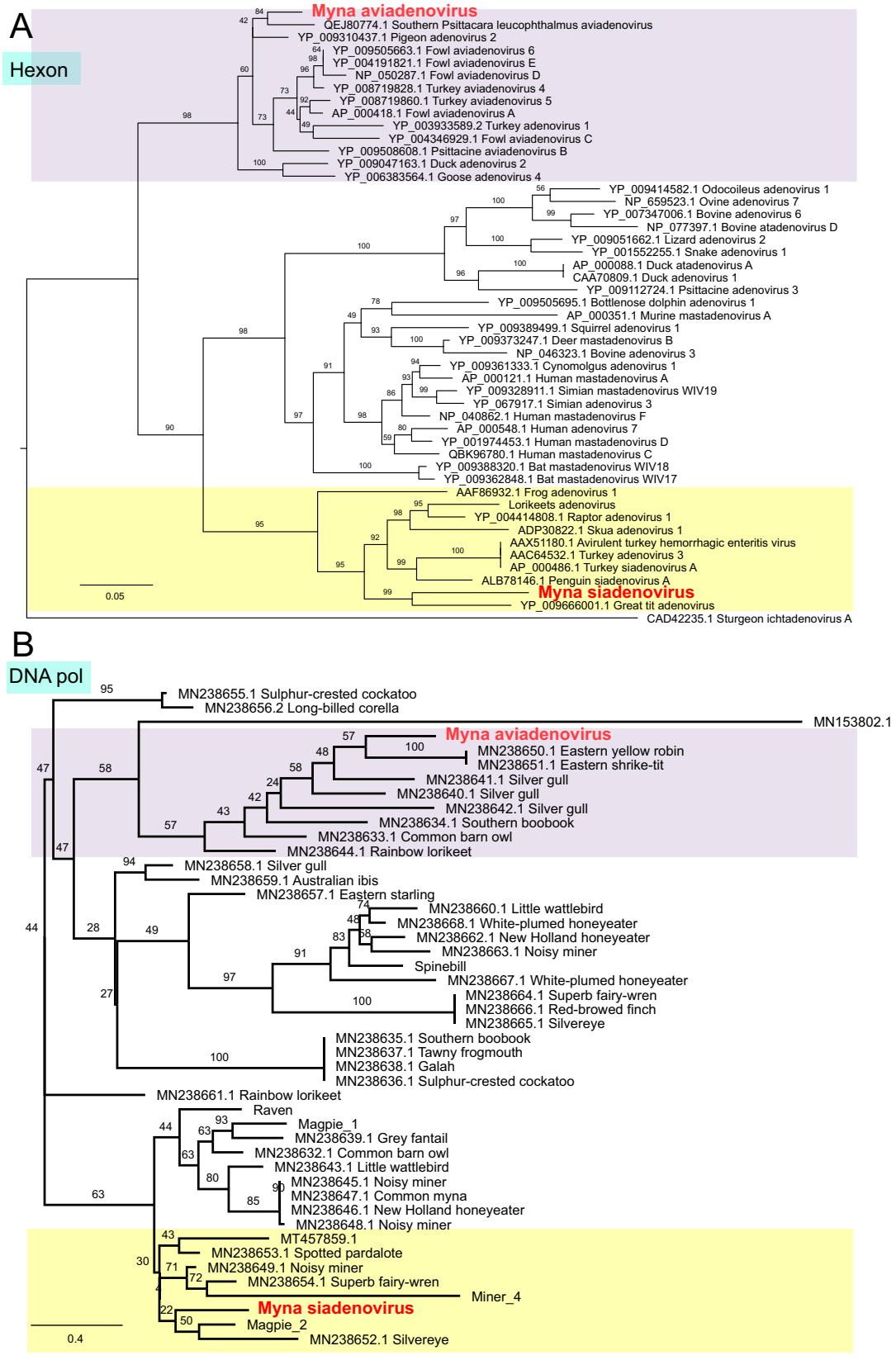

Fig. 4. Phylogenetic relationships of the myna adenoviruses. (A) Amino acid phylogenetic tree of the hexon protein of myna siadenovirus and myna aviadenovirus (bold red font). (B) Phylogenetic tree of DNA polymerase (~232 nt) of the myna aviadenovirus and myna siadenovirus (bold red font). The yellow box shading indicates the avian siadenoviruses while the purple box denotes the avian aviadenoviruses. The scale bar represents the number of substitutions per site and bootstrap values are shown. Both trees are mid-point rooted for clarity only. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Phylogenetic tree of the non-structural gene (NS) of parvoviruses (*Parvoviridae*). The genome structure and sequence coverage of myna chaphamaparvovirus. The yellow shading denotes the subfamily *Hamaparvovirinae* that contains the novel myna chaphamaparvovirus (bold red font). The scale bar represents the number of amino acid substitutions per site. The tree is mid-point rooted for clarity only. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Isospora, termed *Isospora greineri* strain/vert71len6461 to reflect its closest match to known taxa. The genome of this *Isospora* strain (6461 nt) encoded typical features of mitochondrial protein coding genes, including the cytB, COX as well as LSU and SSU regions. The full genome showed 99.2% nucleotide similarity to *Isospora greineri* voucher MAH-2013a_MTZ1 (accession number: KR108298.1) [38]. A near complete fragment of *Isospora superbusi* strain/vert78len2672 (5770 bp) was also identified, sharing 98.78% nt similarity with strain MTZ2 (accession number: KT203396.1) [38]. The cytochrome c oxidase subunit 1 (COX1) gene regions of these coccidian protozoa were then utilized for phylogenetic analysis. Notably, both the *Isospora* sequences identified in our myna samples were closely related to *Isospora greineri* (98.96% nt identity) and *Isospora superbi* (99.38% nt identity) isolated from superb glossy starlings (*Lamprotornis superbus*) from Canada [38], clustering in the same clade as *Isospora* sp. identified in other Passeriformes.

4. Discussion

The Indian myna bird is a highly invasive avian species in urban Australia that frequently interacts with native species, peri-urban and rural agricultural species, and humans. Herein, we present the first metagenomic survey of the virome of highly invasive Indian myna birds

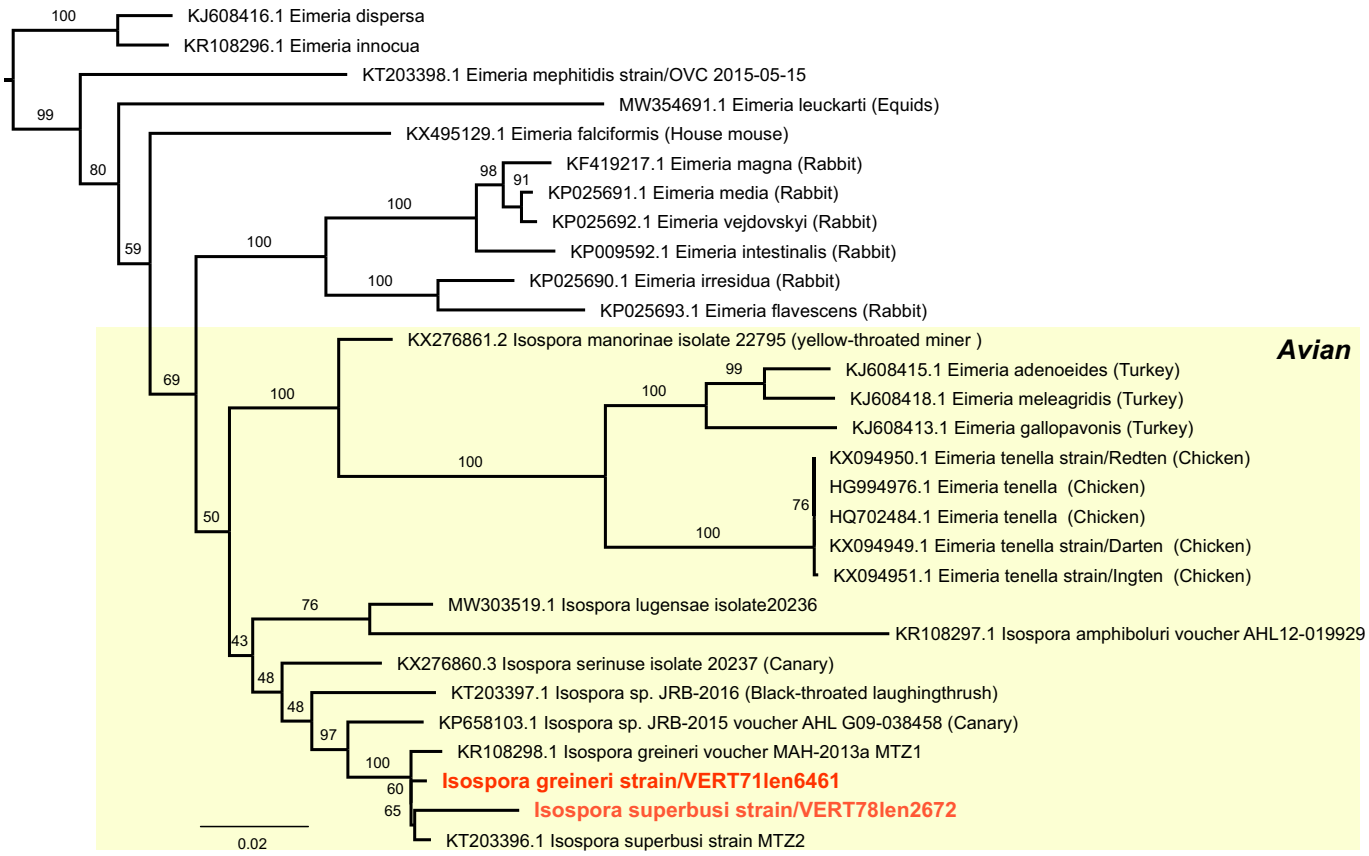

Fig. 6. Phylogenetic tree of the cytochrome C oxidase subunit 1 (COX1) gene of *Isospora* sp. Two abundant isosporan transcripts (bold red font) from the Indian myna showed high sequence similarity to *Isospora greineri* and *Isospora superbusi*. Yellow shading indicates the avian-associated isosporan coccidial parasites. The scale bar denotes the number of nucleotide substitutions per site and bootstrap values are shown. The tree is mid-point rooted for clarity only. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in Australia, revealing a number of novel myna-associated RNA viruses (pegivirus, hepatovirus, and caliciviruses), DNA viruses (adenoviruses and chaphamaparvovirus), potentially pathogenic bacteria (belonging to *Mycoplasmataceae* and *Helicobateraceae*) and protozoan parasites (*Isospora* sp.). No birds exhibited clinical signs or gross lesions suggestive of illness at the time of sampling. Importantly, although these and other data demonstrate that invasive mynas in Australia carry a diverse group of avian viruses and other potential pathogens [39], we found no evidence of direct virus exchange between invasive and native species. Indeed, all the viruses identified here were genetically highly distinct. For example, while the myna hepatovirus newly identified here clustered with its closest relative, Garigal virus, a lorikeet hepatovirus previously identified in Australia [19], it only shared ~45% amino acid similarity in the polyprotein sequence, suggesting a long period of independent evolution. However, the apparent lack of virus transmission from invasive to native species may simply reflect a lack of sampling. Accordingly, future work should increase both the sample size and the number of sites, addressing whether there are host genetic or environmental barriers to successful cross-species virus transmission.

As in many recent avian virome studies, most of the newly identified viruses were from the families *Picornaviridae*, *Parvoviridae*, *Circoviridae*, and *Caliciviridae* [40]. Generally, all the closest relatives of the viruses discovered in this study were avian viruses, indicative of a relatively long evolutionary history in birds. The novel Pin virus described here is of particular interest as this represents only the second pegivirus from an avian species. Goose pegiviruses isolated from China showed lymphotropic pathogenicity and a high rate of co-infection with parvoviruses, circoviruses and astroviruses, with a co-infection frequency of 24.5% between goose pegivirus and goose parvovirus [41]. Of note, Pin virus exhibited higher abundance in liver libraries in comparison to other organs, suggesting its potential hepatotropism or lymphotropism in accordance with those pegiviruses found in pigs, horses and cetaceans [10,42,43]. Coincidently, we identified a novel myna chaphamaparvovirus (*Parvoviridae*) in the same sequencing library as Pin pegivirus. It is unclear whether this coinfection is of clinical or epidemiological importance, or whether Pin virus alters host fitness in mynas and other birds.

Many studies have revealed a high diversity of adenoviruses in a range of Australian birds [6,36,44,45]. In addition, recent studies have shown that adenoviruses frequently jump species boundaries to emerge in new hosts [46], although aviadenoviruses identified from Psittaciformes tend to cluster together in phylogenetic trees [35]. Interestingly, the partial DNA polymerase sequence previously detected in an Indian myna [6] (NCBI accession: MN238647) was identical to those found in native Australian species (honeyeater), yet only shared 65% nucleotide similarity with the myna siadenovirus identified in our study. Although the polymerase region used for phylogenetic analysis is short (232 nt), this result suggests that this invasive species might be susceptible to infection by enzootic avian siadenoviruses, and that additional adenoviruses are likely to be present in this species. Importantly, however, we found no evidence of clustering of myna-associated viruses with those previously sampled from native bird species.

In addition to viruses, our meta-transcriptomic analysis of mynas revealed multiple bacteria, fungi and protozoa. Of particular interest was the identification of two mitochondrial genomes of isosporan coccidial parasites. Over 90% of the described coccidia infecting wild birds belong to the genus *Isospora*, either sub-clinically or in association with anorexia and enteritis [47]. It has been suggested that *Isospora* species

are highly host specific at the genus level [48]. However, their phylogenetic position in our study suggests possible isosporan transmission between bird species within the same family, since the myna and superb glossy starling are both members of *Sturnidae*.

More broadly, this study demonstrates the value of performing transcriptome sequencing to simultaneously detect an array of viruses, bacteria, and eukaryotes (fungi and protozoa). Notably, economically important bacterial zoonotic pathogens such as *Salmonella typhimurium* DT160 [49], *Chlamydia psittaci* [50] and avian pathogenic *Escherichia coli* [51,52], which have been previously reported to cause zoonoses and mortality outbreaks in wild and domestic avian species, were not detected in our genomic data. Avian malarial parasites (*Plasmodium* spp. *and Haemoproteus spp.*) have previously been demonstrated to be relatively abundant in mynas [4]. Interestingly, we detected abundant *Plasmodium* reads in the liver of mynas, although no *Haemoproteus* reads were obtained. *Haemoproteus* spp. were mostly recorded in the primary range of the myna and not at their secondary expansion sites [4], which is compatible with their absence from Sydney, Australia.

The surveillance of invasive species in urban areas is central to the identification of known and novel microbes of significant concern to the health of wildlife, domestic animals and humans, and providing important evolutionary context. Given the high density and invasiveness of myna birds, additional studies, including the characterization of myna-associated microbes in different geographic areas, expanding the sample sites from introduced and native ranges of mynas, and in various environments, are needed to better evaluate the prevalence, route of transmission, tissue tropism, and pathobiology of identified microbes to improve animal and public health.

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Data availability statement

The raw sequence reads are available at BioProject PRJNA756632. Consensus sequences of the new viruses identified in this study are available on GenBank under accession numbers OK334621 to OK334628.

Declaration of competing interest

The authors declare no conflict of interest.

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