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| **Table S2.** Characterization of high frequency single nucleotide variants, defined as having an intrahost frequency greater than 2%, that arose during West Nile virus passaging in wild-caught birds, young chickens and *Culex quinquefasciatus* mosquitoes. | | | | | | | | |
| **Site** | **Coding region** | **Nucleotide change** | **Ts/Tv** | **Codon position** | **S/N** | **Amino acid change** | **Highest**  **frequency** | **Passage of highest frequency** |
| 101 | C | CT | Ts | 2 | N | S2F | 0.225 | sparrpw p2ca |
| 111 | C | AT | Tv | 3 | S |  | 0.190 | sparrow p1a |
| 148 | C | AG | Ts | 1 | N | K18E | 0.022 | crow p2c |
| 202 | C | AT | Tv | 1 | N | S36C | 0.024 | robin p2c |
| 340 | C | CT | Ts | 1 | S |  | 0.042 | robin p4b |
| 369 | C | GA | Ts | 3 | S |  | 0.715 | sparrow p5a |
| 407 | C | AG | Ts | 2 | N | K104R | 0.042 | robin p4b |
| 432 | C | AG | Ts | 3 | S |  | 0.020 | sparrow p4b |
| 539 | prM | TC | Ts | 2 | N | I25T | 0.020 | robin p3b\* |
| 547 | prM | GA | Ts | 1 | N | A28T | 0.020 | robin p5a |
| 683 | prM | CT | Ts | 3 | N | A73V | 0.030 | crow p2c |
| 779 | prM | CT | Ts | 2 | N | A105V | 0.023 | crow p5a |
| 818 | prM | CT | Ts | 3 | N | T118I | 0.036 | crow p3c |
| 925 | prM | GA | Ts | 1 | N | V154M | 0.021 | crow p5a |
| 945 | prM | TC | Ts | 3 | S |  | 0.077 | robin p5a |
| 951 | prM | GA | Ts | 3 | S |  | 0.032 | crow p5b |
| 997 | E | TC | Ts | 1 | N | F11L | 0.028 | robin p3b |
| 1032 | E | TA | Tv | 3 | N | D22E | 0.049 | mosquito p1a |
| 1071 | E | TC | Ts | 3 | S |  | 0.832 | robin p5c |
| 1102 | E | AG | Ts | 1 | N | M46V | 0.293 | robin p5a |
| 1117 | E | GA | Ts | 1 | N | A51T | 0.088 | robin p5a |
| 1170 | E | CT | Ts | 3 | S |  | 0.032 | crow p4b |
| 1179 | E | AG | Ts | 3 | S |  | 0.077 | crow p1a |
| 1205 | E | CT | Ts | 2 | N | A80V | 0.191 | robin p5b |
| 1238 | E | TC | Ts | 2 | N | V91A | 0.023 | mosquito p1b |
| 1272 | E | CT | Ts | 3 | S |  | 0.046 | chicken p1a |
| 1395 | E | CT | Ts | 3 | S |  | 0.101 | sparrpw p4c |
| 1440 | E | GT | Tv | 3 | N | Q158H | 0.037 | robin p3b |
| 1483 | E | GA | Ts | 1 | N | A173T | 0.031 | sparrow p3a |
| 1491 | E | AG | Ts | 3 | S |  | 0.020 | sparrpw p1c |
| 1496 | E | CT | Ts | 2 | N | T177I | 0.043 | robin p3c |
| 1545 | E | GA | Ts | 3 | S |  | 0.728 | sparrow p5a |
| 1557 | E | TC | Ts | 3 | S |  | 0.672 | sparrow p2a |
| 1567 | E | TC | Ts | 1 | N | Y201H | 0.056 | robin p2c |
| 1569 | E | CT | Ts | 3 | S |  | 0.357 | crow p4b |
| 1661 | E | TC | Ts | 2 | N | V232A | 0.028 | robin p4c |
| 1701 | E | AG | Tv | 3 | S |  | 0.027 | robin p4c |
| 1893 | E | CT | Ts | 3 | S |  | 0.023 | crow p5b |
| 1938 | E | CT | Ts | 3 | S |  | 0.097 | sparrpw p4c |
| 1974 | E | TC | Ts | 3 | S |  | 0.160 | chicken p1b |
| 2007 | E | CT | Ts | 3 | S |  | 0.048 | crow p5c |
| 2029 | E | TC | Ts | 1 | S |  | 0.021 | chicken p1a |
| 2077 | E | GT | Tv | 1 | N | V371F | 0.036 | robin p3b |
| 2114 | E | AT | Tv | 2 | N | Y383F | 0.024 | chick p1a |
| 2152 | E | CT | Ts | 1 | N | H396Y | 0.020 | sparrpw p2c |
| 2202 | E | CT | Ts | 3 | S |  | 0.022 | sparrpw p3c |
| 2321 | E | TC | Ts | 2 | N | L452S | 0.032 | crow p5a |
| 2345 | E | TC | Ts | 2 | N | I460T | 0.041 | robin p3b |
| 2346 | E | AG | Tv | 3 | N | I460M | 0.814 | robin p5c |
| 2518 | NS1 | AC | Tv | 1 | N | S17R | 0.515 | crow p1a |
| 2601 | NS1 | GA | Ts | 3 | S |  | 0.023 | sparrow p3b |  |
| 2607 | NS1 | TC | Ts | 3 | S |  | 0.058 | robin p3c |  |
| 2648 | NS1 | TC | Ts | 2 | N | V60A | 0.030 | robin p3b |  |
| 2679 | NS1 | AT | Tv | 3 | S |  | 0.035 | crow p2a |  |
| 2688 | NS1 | CA | Tv | 3 | N | D73E | 0.032 | sparrpw p1c\* |  |
| 2734 | NS1 | GT | Tv | 1 | N | V89L | 0.067 | robin p3b |  |
| 2833 | NS1 | AG | Ts | 1 | N | I122V | 0.054 | robin p5a |  |
| 2856 | NS1 | CT | Ts | 3 | S |  | 0.151 | sparrpw p5c |  |
| 3123 | NS1 | TG | Tv | 3 | S |  | 0.020 | sparrow p3a |  |
| 3156 | NS1 | TC | Ts | 3 | S |  | 0.028 | robin p4a |  |
| 3162 | NS1 | GA | Ts | 3 | S |  | 0.047 | crow p5b |  |
| **Table S2 continued.** | | | | | | | | |
| **Site** | **Coding region** | **Nucleotide change** | **Ts/Tv** | **Codon position** | **S/N** | **Amino acid change** | **Highest**  **frequency** | **Passage of highest frequency** |
| 3186 | NS1 | TC | Ts | 3 | S |  | 0.034 | robin p3b |
| 3219 | NS1 | AC | Tv | 3 | S |  | 0.054 | mosquito p1a |
| 3243 | NS1 | TC | Ts | 3 | S |  | 0.033 | mosquito p1bb\* |
| 3300 | NS1 | CT | Ts | 3 | S |  | 0.023 | crow p5c |
| 3306 | NS1 | CT | Ts | 3 | S |  | 0.037 | crow p2c |
| 3318 | NS1 | TC | Ts | 3 | S |  | 0.029 | robin p5a |
| 3327 | NS1 | CT | Ts | 3 | S |  | 0.119 | robin p5ac |
| 3340 | NS1 | TG | Tv | 1 | N | C291G | 0.048 | robin p4c |
| 3467 | NS1 | TC | Ts | 2 | N | M333T | 0.030 | mosquito p1b |
| 3555 | NS2A | GA | Ts | 3 | S |  | 0.056 | crow p5b |
| 3561 | NS2A | CT | Ts | 3 | S |  | 0.117 | robin p5a |
| 3684 | NS2A | TC | Ts | 3 | S |  | 0.027 | sparrow p3ad |
| 3749 | NS2A | TC | Ts | 2 | N | V75A | 0.047 | sparrow p4b |
| 3819 | NS2A | AG | Ts | 3 | S |  | 0.027 | sparrow p3b |
| 3852 | NS2A | GA | Ts | 3 | S |  | 0.039 | crow p4b |
| 3861 | NS2A | TC | Ts | 3 | S |  | 0.042 | robin p5a |
| 3893 | NS2A | AG | Ts | 2 | N | Q123R | 0.080 | sparrow p3be |
| 3899 | NS2A | TC | Ts | 2 | N | L125P | 0.056 | sparrow p3a |
| 3952 | NS2A | CT | Ts | 1 | S |  | 0.832 | robin p5c |
| 3997 | NS2A | CT | Ts | 1 | S |  | 0.048 | mosquito p1b |
| 4110 | NS2A | AG | Ts | 3 | S |  | 0.139 | crow p5b |
| 4221 | NS2B | AG | Ts | 3 | S |  | 0.129 | robin p5c |
| 4230 | NS2B | AG | Ts | 3 | S |  | 0.030 | crow p4b |
| 4318 | NS2B | AG | Ts | 1 | N | T34A | 0.026 | sparrpw p2c |
| 4332 | NS2B | CT | Ts | 3 | S |  | 0.021 | mosquito p1b |
| 4340 | NS2B | CT | Ts | 2 | N | A41V | 0.027 | sparrow p3a |
| 4392 | NS2B | GA | Ts | 3 | S |  | 0.037 | sparrpw p2c |
| 4484 | NS2B | AG | Ts | 2 | N | N89S | 0.020 | crow p5b |
| 4527 | NS2B | CT | Ts | 3 | S |  | 0.195 | robin p5b |
| 4530 | NS2B | TC | Ts | 3 | S |  | 0.719 | sparrow p5a |
| 4573 | NS2B | GA | Ts | 1 | N | V119I | 0.116 | robin p5a |
| 4596 | NS2B | CT | Ts | 3 | S |  | 0.054 | crow p3b |
| 4668 | NS3 | CT | Ts | 3 | S |  | 0.032 | chicken p1a |
| 4704 | NS3 | CT | Ts | 3 | S |  | 0.020 | crow p4c |
| 4749 | NS3 | CT | Ts | 3 | S |  | 0.086 | chick p1a |
| 4813 | NS3 | TC | Ts | 1 | N | Y68H | 0.027 | mosquito p1a |
| 4998 | NS3 | CT | Ts | 3 | S |  | 0.184 | robin p3a |
| 5002 | NS3 | CT | Ts | 1 | N | P131S | 0.020 | sparrpw p3c |
| 5136 | NS3 | CT | Ts | 3 | S |  | 0.023 | crow p5b |
| 5157 | NS3 | GT | Tv | 3 | N | E182D | 0.041 | robin p1b |
| 5184 | NS3 | AG | Ts | 3 | S |  | 0.025 | robin p2c |
| 5240 | NS3 | AG | Ts | 2 | N | K210R | 0.025 | robin p2a |
| 5469 | NS3 | GA | Ts | 3 | S |  | 0.023 | crow p5b |
| 5472 | NS3 | TC | Ts | 3 | S |  | 0.497 | sparrpw p5c |
| 5629 | NS3 | AG | Ts | 1 | N | I340V | 0.028 | robin p3b |
| 5668 | NS3 | AT | Tv | 1 | N | T353S | 0.020 | robin p1a |
| 5801 | NS3 | AG | Ts | 2 | N | K397R | 0.023 | chicken p1a |
| 5816 | NS3 | AT | Tv | 2 | N | D402V | 0.541 | mosquito p1b |
| 5874 | NS3 | CT | Ts | 3 | S |  | 0.034 | crow p4b |
| 5889 | NS3 | CT | Ts | 3 | S |  | 0.020 | crow p4c |
| 6393 | NS3 | GT | Tv | 3 | S |  | 0.032 | sparrpw p1c |  |
| 6410 | NS3 | GT | Tv | 2 | N | R600M | 0.027 | robin p3b |  |
| 6420 | NS3 | GA | Ts | 2 | S |  | 0.039 | robin p5c |  |
| 6481 | NS4A | CT | Ts | 1 | N | L5F | 0.038 | robin p5a\* |  |
| 6543 | NS4A | CT | Ts | 3 | S |  | 0.125 | robin p5a |  |
| 6644 | NS4A | TC | Ts | 2 | N | L59S | 0.025 | crow p5a |  |
| 6672 | NS4A | CT | Ts | 3 | S |  | 0.063 | crow p5b |  |
| 6711 | NS4A | TA | Tv | 3 | S |  | 0.133 | sparrow p4a |  |
| 6734 | NS4A | TC | Ts | 2 | N | V89A | 0.641 | mosquito p1a |  |
| 6780 | NS4A | CT | Ts | 3 | S |  | 0.047 | crow p4b |  |
| 6849 | NS4A | GA | Ts | 3 | S |  | 0.033 | sparrpw p4c |  |
| 6871 | 2K | GA | Ts | 1 | N | V13M | 0.139 | sparrow p2a |  |

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| **Table S2 continued.** | | | | | | | | | |
| **Site** | **Coding region** | **Nucleotide change** | **Ts/Tv** | **Codon position** | **S/N** | | **Amino acid change** | **Highest**  **frequency** | **Passage of highest frequency** |
| 6879 | 2K | GA | Ts | 3 | S | |  | 0.043 | sparrpw p1c |
| 7095 | NS4B | CT | Ts | 3 | S | |  | 0.027 | robin p4c\* |
| 7236 | NS4B | CT | Ts | 3 | S | |  | 0.025 | robin p5a |
| 7320 | NS4B | CT | Ts | 3 | S | |  | 0.125 | robin p5a |
| 7362 | NS4B | TC | Ts | 3 | S | |  | 0.022 | sparrpw p2c |
| 7648 | NS4B | AT | Tv | 1 | N | | I245L | 0.718 | sparrow p5a |
| 7754 | NS5 | CT | Ts | 2 | N | |  | 1.000 | mosquito p1b |
| 7825 | NS5 | GA | Ts | 1 | N | | A49I | 0.048 | robin p4b |
| 7826 | NS5 | CT | Ts | 2 | N | | A49I | 0.048 | robin p4b |
| 8076 | NS5 | GA | Ts | 3 | S | |  | 0.027 | crow p1a |
| 8121 | NS5 | CT | Ts | 3 | S | |  | 0.042 | crow p5b |
| 8148 | NS5 | TC | Ts | 3 | S | |  | 0.023 | sparrow p3a |
| 8220 | NS5 | CT | Ts | 3 | S | |  | 0.123 | sparrpw p5c |
| 8261 | NS5 | AG | Ts | 2 | N | | K194R | 0.078 | robin p4c |
| 8289 | NS5 | GT | Tv | 3 | S | |  | 0.023 | robin p3b |
| 8499 | NS5 | CT | Ts | 3 | S | |  | 0.028 | robin p3b |
| 8553 | NS5 | TC | Ts | 3 | S | |  | 0.038 | crow p5c |
| 8679 | NS5 | AC | Tv | 3 | S | |  | 0.062 | sparrow p1b |
| 8883 | NS5 | CT | Ts | 3 | S | |  | 0.032 | crow p2c |
| 9039 | NS5 | TC | Ts | 3 | S | |  | 0.038 | crow p2a |
| 9054 | NS5 | AG | Ts | 3 | S | |  | 0.021 | robin p5a |
| 9086 | NS5 | AG | Ts | 2 | N | | K469R | 0.671 | mosquito p1a |
| 9136 | NS5 | CT | Ts | 1 | S | |  | 0.022 | robin p5a |
| 9285 | NS5 | TC | Ts | 3 | S | |  | 0.038 | chick p1c |
| 9704 | NS5 | TC | Ts | 2 | N | | L675P | 0.035 | sparrow p3a |
| 9730 | NS5 | CT | Ts | 1 | N | | H684Y | 0.030 | robin p1c |
| 9759 | NS5 | CT | Ts | 3 | S | |  | 0.026 | crow p5c |
| 9783 | NS5 | GA | Ts | 3 | S | |  | 0.033 | robin p2c |
| 10243 | NS5 | CT | Ts | 1 | S | |  | 0.681 | sparrow p5a |
| 10326 | NS5 | GA | Ts | 3 | S | |  | 0.089 | sparrow p5a |
| 10339 | NS5 | TC | Ts | 1 | N | | Y887H | 0.106 | robin p5bf |
| 10375 | NS5 | TC | Ts | 1 | S | |  | 0.029 | sparrow p3a |
|  | | | | | |

C, capsid; prM, pre-membrane; E, envelope; NS, nonstructural; Ts, transition; Tv, transversion; N, nonsynonymous; S, synonymous.\*, also detected in input p0 virus. a, also detected in sparrow replicate “a”. b, also detected in robin replicate “b”. c, also detected in sparrow replicate “c”. d, also detected in crow replicate “c”. e, also detected in sparrow replicate “a”. f, also detected in crow replicate “b”.