Comparison of the prevalence of *Carnivore protoparvovirus 1* in live-captured and road-killed wild carnivore in Taiwan

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Carnivore protoparvovirus 1 can infect wide host range of Carnivores, the main species are canine parvovirus 2 (CPV-2) and feline panleukopenia virus (FPV). While FPV has been endemic in cat, CPV-2 can classify in to 3 antigenic variants, including CPV-2a, b and c. They emerged as a pathogen for domestic dog and cause serious hemorrhagic enteritis. Cross-species transmission of protoparvovirus could be a potential threat to wild-carnivore, so aim of our study is to investigate the prevalence of *Carnivore protoparvovirus* and hazard to wild-carnivore. During 2015 to 2018 we totally collected 131 individuals of ferret badgers (*Melogale moschata*), masked palm civet (*Paguma larvata*) and crab-eating mongoose (*Herpestes urva Hodgson*). Of all, 99 individuals were live-trapped and 32 were road-killed. We adopted polymerase chain reaction (PCR) for detecting partial VP2 gene. The PCR products were sequenced, and CPV-2a, CPC-2b, CPV-2c, and FPV, were identified. CPV-2a was the primary variant found in both road-killed and live-trapped with 46.67 % and 40 % of prevalence, respectively, followed by CPV-2c (33.33 %, 30 %), FPV (13.33 %, 10 %) and CPV-2b (6.67 %, 0 %). The prevalence of road-killed was 46.88% (95% Confidence Interval: 29.58 - 64.17 %), which was significantly higher than the 10.1% of prevalence of live-trapped (95% CI: 4.16 - 16.4 %). The protoparvovirus-positive of road-killed also exhibited enteritis, revealed the possibility that protoparvovirus might be threat to wild-carnivore. To our knowledge, this is the first molecular characterization of protoparvoviruses in Taiwan wild-carnivore and more studies are needed to elucidate the distribution of these viruses in the future.

Keywords: Parvovirus, Cross-species transmission, Wildlife conservation, Road-killed

Distribution of Carnivore protoparvovirus 1 in free-living leopard cats (*Prionailurus bengalensis chinensis*) and its association with road kill in Taiwan

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Abstract

Road kill is a factor threatening the sustainability of populations of wild mammals, particularly those of endangered species in rural areas. However, evidence suggests that disease-induced changes in animal behavior and cognition may increase the risk of collisions with vehicles. Carnivore protoparvovirus 1 (CPPV-1) is widespread among free-living carnivores, and CPPV-1 infection may result in secondary road kill. In this study, we used molecular screening of *VP2* from 2015 to 2017, to assess the incidence of CPPV-1 infection in 9 live-trapped (LT) and 17 vehicle collision (VC)-affected free-living leopard cats (*Prionailurus bengalensis chinensis*). In addition, we evaluated the effects of CPPV-1 infection on the predisposition to VC. The prevalence of CPPV-1 infection in the LT and VC-affected samples was 33.3% and 82.4%, respectively. Our results indicated an association between road kill and CPPV-1 infection in the free-living leopard cats. We identified circulation of feline parvovirus and variants of canine parvovirus (CPV), including CPV-2a, CPV-2b, and CPV-2c, in the free-living leopard cat population. The partial sequences of different variants of *VP2* obtained from the leopard cats were identical with those obtained from the domestic dogs and cats in Taiwan. Based on the results of our study, we recommend that efforts to manage CPPV-1 infection in the leopard cat population focus on vaccination programs for

the leopard cats and measures for controlling the free-roaming dog and cat population. In addition, road development might increase the likelihood of contact and disease transmission between free-roaming domestic animals and wild animals. These effects of road development should be considered in wildlife management.

Introduction

The leopard cat (*Prionailurus bengalensis chinensis*) is an endangered felid species, which is distributed in East, Southeast, and South Asia [1]. It was previously commonly distributed in the lowland habitats throughout the island of Taiwan [2, 3]. However, owing to the island-wide decline in the population of this species, it was listed as an endangered species under the Wildlife Conservation Act in Taiwan in 2009 [4]. Currently, the distribution of Taiwanese leopard cats is restricted to small areas in 3 counties, namely Miaoli, Nantou, and Taichung City, in Central Taiwan. Studies in the Miaoli County suggested that road kill, habitat fragmentation and degradation, illegal trapping, and poisoning are the major threats to the sustainability of the leopard cat population [5].

Road kill is considered a factor threatening the sustainability of wild mammal populations, particularly that of populations of endangered species in rural areas [6, 7]. Based on our records, at least 50 cases of leopard cat road kill were recorded from 2012 to 2017 in Taiwan [5].

Previously, disease-induced changes in behavior and cognition were suggested to increase the risk of vehicle collision (VC) [8]. Krumm et al. [9] compared the prevalence of chronic wasting disease among road-killed mule deer (*Odocoileus hemionus*) and mule deer sampled in the vicinity of VC sites. They found that the

prevalence of chronic wasting disease was significantly higher in the road-killed deer than in the deer sampled in the collision vicinity. In addition, Hollings et al. [10] found that road-killed Tasmanian pademelons (*Thylogale billardierii*) exhibited a significantly higher seroprevalence of *Toxoplasma gondii* than did culled individuals. In addition, a high prevalence of parvovirus DNA was detected among road-killed carnivores in Portugal [11].

Carnivore protoparvovirus 1 (CPPV-1), belonging to the genus *Protoparvovirus* of the family Parvoviridae, can cause life-threatening pathogenic infections in many carnivores [12]. According to the International Committee on Taxonomy of Viruses, numerous phylogenetically closed viral strains that infect carnivore species are classified under CPPV-1, including feline parvovirus, variants of canine parvovirus (CPVincluding CPV-2a, CPV-2b, and CPV-2c), mink enteritis virus, and raccoon parvovirus [13]. Infections of variants of CPV-2a, CPV-2b, CPV-2c, and feline parvovirus among domestic and wild felids have been commonly documented [12, 14, 15]. In Taiwan, the infection with CPV-2a has been reported in apparently healthy captive leopard cats [16, 17]. Furthermore, CPV-2b and CPV-2c were isolated from leopard cats in Vietnam [17]. Felids infected with feline parvovirus exhibit acute depression, diarrhea, vomiting, and panleukopenia [18]. No evidence supports hypothesis that the disease are induced by CPV-2 derived variants (2a, 2b,

and 2c) in leopard cats. However, in several studies,domestic cats infected with CPV-2a and CPV-2c developed clinical signs of panleukopenia [19-21].

Stray and free-roaming domestic animals, particularly dogs and cats, can adversely affect wildlife conservation through predation, competition, hybridization, and disease transmission [22, 23]. Free-roaming domestic dogs and cats have been commonly seen in the main habitat of the leopard cat in Taiwan [5, 24]. In addition, CPV-2 derived variants and feline parvovirus have been documented in the population of domestic dogs and cats, respectively, have been documented [25-27]. Therefore, transmission of CPPV-1 between domestic dogs, cats, and leopard cats is highly possible. Nevertheless, the effects of parvovirus infection on the leopard cat population may be overlooked or obscured by other factor, such as road kill.

In the present study, free-living leopard cats were screened for CPPV-1 infection, and the contribution of CPPV-1 infection to the risk of VC for the leopard cats was evaluated. We compared the prevalence of CPPV-1 infection in live-trapped (LT) and VC-affected leopard cats by using a nested polymerase chain reaction (nested PCR) method. Furthermore, the nested PCR amplicons were sequenced for phylogenetic analysis and for identifying the possible source of parvovirus. Our hypothesis was that VC-affected leopard cats exhibit a higher prevalence of CPPV-1 infection than LT leopard cats because the infection and

98 diseases induced by the CPPV-1 increase the likelihood of VC.

99

100 Materials and methods

101 Study area

102 All the leopard cats sampled were from Miaoli County in northwestern Taiwan
103 (Fig 1). The sampling area has a hilly landscape with an elevation of less than 320 m
104 above sea level. The total area of Miaoli County is 1820 km^2, which consists of
105 1245.3 km^2 of forests (68.8%), 291.2 km^2 of agricultural land (16.1%), and 132.6
106 km^2 of man-made construction (7.3%) (Fig 1). A well-developed road system, which
107 includes a primary road (approximately 25 m wide), secondary roads (approximately
108 10 m wide), and tertiary roads (about 5 m wide), and human encroachment have
109 fragmented the wildlife habitat in this rural area. The region has a subtropical
110 climate with hot and wet summers and cold and dry winters. The wet season lasts
111 from March to September (mean monthly rainfall = 293 mm; mean monthly
112 temperature = 25.4°C), while the dry season lasts from October to February (mean
113 monthly rainfall = 72 mm; mean monthly temperature = 19.2°C) [28].

114 Although an estimate of the population of the stray or free-roaming dogs and cats
115 was not available, they were commonly observed and were sympatric with the
116 leopard cats in the study area [29].

FIG 1. Sampling sites of leopard cats in Miaoli County. Circles and triangles denote live-trapped and vehicle-collision-affected leopard cats, respectively. White and black denote Carnivore protoparvovirus 1 (CPPV-1) positive and CPPV-1 negative leopard cats, respectively. The variant identified in each isolation is labeled beside the symbol.

Sample collection

Free-living leopard cats were trapped for radio telemetry tracking or relocation of the leopard cats that invaded poultry farms. Permission for this study was issued by the Forest Bureau (Permit no.: COA, Forestry Bureau, 1061702029). We used steel-mesh box traps (108-Rigid Trap, Tomahawk Live Trap, LLC., Hazelhurst, Wisconsin, USA) baited with live quails. The trapped leopard cats were anesthetized by a veterinarian by using a mixture of medetomidine hydrochloride (50 μg/kg) and tiletamine HCl/zolazepam HCl (2 mg/kg). The procedures for leopard cat trapping, anesthesia administration, and sample collection were approved by Institutional Animal Care and Use Committee of National Pingtung University of Science and Technology (Approval no.: NPUST-106-014).

The carcasses of VC-affected individuals were collected and submitted by the

136 county government of Miaoli for additional necropsy and sample collection.
137 However, because of severe autolysis and physical trauma in most of the
138 VC-affected individuals, histopathological examinations of lesions induced by
139 CPPV-1 infections were inconclusive.
140 We collected whole blood samples and rectal swabs of the LT leopard cats and
141 spleen tissue and rectal swabs of the VC-affected leopard cats for further nested
142 PCR diagnosis, respectively. A staff member of the Miaoli Animal Care and Health
143 Office collected whole blood and rectal swab samples of free-roaming dogs and cats
144 in the same study area on the same day as the trapping of free-roaming dogs and cats
145 and their transfer to the rescue center. The samples from the dogs and cats were used
146 to compare the DNA sequences of CPPV-1 in domestic animals with CPPV-1
147 isolated from the leopard cats. The samples collected from the LT and VC-affected
148 leopard cats for parvovirus detection were different because of the limitation of our
149 target species. The evidence indicated that the quantity and distribution of the
150 CPPV-1 strains were similar between the blood and spleen and between the intestine
151 and feces samples [30, 31]. We recorded the global positioning system (GPS)
152 locations of all the LT leopard cats or those that were found dead as well as the
153 township locations of the dogs and cats for further spatial analysis.
154

PCR screening and phylogenetic analysis of the CPPV-1

We performed nested PCR for CPPV-1 screening using consensus primers that targeted *VP2*, which is the gene that codes for the outer capsid protein of the CPPV-1. The primers were designed by Steinel et al. [32]. In the first round of nested PCR, the forward primer M10 (5′-ACACATACATGGCAAACAAATAGA-3′) and reverse primer M11 (5′-ACTGGTGGTACATTATTTAATGCAG-3′) were used. In the second round, the forward primer M13 (5′-AAATAGAGCATTGGGCTTACCACCATTTTT-3′) and reverse primer M14 (5′-ATTCCTGTTTTACCTCCAATTGGATCTGTT-3′). Total DNA was extracted from the whole blood, spleen, and small intestine samples by using a DNeasy blood and tissue kit (Qiagen, Valencia, CA) and from the rectal swabs by using a QIAamp DNA Stool Mini Kit (Qiagen); the manufacturer's instructions were followed for both types of samples. The conditions of nested PCR amplification mainly followed the protocol described by Steinel et al. [32], with minor modifications. Briefly, viral templates were amplified in a 20-μL reaction mixture, which contained PCR buffer (1.5 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 200 M, and Hot-StarTaq Master Mix [Qiagen]), 0.2 μM of each PCR primer, and 2 μL of the DNA templates. The amplification procedure was as follows: 15 min at 95°C; 35 cycles of 30 s at 94°C, 30 s at 47°C, and 60 s at 72°C, and a final extension for 7

174 min at 72°C. The second round of nested PCR was performed using the same
175 conditions. The expected size of the nested PCR product was 482 bp.
176 The PCR amplicons were sequenced in an ABI377 sequencer by using an ABI
177 PRISM dye-terminator cycle sequencing ready reaction kit with Amplitaq DNA
178 polymerase (Perkin-Elmer, Applied Biosystems). To search for sequences similar to
179 those of the amplicons, a BLAST search was conducted using GeneBank with the
180 nt/nr database available on the BLAST website (BLAST;
181 https://blast.ncbi.nlm.nih.gov/Blast.cgi). In addition, the viral strains isolated from
182 leopard cats and domestic animals were classified according to the amino acid
183 variation in *VP2* [14].
184 The nucleotide sequences were aligned with CLUSTALW [33] in the software
185 MEGA version 6 [34]. The maximum-likelihood method [35] was used to model the
186 phylogenetic relationship among the various CPPV-1 strains isolated from the
187 leopard cats, dogs, and cats. Prior to the construction of a maximum-likelihood tree,
188 the most suitable model was determined using MEGA 6.0. Consequently, the
189 Tamura three-parameter model was selected on the basis of the lowest Bayesian
190 information criterion value [36]. Finally, a phylogenetic tree was constructed using
191 1000 bootstrap iterations.
192

Road coverage in the home range

In this study, we considered road coverage in the home range of each leopard cat as a confounder that might increase the risk of road kill as well as that of parvovirus infection because the movement area of free-roaming domestic dogs and cats mainly depends on road coverage [37], and CPPV-1 transmission to other sympatric wild carnivores occurs within this movement area.

For clarifying the relationship between road coverage and the risk of road kill, a buffer zone of 5 km^2 with a 1262-m radius was assigned as the mean home range size [5], based on the GPS location, to each LT or VC-affected leopard cat. The land use dataset in vector format was acquired from the Land Use Investigation of Taiwan conducted by the National Land Surveying and Mapping Center. The investigation of land use in our study area was conducted and collated in 2017. The vector format of the land use dataset was transformed into the raster format with a grid size of 10×10 m^2. We obtained road coverage data of primary, secondary, and tertiary roads in the buffer zone of each leopard cat from the land use layer using LecoS, a QGIS plugin [38]. QGIS was used to manipulate all the spatial data [39].

Data analysis

We first estimated the prevalence of CPPV-1 infection and its 95% confidence

interval (CI) in the VC-affected and LT leopard cats. The Fisher exact test was applied to compare the prevalence of CPPV-1 infection between the VC-affected and LT leopard cats. Furthermore, we used logistic regression to analyze potential risk factors inducing VC. We used VC or non-VC as the dependent variable. In addition, the status of parvovirus infection and road coverage were treated as explanatory variables. Explanatory variables were selected in the final logistic models depending on the p values; the significance threshold was set at $p = 0.05$. In addition, the values of the Akaike information criterion corrected for small sample sizes (AICc) and Akaike weights were computed for each model and used to assess the fit of models [40]. The computing environment R was used for statistical analysis [41].

Results

Leopard cat sample collection and distribution in Miaoli County

From 2015 to 2017, we collected samples from 26 leopard cats of which 9 were LT and 17 were VC-affected individuals (S1 Table). The samples collected from the leopard cats that were distributed in the western Miaoli County (Fig 1).

CPPV-1 screening and viral strain identification

The screening of the samples collected from the 26 leopard cats revealed that the overall prevalence of CPPV-1 infection was 65.4% (95% CI 47.1%–83.7%). However, the prevalence in the LT leopard cats was 33.3% and that in the VC-affected leopard cats was 82.4% (Table 1). The Fisher exact test results indicated that the prevalence of CPPV-1 infection in the LT group was significantly lower than that in the VC-affected group ($p = 0.027$). Based on the partial VP2 amino acid sequences obtained from the 17 CPPV-1 positive leopard cats, we determined that 7, 7, 1, and 2, leopard cats were infected with CPV-2a, CPV-2b, CPV-2c, and feline parvovirus, respectively (Table 2). The partial VP2 amino acid sequences of CPV-2a and were identical among the leopard cats, domestic dogs, and domestic cats. However, for CPV-2b, we found that Tyr at position 324 was substituted by Ile in 2 samples isolated from the leopard cats. In addition, a substitution of Asn to Lys at position of 321 was found in all the samples isolated from domestic dogs infected with CPV-2b but not in those isolated from the leopard cats. Only one isolate of CPV-2c was obtained from the leopard cats in our study and was identical with one of the genotypes currently circulating in Taiwan (Table 2).

Table 1. Samples from live-trapped or vehicle-collision-affected leopard cats and the result of parvovirus screening and mean road coverage in the home range

Type of sample[a]	No. Individuals	Parvovirus[b]	Prevalence	95% CI[c]		Mean road coverage (m^2)
				lower	upper	
LT	9	3/9	33.3%	2.53%	64.13%	66600
VC	17	14/17	82.4%	64.23%	100%	106305.9

[a]Leopard cat specimen: LT: live-trapped leopard cats; VC: vehicle-collision-affected leopard cats.

[b]Results of parvovirus screening, positive/total individuals

[c]CI: confidence interval

Table 2. Multiple alignment of partial VP2 amino acid sequence of Carnivore protoparvovirus 1 isolated from leopard cats, domestic dogs and cats, and sequences downloaded from the National Center for Biotechnology Information. The species and virus strains are listed and the accession numbers are presented in parentheses. Bold font with gray background indicate that the sample was collected in the sampling area of this study

Isolations	**Amino acid position of VP2 gene**												
	300	**305**	**321**	**323**	**324**	**339**	**353**	**370**	**383**	**410**	**413**	**420**	**426**
P.bengalensis/c2015100901/CPV-2a	**G**	**Y**	**N**	**N**	**I**	**S**	**F**	**Q**	**Q**	**P**	**D**	**F**	**N**
P.bengalensis/2015110601/CPV-2a	.	.	.	.	.	.	.	.	.	.	.	.	.
P.bengalensis/c2016010401/CPV-2a	.	.	.	.	.	.	.	.	.	.	.	.	.
P.bengalensis/c2016030101/CPV-2a	.	.	.	.	.	.	.	.	.	.	.	.	.
P.bengalensis/2017100201/CPV-2a	.	.	.	.	.	.	.	.	.	.	.	.	.
P.bengalensis/2017112501/CPV-2a	.	.	.	.	.	.	.	.	.	.	.	.	.
P.bengalensis/2017110701/CPV-2a	.	.	.	.	.	.	.	.	.	.	.	.	.
952/Cat/CPV-2a	.	.	.	.	.	.	.	.	.	.	.	.	.
953/Cat/CPV-2a	.	.	.	.	.	.	.	.	.	.	.	.	.
954/Cat/CPV-2a	.	.	.	.	.	.	.	.	.	.	.	.	.
1019/Dog/CPV-2a	.	.	.	.	.	.	.	.	.	.	.	.	.
1026/Dog/CPV-2a	.	.	.	.	.	.	.	.	.	.	.	.	.

1036/Dog/CPV-2a	.	.	.	.	.	.	.	.	.	.	.	.	.
1037/Dog/CPV-2a	.	.	.	.	.	.	.	.	.	.	.	.	.
1038/Dog/CPV-2a	.	.	.	.	.	.	.	.	.	.	.	.	.
CPV-2a(KX396349)	.	.	.	.	.	.	.	.	.	.	.	.	.
CPV-2a(KX396353)	.	.	.	.	.	.	.	.	.	.	.	.	.
CPV-2a(KX396376)	.	.	.	.	.	.	.	.	.	.	.	.	.
P.bengalensis/c2015011901/CPV-2b	.	.	.	.	Y	.	.	.	.	.	.	.	D
P.bengalensis/c2015111901/CPV-2b	.	.	.	.	.	.	.	.	.	.	.	.	D
P.bengalensis/c2015110501/CPV 2b	.	.	.	.	Y	.	.	.	.	.	.	.	D
P.bengalensis/c2015110502/CPV-2b	.	.	.	.	Y	N	.	.	.	.	.	.	D
P.bengalensis/c2015110503/CPV-2b	.	.	.	.	.	.	.	.	.	.	.	.	D
P.bengalensis/c2016012801/CPV-2b	.	.	.	.	Y	.	.	.	.	.	.	.	D
P.bengalensis/c2016112701/CPV-2b	.	.	.	.	Y	.	.	.	.	.	.	.	D
1020/Dog/CPV-2b	.	.	K	.	Y	.	.	.	.	.	.	.	D
1023/Dog/CPV-2b	.	.	K	.	Y	.	.	.	.	.	.	.	D
1025/Dog/CPV-2b	.	.	K	.	Y	.	.	.	.	.	.	.	D
1027/Dog/CPV-2b	.	.	K	.	Y	.	.	.	.	.	N	.	D
1028/Dog/CPV-2b	.	.	K	.	Y	.	.	.	.	.	.	.	D
CPV-2b(KX396348)	.	.	K	.	Y	.	.	.	.	.	.	.	D
CPV-2b(KX396361)	.	.	.	.	Y	.	.	.	.	.	.	.	D

P.bengalensis/2017090801/CPV-2c	.	.	.	.	.	.	.	R	.	.	.	.	E
CPV-2c(KX396355)	.	.	.	.	.	.	.	R	R	L	.	.	E
CPV-2c(KX396395)	.	.	.	.	.	.	.	R	.	.	.	.	E
CPV-2c(KX396398)	.	.	.	.	.	.	.	R	.	.	.	S	E
P.bengalensis/2016120301/FPV[1]	A	D	.	D	Y	.	.	.	.	.	.	.	.
P.bengalensis/c2017110701/FPV	A	D	.	D	Y	.	.	.	.	.	.	.	.
1018/Cat/FPV	A	D	.	D	Y	.	.	.	.	.	.	.	.
FPV(JX048608)	A	D	.	D	Y	.	.	.	.	.	.	.	.
FPV(AF015223)	A	D	.	D	Y	.	L	.	.	.	.	.	.

FPV: feline parvovirus

Phylogenetic analysis of the CPPV-1 strains circulating in the leopard cat, domestic dog, and domestic cat populations

To estimate evolutionary divergence, we grouped the CPPV-1 sequences according to source (leopard cats, domestic dogs, and cats) in the study area and compared them with sequences retrieved from the nucleotide database (NCBI GenBank). The database sequences had been isolated from domestic dogs and cats in other regions of Taiwan. All CPPV-1 sequences registered in GenBank after 2014, except the feline parvovirus sequences, were retrieved for this study. In total, 42 sequences (414 bp) were included for phylogenetic analysis (S2 Table). The CPV-2a sequences isolated from different hosts in the study area and those retrieved from GenBank were identical. We constructed a phylogenetic tree by using the partial nucleotide sequences of *VP2* from the 17 leopard cat isolations, 15 domestic dog and cat isolations, and 10 registered data. The sequences of CPV-2a, CPV-2c, and feline parvovirus included in the analysis were grouped into distinct clusters based on the variants (Fig 2). A unique cluster of CPV-2b from the leopard cat isolations was found, which was different from the other CPV-2b clusters observed in wild carnivores and domestic animals.

Fig 2. Molecular phylogenetic relationship of the partial *VP2* sequences of the Carnivore protoparvovirus 1 isolated from leopard cats, domestic dogs, domestic cats, and sequences accessed from GenBank. This phylogenetic tree was constructed using the maximum-likelihood method. The bootstrap value is shown next to the node with 1,000 replicates. The GenBank accession numbers, virus strains, and animal are indicated.

Association between road kill and parvovirus infection

Logistic regression analysis results indicated that CPPV-1 infection was significantly associated with road kill but not with road coverage (Table 3). The model fitted with the explanatory variables of CPPV-1 infection and road coverage exhibited similar values of AICc and Akaike weights compared with the model fitted with CPPV-1 infection only (Table 4). The final fitted model included only CPPV-1 infection in the model with an odds ratio of 9.33 (95% CI: 1.59–71.74) (Table 3). This result indicated that CPPV-1 positive leopard cats had a significantly higher odds of VC than did CPPV-1 negative leopard cats.

301 Table 3. Logistic regression statistics of each explanatory variable in the full model and

302 final fitted model

Variables	Coefficient	SE[1]	p value	Odds ratio	95% CI of odd ratio	
					Lower	Upper
Full model						
Intercept	-2.365	1.395	0.0911	-	-	-
CPPV-1[2] infection	2.457	1.083	0.0233	11.67	1.67	138.62
Road coverage	0.000008	0.000005	0.1333	1.0	0.9999	1.00002
Fitted model						
Intercept	-0.693	0.707	0.32	-	-	-
CPPV-1 infection	2.234	0.951	0.0189	9.33	1.59	71.74

303 [1]standard error.[2]Carnivore protoparvovirus 1

304 Table 4. Comparison of the Akaike information criteria and Akaike weights for logistic

305 models fitted with different combinations of explanatory variables

Variables in the model	AIC	AICc[2]	△AIC	Akaike weight
CPPV-1[3] infection, RC[4]	30.12	32.02	0	0.499
CPPV-1 infection[1]	**31.3**	**32.39**	**0.37**	**0.414**
RC	34.42	35.51	3.49	0.087

306 [1]Bold font indicates the final fitted model.

307 [2]AICc denotes AIC value corrected for small sample sizes; ΔAIC, difference in AIC value

308 from final fitted model.

309 [3]Carnivore protoparvovirus 1

310 [4]RC denotes the road coverage variable.

Discussion

In this study, circulation of CPPV-1, including the variants of feline parvovirus, CPV-2a, CPV-2b, and CPV-2c, was identified in the free-living leopard cat population. Except a distinct subclade of CPV-2b isolated from the leopard cats, the partial sequence of the variants of *VP2* isolated from the leopard cats were identical with those isolated from the domestic dogs and cats. Furthermore, we determined an association between the risk of VC and CPPV-1 infection in the free-living leopard cats. This study is the first to report CPPV-1 infection in free-living leopard cats, but CPPV-1 infection has been previously reported in captive leopard cats [16] and various carnivores [11, 32, 42].

The nucleotide sequences of the CPPV-1 isolates suggested a high likelihood that variants of CPPV-1 had been transmitted between the domestic animals and the leopard cats. Cross-species transmission of CPPV-1 between domestic and free-living carnivores has been demonstrated or suspected in various countries [11, 42]. Allison et al. [43] found that the amino acid at VP2 position 300 is a key determinant of the host range. The receptor-binding ability and infectivity of VP2 position 300 mutants are differ substantially. Therefore, mutations at VP2 position 300 affect the susceptibility and resistance of a host to infection by the virus. The amino acid at VP2 position 300 of CPV-variants isolated in our study was glycine

(Table 2), which infects the Canidae and Felidae species. In addition, the amino acid at VP2 position 300 of the feline parvovirus strain isolated in our study was alanine, which infects the Felidae species [43]. We found a distinct genotype of CPV-2b isolated from the leopard cats. This result might imply the evolutionary isolation of the genotype in the leopard cat population. However, owing to the limitation of sample size, the analysis of the distribution of this distinct CPV-2b genotype was inconclusive. Furthermore, the amino acid at VP2 position 300 of this CPV-2b genotype was glycine, which indicated the possibility of transmission between the domestic animals and leopard cats [43-45]. The primary reservoir of CPPV-1 was not possible to determine based on the results of our study. Nevertheless, the leopard cat is a critical endangered species in Taiwan and sustained CPPV-1 transmission in this low-density population is questionable [46]. We considered the stray dogs and cats, which exhibited the highest abundance among carnivores in study area, as the primary reservoirs.

The distribution of free-roaming dogs and cats in our study area was similar to that in other rural areas in Taiwan [47, 48]. In addition to a high population density, the vaccination and neutering coverage of free-roaming dogs and cats is usually low in Taiwan [49]. Groups of free-roaming dogs are active in areas with well-developed road systems [37, 50]; this aggravates the transmission of pathogens between dogs

as well as the flow of pathogens into the habitat of leopard cats. The flow of pathogens is highly plausible, particularly for stable pathogens such as CPPV-1, which can retain their infectiousness in the environment for a long period [11]. In this study, we could not discover any associations among the density of free-roaming domestic carnivores, road coverage, and CPPV-1 infection in the leopard cats because of the limitation of our leopard cat sample size and lack of distribution information on free-roaming domestic carnivores. Future studies shouldexamine domestic carnivores' influence on the transmission of CPPV-1 in the habitat.

Relationships between diseases and road kill have been reported [9, 10]. The association between road kill and CPPV-1 infection of the leopard cats implied that CPPV-1 infection reduced the leopard cats' awareness or speed of response to their environment. Except for mass mortality, revealing the effects of diseases on free-living wild animals is challenging and is usually obscured by other apparent factors such as road kill and predation. Even subclinical infections that induce behavioral changes may reduce survival and fitness and make infected animals more susceptible to predation than noninfected ones [10, 51], particularly for endangered species. Most of the VC-affected leopard cats included in our study exhibited severe autolysis. Therefore, analyses of lesions and possible clinical signs induced by

CPPV-1 infection were inconclusive. Nonetheless, histopathological changes and lymphoid cell depletion in the spleen and lymph nodes were commonly observed in the CPPV-1 positive leopard cats. Lymphoid cell depletion is a cause of lesions and is often observed in instances of CPPV-1 infection in the other carnivores [12, 52].

The association between diseases and increased risk of VC has been documented in various host pathogen relationships. However, no specific lesion or abnormal behavior has been identified as associated with the risk of VC. Skin irritation and lesions caused by sarcoptic mange infestation can obstruct hunting behavior and slow down the reaction in coyote, which in turn increasing the risk of VC [51]. Mule deer with chronic wasting disease and Tasmanian pademelons infected with *Toxoplasma gondii* may be at relatively high risk of VC, because the pathogens cause damage to their central nervous system [9, 10]. Additionally, a study determined that humans with asymptomatic infection of *T. gondii* had a higher risk of car accidents compared with the noninfected individuals [53]. We were not able to evaluate lesions from CPPV-1 infection in this study. However, according to the characteristic clinical signs, CPPV-1 infection in various carnivores may dramatically weaken the infected animal and impair its ability to respond quickly to approaching vehicles. Additional studies should be conducted to further evaluate the clinical and pathological effects of CPPV-1 on leopard cats.

Our study revealed CPPV-1 infection in free-living leopard cats. We strongly recommend establishment of efforts to manage of CPPV-1 in the leopard cat habitat with an emphasis on vaccination programs and population control measures for free-roaming dogs and cats. Additionally, previous studies have indicated that because of antigenic differences among CPPV-1 variants, new vaccines that also provide protection against the CPV-2c variantmust be developed [54, 55]. Although we identified an association between road kill and CPPV-1 infection, road development may also negatively affect the leopard cat population. The movement of free-roaming dogs is based on the road system [37, 56]. From the disease transmission perspective, road development may also increase the likelihood of contact and disease transmission between free-roaming domestic animals and wildlife (Fig 3).

Fig 3. Possible relationship of Carnivore protoparvovirus 1 transmission between domestic dogs, cats, and leopard cats.

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Supporting information

S1 Table. Individual information for sampled leopard cats.

S2 Table. Sequences of partial *VP2* included in this study. Sequences were isolated from the leopard cats, domestic dogs and cats, and retrieved from Genbank with accession numbers provided in parenthesis.