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# Phylogenetic Analysis of Feline Immunodeficiency Virus in Feral and Companion Domestic Cats of New Zealand▽

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**Nested PCR was used to amplify envelope V3-V6 gene fragments of feline immunodeficiency virus (FIV) from New Zealand cats. Phylogenetic analyses established that subtypes A and C predominate among New Zealand cats, with clear evidence of intersubtype recombination. In addition, 17 sequences were identified that were distinct from all known FIV clades, and we tentatively suggest these belong to a novel subtype.**

Feline immunodeficiency virus (FIV) is a lentivirus that infects the domestic cat (*Felis catus*), causing progressive immunodeficiency analogous to AIDS in humans (21). Five distinct FIV subtypes have been identified, based on sequence diversity within the V3 to V5 region of the envelope gene (13, 20, 29). A recent study identified a distinct group of FIV isolates from Texas that possibly represent a new subtype (32). In addition, intersubtype recombination has been detected in natural populations (1, 4, 23).

The only previously published study on FIV in New Zealand cats examined the infection status of 250 domestic cats of different sex, age, breed, and health condition (30). In the present study, New Zealand cats were sampled from April 2003 to January 2006. Lymph nodes were dissected from 334 New Zealand feral cats and 28 New Zealand stray cats obtained from seven locations throughout both the North and South Islands of New Zealand. Feral cats are defined as unowned cats that inhabit rural areas, including wooded countryside and parkland, whereas stray cats inhabit urban areas. Blood samples from 48 FIV-symptomatic domestic New Zealand cats were also obtained from a local veterinary diagnostic facility, and a further 29 seropositive New Zealand domestic cat blood samples were sourced from veterinarians. Genomic DNA was extracted from lymph nodes by using QIAamp DNA Minikit (QIAGEN) and from blood samples by using QIAamp DNA blood minikit (QIAGEN).

Nested PCR was used to amplify 858 bp of the FIV envelope gene within the V3 to V6 region. Briefly, a 25-μl reaction containing 0.4 μM concentrations of each primer, 0.2 mM concentrations of each deoxynucleoside triphosphate, and 0.625 U of Platinum *Taq* DNA polymerase was used. In the first round, 2 μl of genomic DNA was added. In the second round, 1 μl from the first round tube was added. The reactions were run on a Biometra T1 thermal cycler. The first-round primers, VE1R and VE1S, amplified a 1,230-bp fragment containing the annealing sites for the second-round primers, VE2R and VE2S (17). Thermal cycling parameters used were as follows: 3 min at 94°C, followed by 5 cycles of 1 min at 94°C, 1 min at 57°C, and 2 min at 72°C, followed in turn by 31 cycles of 15 s at 94°C, 45 s at 57°C, and 1 min at 72°C, followed finally by extension for 2 min at 72°C. PCR products were visualized by electrophoresis on a 1.6% agarose gel at 100 V.

Direct sequencing of PCR products was performed by using a BigDye terminator version 3.1 ready reaction cycle sequencing kit and an Applied Biosystems genetic analyzer AB13730. Sequences were edited in Sequencher v4.1 (Genecodes Corp.). A multiple alignment was constructed by using CLUSTALX v1.81 (11, 12). In Se-Al v2.0a11 Carbon (A. Rambaut, University of Oxford) a 33-bp ambiguously aligned region of V5 was deleted to exclude it from subsequent analyses. The best model of evolution for our data was determined by using Modeltest v3.7 (22). This model, TVM+I+G, allowing for variable rates across sites and a proportion of invariant sites, was used to construct a neighbor-joining (NJ) tree (27) with PAUP* v4.0b10 (31). A nonparametric bootstrap (6) with 1,000 replicates was conducted.

Novel sequences from New Zealand cats were submitted to GenBank (accession numbers EF153955 to EF154083).

FIV subtypes A and C predominate within infected New Zealand cats (Fig. 1). The principal subtype is C, comprising 48% (±4.4%) of all sequences (Table 1). Although there is some clustering of sequences from the same location, such as that seen from Great Barrier Island in subtype A, there is no obvious geographical pattern to the subtypes found throughout NZ. Australia, the country closest to New Zealand, has only subtypes A and, more rarely, B (14). Thus, the high prevalence of FIV-C in New Zealand cats was unexpected. FIV subtype A sequences from three infected Australian cats were included in Fig. 1 to determine whether Australia is a potential country of origin of New Zealand FIV-A. These Australian sequences group within the New Zealand subtype A clade, as did reference FIV-A sequences from other countries; thus, there is no firm evidence that Australia is the origin of New Zealand FIV-A.

A group of 17 New Zealand sequences, 11 of which are from feral cats from one location, did not group with any known subtype on the phylogenetic tree. Fifteen sequences from this group are very closely related (91.0% similarity), suggesting their recent spread in New Zealand cats. All 17 sequences were

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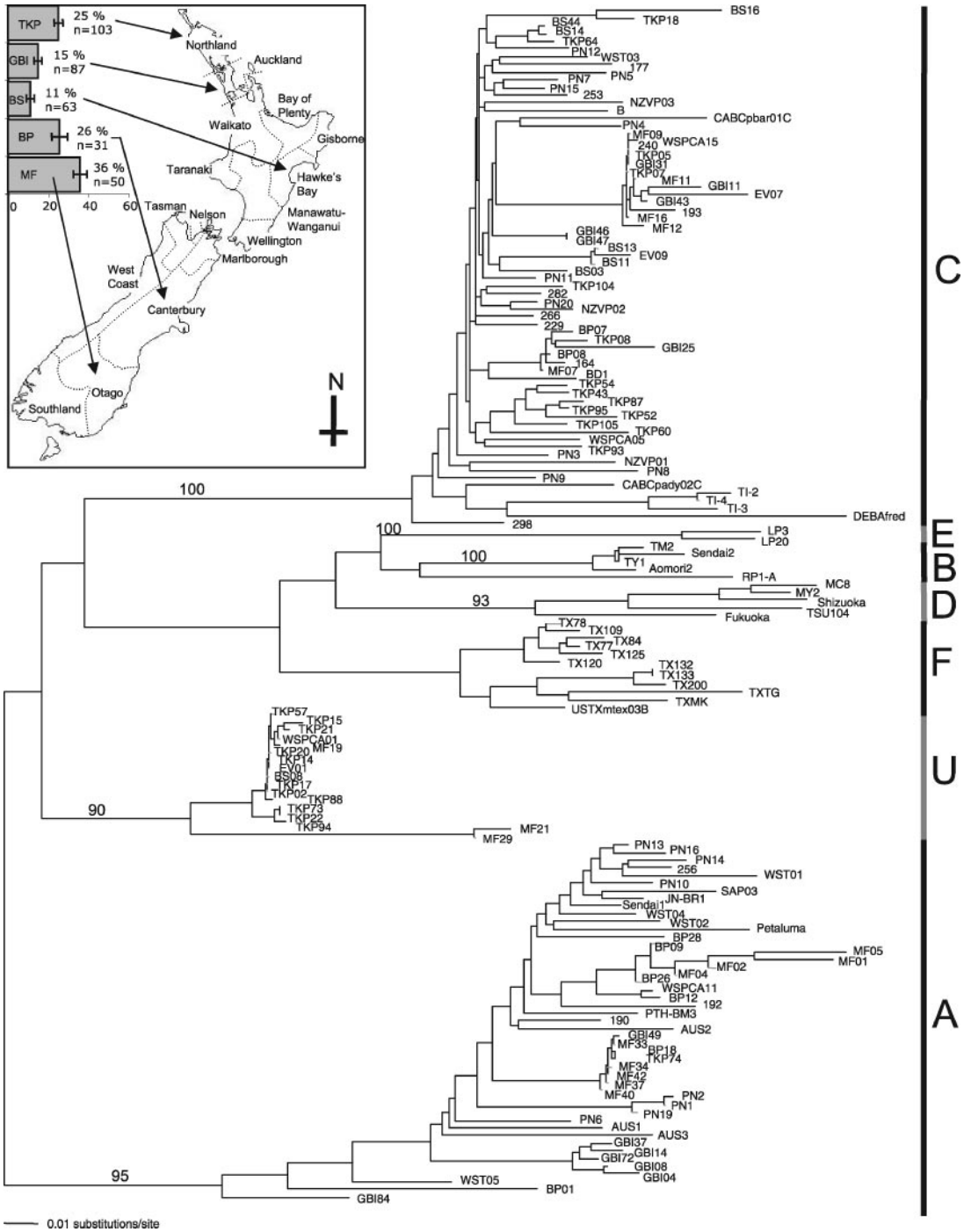


FIG. 1. Midpoint-rooted neighbor-joining tree with map of New Zealand showing regions and feral cat prevalence data. Subtypes are shown along the right side of the tree. F represents sequences from a recent study in Texas (32) with accession numbers U02422, AY139094, AY139095, AY139096, AY139097, AY139098, AY139099, AY139100, AY139101, AY139102, AY139103, and AY139104. U represents sequences from New Zealand that do not group with a known subtype. Three subtype A Australian sequences are included (AUS1, AUS2, and AUS3). The reference sequences downloaded from GenBank include subtype A: JN-BR1 (D67052, Japan), Petaluma (M25381, United States), PTH-BM3 (AB010401, Japan), SAP03 (AB010404, Japan), Sendai1 (D37813, Japan); subtype B: Aomori2 (D37817, Japan), RP1-A (AJ304988, Portugal), Sendai2 (D37814, Japan), TM2 (M59418, Japan), and TY1 (D67064, Japan); subtype C: CABCpady02C (U02392, Canada), CABCpbar01C (U02393, Canada), DEBAfred (U57020, Germany), TI-2 (AB016026, Taiwan), TI-3 (AB016027, Taiwan), and TI-4 (AB016028, Taiwan); subtype D: Fukuoka (D37815, Japan), MC8 (D67062, Japan), MY2 (D67063, Japan), Shizuoka (D37811, Japan), and TSU104 (AB021111, Japan); subtype E: LP3 (D84496, Argentina) and LP20 (D84498, Argentina). Numbers are bootstrap values based on 1,000 replicates.

TABLE 1. FIV-infected NZ cat samples, showing cat lifestyle, location, and FIV subtype

| Sample | Lifestyle | Location[a] | Subtype[b] | Sample | Lifestyle | Location[a] | Subtype[b] |
|---|---|---|---|---|---|---|---|
| 190 | Domestic | Auckland | A | EV09 | Stray | Auckland | C |
| 192 | Domestic | Canterbury | A | WSPCA05 | Stray | Northland | C |
| 256 | Domestic | Hawke's Bay | A | WSPCA15 | Stray | Northland | C |
| PN1 | Domestic | Wellington | A | BP07 | Feral | Canterbury | C |
| PN2 | Domestic | Wellington | A | BP08 | Feral | Canterbury | C |
| PN6 | Domestic | Manawatu-Wanganui | A | BS03 | Feral | Hawke's Bay | C |
| PN10 | Domestic | Wellington | A | BS11 | Feral | Hawke's Bay | C |
| PN13 | Domestic | Taranaki | A | BS13 | Feral | Hawke's Bay | C |
| PN14 | Domestic | Manawatu-Wanganui | A | BS14 | Feral | Hawke's Bay | C |
| PN16 | Domestic | Taranaki | A | BS16 | Feral | Hawke's Bay | C |
| PN19 | Domestic | Wellington | A | BS44 | Feral | Hawke's Bay | C |
| WST01 | Domestic | West Coast | A | GBI11 | Feral | Auckland | C |
| WST02 | Domestic | West Coast | A | GBI25 | Feral | Auckland | C |
| WST04 | Domestic | NA | A | GBI31 | Feral | Auckland | C |
| WST05 | Domestic | NA | A | GBI43 | Feral | Auckland | C |
| WSPCA11 | Stray | Northland | A | GBI46 | Feral | Auckland | C |
| BP01 | Feral | Canterbury | A | GBI47 | Feral | Auckland | C |
| BP09 | Feral | Canterbury | A | MF07 | Feral | Otago | C |
| BP12 | Feral | Canterbury | A | MF09 | Feral | Otago | C |
| BP18 | Feral | Canterbury | A | MF11 | Feral | Otago | C |
| BP26 | Feral | Canterbury | A | MF12 | Feral | Otago | C |
| BP28 | Feral | Canterbury | A | MF16 | Feral | Otago | C |
| GBI04 | Feral | Auckland | A | TKP05 | Feral | Northland | C |
| GBI08 | Feral | Auckland | A | TKP07 | Feral | Northland | C |
| GBI14 | Feral | Auckland | A | TKP08 | Feral | Northland | C |
| GBI37 | Feral | Auckland | A | TKP18 | Feral | Northland | C |
| GBI49 | Feral | Auckland | A | TKP43 | Feral | Northland | C |
| GBI72 | Feral | Auckland | A | TKP52 | Feral | Northland | C |
| GBI84 | Feral | Auckland | A | TKP54 | Feral | Northland | C |
| MF01 | Feral | Otago | A | TKP60 | Feral | Northland | C |
| MF02 | Feral | Otago | A | TKP64 | Feral | Northland | C |
| MF04 | Feral | Otago | A | TKP87 | Feral | Northland | C |
| MF05 | Feral | Otago | A | TKP93 | Feral | Northland | C |
| MF33 | Feral | Otago | A | TKP95 | Feral | Northland | C |
| MF34 | Feral | Otago | A | TKP104 | Feral | Northland | C |
| MF37 | Feral | Otago | A | TKP105 | Feral | Northland | C |
| MF40 | Feral | Otago | A | 168 | Domestic | West Coast | PR |
| MF42 | Feral | Otago | A | 197 | Domestic | Nelson | PR |
| TKP74 | Feral | Northland | A | 214 | Domestic | Waikato | PR |
| 164 | Domestic | Wellington | C | 258 | Domestic | Bay of Plenty | PR |
| 177 | Domestic | Southland | C | 259 | Domestic | Taranaki | PR |
| 193 | Domestic | Canterbury | C | 260 | Domestic | Bay of Plenty | O |
| 229 | Domestic | Auckland | C | PN17 | Domestic | Wellington | PR |
| 240 | Domestic | NA | C | PN21 | Domestic | Manawatu-Wanganui | PR |
| 253 | Domestic | Waikato | C | PN22 | Domestic | Manawatu-Wanganu | O |
| 266 | Domestic | Taranaki | C | PN23 | Domestic | Wellington | PR |
| 282 | Domestic | NA | C | MF14 | Feral | Otago | PR |
| 298 | Domestic | NA | C | EV01 | Stray | Auckland | U |
| B | Domestic | Auckland | C | WSPCA01 | Stray | Northland | U |
| BD1 | Domestic | Auckland | C | BS08 | Feral | Hawke's Bay | U |
| NZVP01 | Domestic | Wellington | C | MF19 | Feral | Otago | U |
| NZVP02 | Domestic | Wellington | C | MF21 | Feral | Otago | U |
| NZVP03 | Domestic | NA | C | MF29 | Feral | Otago | U |
| PN3 | Domestic | Hawke's Bay | C | TKP02 | Feral | Northland | U |
| PN4 | Domestic | NA | C | TKP14 | Feral | Northland | U |
| PN5 | Domestic | Wellington | C | TKP15 | Feral | Northland | U |
| PN7 | Domestic | Wellington | C | TKP17 | Feral | Northland | U |
| PN8 | Domestic | Manawatu-Wanganui | C | TKP20 | Feral | Northland | U |
| PN9 | Domestic | Manawatu-Wanganui | C | TKP21 | Feral | Northland | U |
| PN11 | Domestic | Wellington | C | TKP22 | Feral | Northland | U |
| PN12 | Domestic | Manawatu-Wanganui | C | TKP57 | Feral | Northland | U |
| PN15 | Domestic | Wellington | C | TKP73 | Feral | Northland | U |
| PN20 | Domestic | Wellington | C | TKP88 | Feral | Northland | U |
| WST03 | Domestic | West Coast | C | TKP94 | Feral | Northland | U |
| EV07 | Stray | Auckland | C | | | | |

[a] NA, not available. Locations by region are shown on the map in Fig. 1.
[b] U, unknown subtype; O, outlier but not labeled putative recombinant since not significant as determined by KH test; PR, putative recombinant.

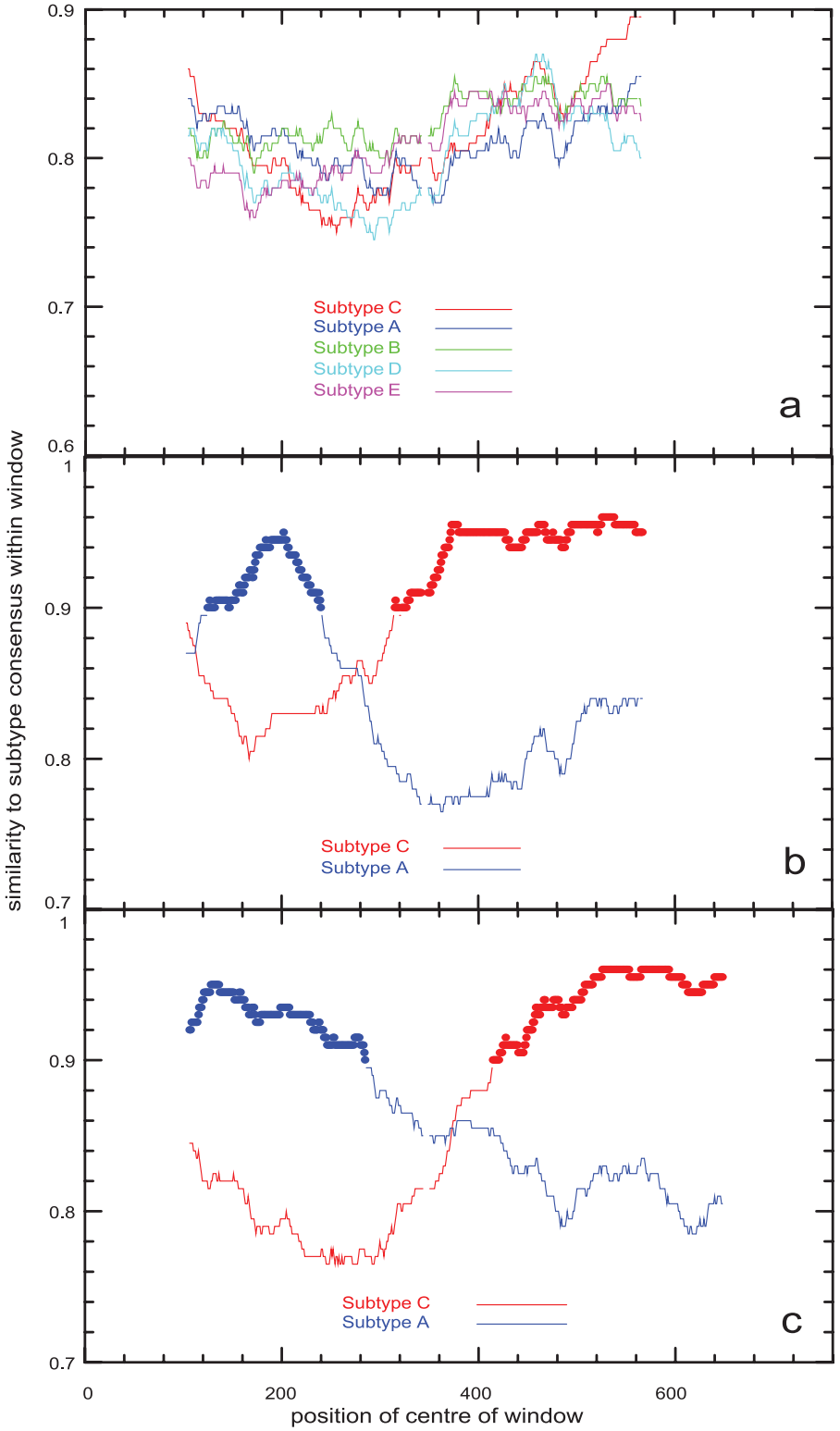


FIG. 2. RIP graphical outputs of TKP21, a sequence from the "unknown" group (a); 214 (b) and 197 (c), two outlier sequences. The plots represent the distance between the query sequence and the subtype A (blue) or subtype C (red) reference sequence, over a sliding window of size 200 bp. In panels b and c, there is a clear point (the putative recombinant crossover point) at which the query sequences cease to be closer to A and become closer to C. In panel a, all five known subtypes are compared to the query sequence, with no significant similarity, although at the very end of the sequenced region there is some similarity to subtype C.

TABLE 2. Kishino-Hasegawa test results of the eleven sequences ambiguously positioned on the phylogenetic tree

| Sequence[a] | Diff −lnL[b] | P[c] |
|---|---|---|
| First[d] | | |
| 168† | 67.635 | 0.000* |
| 197 | 85.935 | 0.000* |
| 214 | 28.094 | 0.006* |
| 258† | 108.153 | 0.000* |
| 259† | 68.407 | 0.000* |
| 260 | 2.656 | 0.344 |
| MF14 | 29.355 | 0.005* |
| PN17 | 72.269 | 0.000* |
| PN21† | 35.537 | 0.002* |
| PN22 | 62.599 | 0.000* |
| PN23† | 66.752 | 0.000* |
| Second[e] | | |
| 168 | 31.741 | 0.006* |
| 197 | 51.234 | 0.000* |
| 214 | 117.730 | 0.000* |
| 258 | 31.743 | 0.002* |
| 259 | 25.720 | 0.035* |
| 260 | 40.658 | 0.000* |
| MF14 | 116.522 | 0.000* |
| PN17 | 23.612 | 0.047* |
| PN21 | 61.732 | 0.000* |
| PN22 | 20.601 | 0.092 |
| PN23 | 27.922 | 0.019* |

[a] †, Samples for which endpoint dilutions were performed.
[b] The difference in log-likelihood scores between the best tree for that subalignment against the best tree for the alternative subalignment.
[c] The probability of obtaining that difference in log-likelihoods by chance alone, given that the true trees are identical. *, *P* values significant at the 0.05 level. Note that, to be conservative, we only identify a sequence as a putative recombinant if both subalignments produce trees that are mutually (statistically) different.
[d] The subalignment of sequences using the query sequence before the putative crossover point identified by RIP.
[e] The subalignment of sequences using the query sequence after the putative crossover point identified by RIP.

analyzed by using a recombination identification program (RIP) (28; http://hivweb.lanl.gov/RIP/RIPsubmit.html) to test for recombination. None of these sequences showed any significant similarity to any previously described subtype (Fig. 2a), nor did they appear to be recombinants of known subtypes. Two lymph node samples, one that yielded FIV sequence of subtype C and another of the "unknown" subtype, were amplified and sequenced independently at the School of Veterinary Science, University of Queensland, in a blind, independent check for contamination. These sequences were almost identical (99%) to those we obtained, thus confirming the novelty of the "unknown" group. We note that the suggested requirements for designating a new human immunodeficiency virus subtype requires three genome sequences from epidemiologically independent infected individuals (24). While FIV subtype designation has been based on V3-V5 *env* sequence (29), we are currently sequencing the *pol* and *gag* genes to provide further sequence data to validate the "unknown" group as a novel NZ-specific subtype.

Eleven other sequences that did not group with any subtype were tested for recombination by using RIP (Fig. 2b and c). A Kishino-Hasegawa (KH) test (9, 10, 15) implemented in PAUP* was used to further investigate recombination. Thirteen reference sequences were used in each KH test: five subtype A sequences, five subtype C sequences, and one sequence of each subtype B, D, and E. The results from this test (Table 2) show that, conservatively, 9 of the 11 samples can be assigned as putative recombinants of subtypes A and C.

To exclude PCR-mediated recombination as a potential source for these putative recombinants (16, 18), we used endpoint dilution (25, 26) on representative samples from which recombinants were detected. Because of limited sample availability, we were only able to perform these tests on five of the nine putative recombinants (Table 2). A fivefold serial dilution was used, and the PCR results were entered into QUALITY (http://ubik.microbiol.washington.edu/computing/quality/jquality.htm) to determine the proviral copy number. Samples were then diluted to an endpoint and amplified to give at least five sequences. In all cases, the endpoint sequences were at least 98% similar to the respective consensus sequences and to each other. Consequently, these recombinants are confirmed as authentic viral sequences, and there is no evidence of dual infection.

Of the 334 feral cats tested, 72 (21.5% ± 2.25%) were positive for FIV proviral DNA. This is generally higher than other feral cat populations globally (2, 8, 19, 33; however, see also reference 3). The FIV prevalence in feral cats from the five New Zealand locations ranges from 11 to 36%, with no obvious geographic pattern (Fig. 1).

More than two-thirds of the FIV-infected feral cats were male, a statistically significant difference ($\chi^2 = 14.16$, df = 1, $P \leq 0.001$), and adult male cats had the highest prevalence of 51% ($n = 70$). This trend is expected because mature male cats demonstrate higher levels of social aggression, caused by competition over mates and territories (5, 7). Thus, mature male cats tend to have a greater number of biting encounters, the typical mode of transmission of FIV (34).

This study is the first to use phylogenetics to analyze FIV in New Zealand cats. Two subtypes and nine putative A/C recombinant strains are identified in New Zealand cats. A novel clade was detected, possibly representing a New Zealand-specific subtype.

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