Co-circulation of both low and highly pathogenic H5 avian influenza viruses in current epidemics in Taiwan

Supplementary Data

Validation of the sequencing approach

We evaluated our sequencing approach by first testing cultured viruses, PR8 (H1N1) and A/Ck/TW/0702/2013 (H6N1). Comparisons of the two viruses' sequences generated by our pipeline with sequences generated by Sanger's method showed that there were ten and zero Single nucleotide variants (SNV), respectively, in whole genomes (~13000 bases) (Supplementary Table 3). Secondly, crude SNV calling from piled-up reads using Samtools v1.9, were compared with SNVs detected by Nanopolish (described in Methods). Since most samples possessed over 100x depth of coverage in each gene, we expected the two methods to produce a similar outcome. As shown in Supplementary Table 3, differences between SNVs found by the two methods were all below 20 among the coding regions in each sample. Third, a number of positions with minor variants, which have support fractions > 0.3 and < 0.6, were reported for each sample, demonstrating little contamination (Supplementary Table 1). Finally, we repeated the sequencing procedure from RNA extraction from A1 sample, where A/chicken/Taiwan/A1/2019 (H5N2) was identified, to compare results from libraries containing different read depths (Supplementary Table 3 & Supplementary Figure 6). The result shows only one difference in SNV between these two conducts (Supplementary Table 3).

Supplementary Table 1. Summary of the hosts and subtypes of avian influenza outbreaks in Taiwan, 2015–2018.

	2015	2016	2017	2018
No of outbreak farms	944	37	182	98
Chicken	145	18	105	33
Duck	98	4	55	46
Goose	681	14	13	12
Turkey	18	1	9	7
Mixed	2	0	0	0
LPAI	60(100%)	5(100%)	19(100%)	26(100%)
H5N2	53(88.3)	2(40%)	12(63.2%)	25(96.2%)
H6N1	1(1.7%)	0(0%)	0(0%)	0(0%)
H5N6	0(0%)	0(0%)	2(10.5%)	1(3.8%)
Non-typing	6(10%)	3(60%)	5(26.3%)	0(0%)
HPAI	944(100%)	37(100%)	182(100%)	98(100%)
H5N2	540 (57.2%)	25 (67.6%)	150 (82.4%)	93 (94.9%)
H5N8	232 (24.6%)	4 (10.8%)	10 (5.5%)	1 (1.0%)
H5N3	21 (2.2%)	0 (0%)	0 (0%)	0 (0%)
H5N6	0 (0%)	0 (0%)	11 (6.0%)	0 (0%)
Mixed H5N2 and H5N8	68 (7.2%)	0 (0%)	3 (1.6%)	0 (0%)
Mixed H5N2 and H5N3	2 (0.21%)	0 (0%)	0 (0%)	0 (0%)
Mixed H5N2 and H5N6	0 (0%)	0 (0%)	1 (0.6%)	0 (0%)
Non-subtyping	81 (8.6%)	8 (21.6%)	7 (3.9%)	4 (4.1%)

Supplementary Table 2. List of database accession numbers from GenBank and GISAID used in the genetic analyses for each gene phylogeny.

PB2	PB1	PA	HA	NP	NA	MP	NS
KT327371	KT327372	KT327373	EPI860712	KT327374	AY573918	KT327376	KT327377
EPI588965	EPI588966	EPI588967	MG965144	EPI588969	KF193388	EPI588971	EPI588972
KC535016	KC535015	KC535014	MG965800	KC535012	AB507266	KC535011	KC535013
AB507255	AB507256	AB507257	MG964808	AB507258	KJ162590	AB507259	AB507260
KF193393	KF193392	KF193391	KX838899	KF193389	KF193396	KF193387	KF193390
AB507261	AB507262	AB507263	KP307957	AB507265	KR137688	AB507267	AB507268
KJ162585	KJ162586	KJ162587	MG964760	KJ162589	KR137680	KJ162591	KJ162592
KF193401	KF193400	KF193399	EPI586534	KF193397	EPI961924	KF193395	KF193398
KR137683	KR137684	KR137685	EPI587521	KR137687	KU646895	KR137689	KR137690
KR137675	KR137676	KR137677	KU201752	KR137679	KJ162806	KR137681	KR137682
EPI961919	EPI961920	EPI961921	EPI953870	EPI961923	KJ162718	EPI961925	EPI961926
KU646890	KU646891	KU646892	KR150901	KU646894	KC785067	KU646896	KU646897
EPI588973	EPI588974	EPI588975	LC026477	EPI588977	KP792310	EPI588979	EPI588980
EPI961928	EPI961930	EPI961931	EPI573638	EPI961934	MK592423	EPI961938	EPI961939
KJ162801	KJ162802	KJ162803	EPI553208	KJ162805	EPI866809	KJ162807	KJ162808
EPI915867	EPI915868	EPI915869	EPI573208	EPI915871	CY146574	EPI915873	EPI915874
KJ162713	KJ162714	KJ162715	KJ509036	KJ162717	EPI866785	KJ162719	KJ162720
KC785066	KC785065	KC785068	EPI547673	KC785071	EPI855811	KC785064	KC785069
KP792305	KP792306	KP792307	EPI555126	KP792309	GU052700	KP792311	KP792312
KT327385	KT327386	KT327387	EPI550848	KT327388	CY005833	KT327390	KT327391
KT388465	KT388466	KT388467	EPI553349	KT388469	JX570864	KT388471	KT388472
KU646930	KU646931	KU646932	EPI573196	KU646934	KU646863	KU646936	KU646937
KT388449	KT388450	KT388451	KJ756630	KT388453	GU050732	KT388455	KT388456
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EPI1119037	EPI1119048	EPI1119057	EPI837564	EPI1119072		EPI1119074	EPI1119075
EPI866831	EPI866832	EPI866830	EPI837584	EPI866827		EPI866829	EPI866828
MK592418	MK592419	MK592420	KJ511812	MK592422		MK592424	MK592425
MN208016	MN208017	MN208014	KJ413842	MN208011		MN208010	MN208015
MF693922	MF694063	MF694032	KJ509164	MF694210		MF694049	MF693947
MN171423	MN171424	MN171425	KJ508988	MN171427		MN171429	MN171430
MK978901	MK978902	MK978903	KT936689	MK978905		MK978907	MK978908
EPI855652	EPI855653	EPI855651	EPI573199	EPI855648		EPI855650	EPI855649
EPI866807	EPI866808	EPI866806	KJ509100	EPI866803		EPI866805	EPI866804
EPI867653	EPI867654	EPI867652	EPI573230	EPI867649		EPI867651	EPI867650
MN378542	KX949434	KX949435	EPI837541	MN378546		KX949439	KX949440
MH579386	MN378543	MN378544	EPI888267	MH579390		MN378548	MN378549
MH579402	MH579387	MH579388	EPI573229	MH579406		MH579392	MH579393
EPI1139887	MH579403	MH579404	EPI837563	EPI1139891		MH579408	MH579409
MG198953	EPI1139888	EPI1139889	EPI888264	MG198937		EPI1139893	EPI1139894
LC339728	MG198949	MG198945	EPI573209	LC339732		MG198929	MG198941
MK592578	LC339729	LC339730	EPI573198	MK592582		LC339734	LC339735

EPI855748	MK592579	MK592580	EPI573206	EPI855744		MK592584	MK592585
KJ200828	EPI855751	EPI855747	EPI573205	KJ200824		EPI855746	EPI855745
EPI1333694	KJ200827	KJ200826	KJ508948	EPI1333697		KJ200822	KJ200825
MH592159	EPI1333695	EPI1333696	KJ508956	MH592283		EPI1333698	EPI1333699
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KJ162825	EPI1226721	EPI1226719	KJ508932	KJ162829		EPI1226718	EPI1226717
KC785037	KJ162826	KJ162827	EPI573197	KC785042		KJ162831	KJ162832
DQ376896	KC785036	KC785039	KY415621	DQ376752		KC785035	KC785040
KF357824	DQ376860	DQ376824	EPI659639	KF357839		DQ376679	DQ376788
KY635469	KF357827	KF357832	EPI659631	KY635523		KF357849	KF357835
MN171439	KY635519	KY635624	EPI659919	MN171443		KY635603	KY635444
CY146569	MN171440	MN171441	KY056286	CY146573		MN171445	MN171446
EPI866783	CY146570	CY146571	MF620123	EPI866779		CY146575	CY146576
KJ907716	EPI866784	EPI866782	EPI1055332	KJ907720		EPI866781	EPI866780
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LC042048	EPI866918	EPI866916	MH685179	LC042051		EPI866915	EPI866914
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KF042281	EPI860646	EPI860644	KR234022	KF042285		EPI860643	EPI860642
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EPI1064656	EPI1123333	EPI1123334	KU201943	EPI1064659		EPI1123338	EPI1123339
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JX570867	GU052665	GU052664	MG964776	JX570870		GU052660	EPI1064662
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	DQ376868	DQ376832	MG965072			DQ376688	JX570869
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			EPI576384				
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			GU052763 GU052651 FJ610108 GU186565 GU052659 FJ610094 GU052667 AB558473 GU052588 GU052596 GU052690 GU186573 KR137670 KR137686 KR137654 KR137662 KR137678 KR137702 KY989967 KF193394 KJ720208 KR137710 KJ162700 KJ162692 KJ162660 KJ162612 KJ162668 KJ162708 KJ162588 AB507264 KJ162620 KF193386 KY989969 AY573917				

Supplementary Table 3. Sequencing statistics.

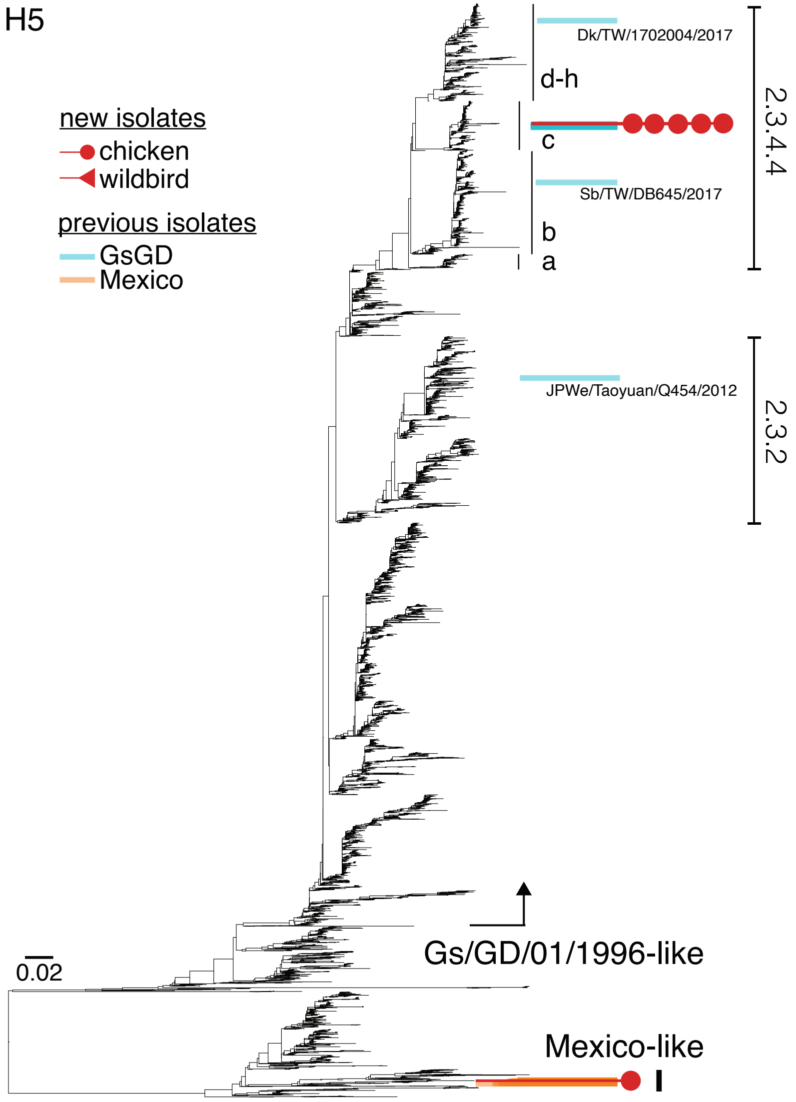
Sample ID	Serotype	Host	Mapped reads	Mean read length (bp)	SNV†	Crude SNV‡	Minor variant	Min. coverage (depth)§
TEST(PR8)	H1N1	-	7812	697	10	13	0	132/65/79/189/246/332/737/965
TEST(0702)	H6N1	-	12967	772	0	0	0	139/66/117/316/582/405/1632/1482
A1 (chicken/Taiwan/A1/2019)	H5N2	chicken	11028	1070	307	322	5	348/183/509/610/892/626/1090/1481
A1 (chicken/Taiwan/A1/2019)*	H5N2	chicken	429268	605	306	323	6	788/230/948/4989/8452/9284/32647/25084
A3 (chicken/Taiwan/A3/2019)	H5N2	chicken	16919	871	215	219	1	234/118/352/202/514/864/2443/4426
A4 (chicken/Taiwan/A4/2019)	H5N2	chicken	9501	1202	212	219	6	553/349/333/552/595/998/1507/1798
A5 (chicken/Taiwan/A5/2019)	H5N2	chicken	51146	793	216	219	0	786/277/670/524/1983/3199/5478/4228
D2 (chicken/Taiwan/D2/2019)	H5N2	chicken	2406	1225	230	243	11	126/86/122/190/189/228/284/433
D9 (chicken/Taiwan/D9/2019)	H5N2	chicken	25792	1226	231	242	9	119/338/1521/1097/2264/4593/6587/5081
WB2477 (pintail/Taiwan/WB2477/2017)	H6N2	pintail	2627	1122	145	156	2	79/40/38/76/178/215/425/577
WB2478 (pintail/Taiwan/WB2478/2017)	H1N3	pintail	54972	1081	139	142	1	2445/841/2278/3240/555/3223/7621/15259

*Another library constructed from the same sample as A1

†Single nucleotide variant detected by Nanopolish

‡Single nucleotide variant called directed from aligned BAM files

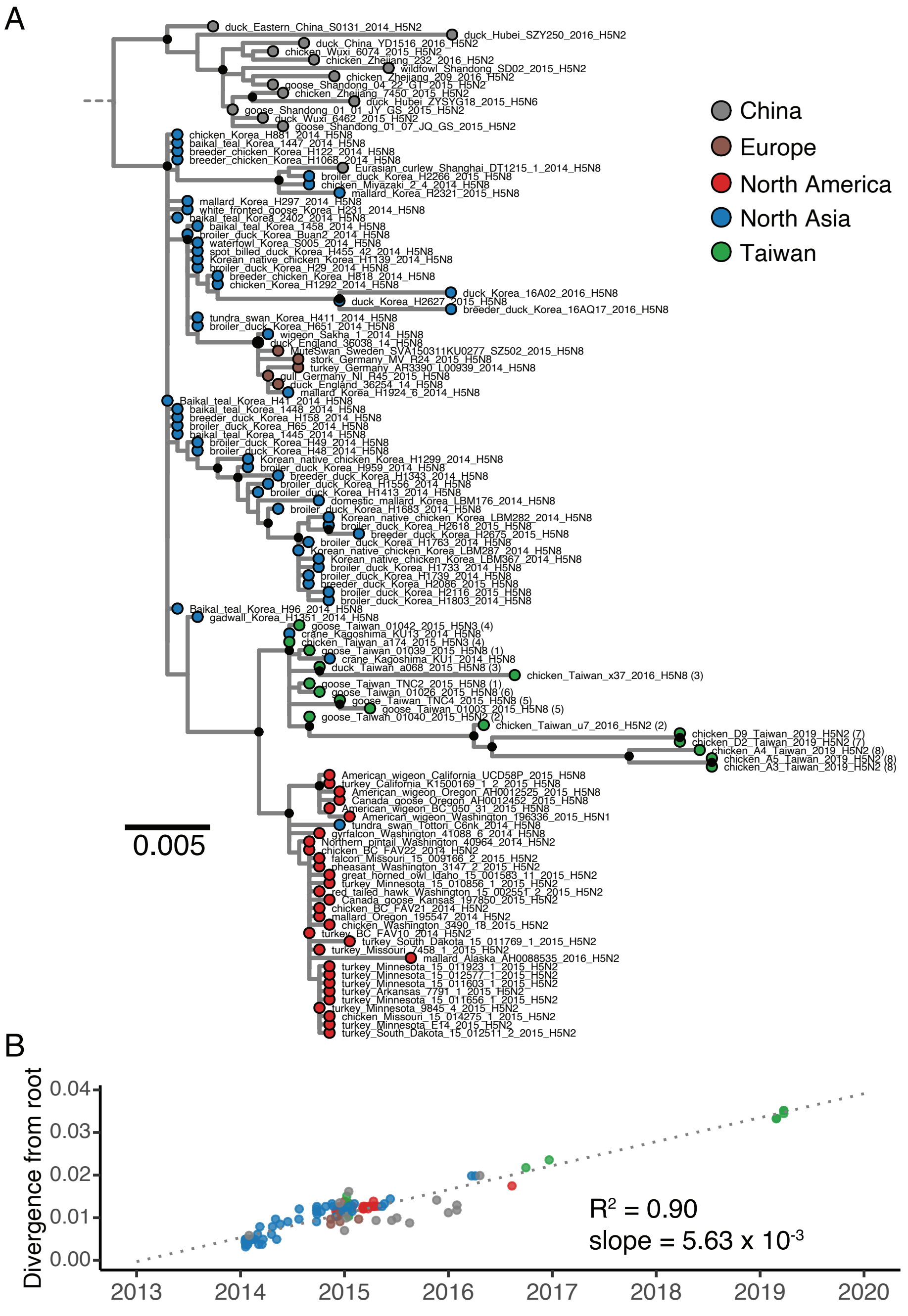
§Minimum coverage in each gene segment (ordered as PB2/PB1/PA/HA/NP/NA/MP/NS)



Supplementary figure S1.

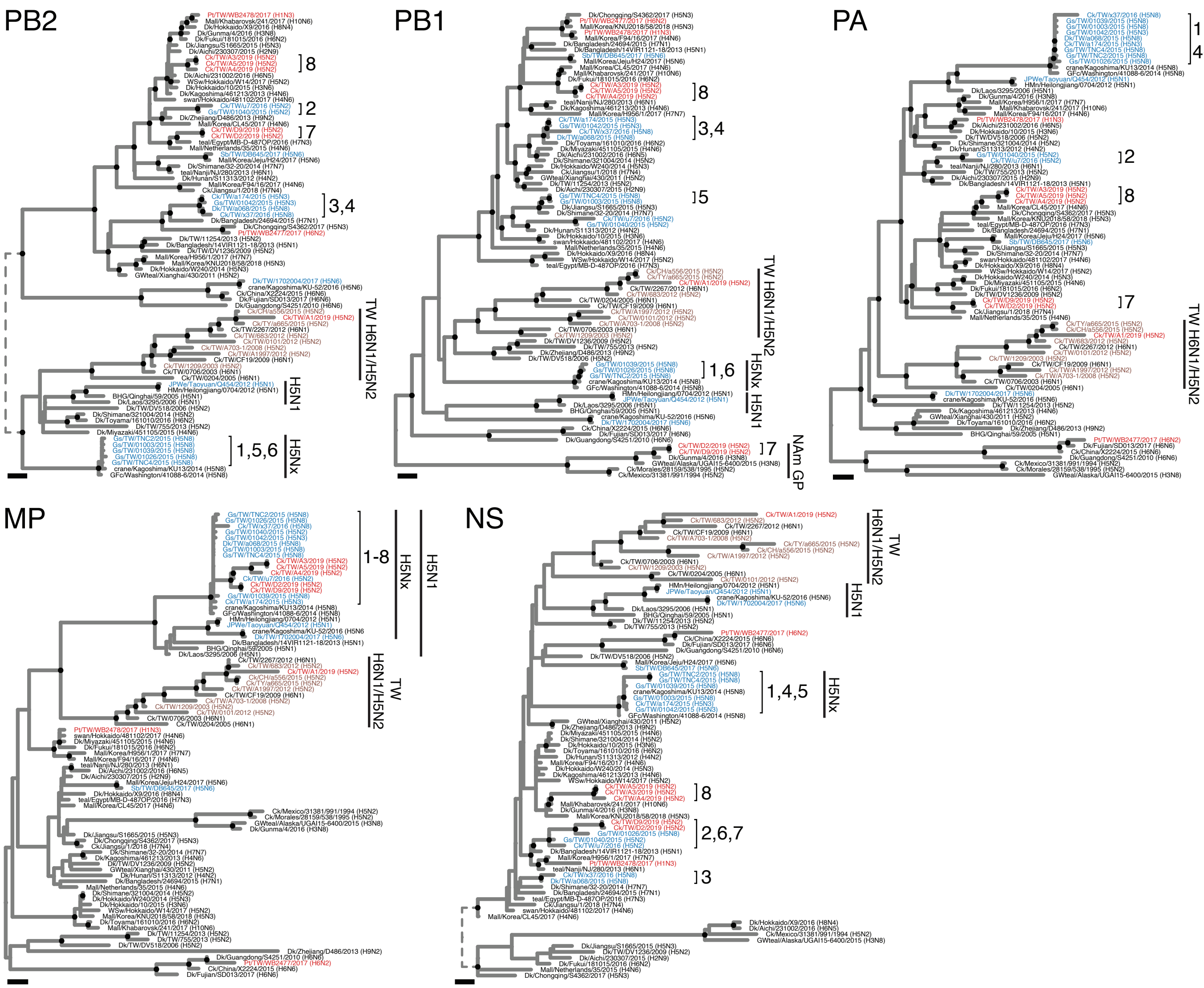
Maximum likelihood (ML) tree of H5 hemagglutinin genes.

Previously identified Gs/GD and Mexico lineage H5 viruses from Taiwan are highlighted with blue and orange bars, respectively, next to the tree. Virus names of three Gs/GD viruses isolated in Taiwan but not belonging to 2.3.4.4c clade are also shown. Viruses isolated in this study are indicated by red lines with circles (chicken) or triangles (wild bird) used to indicate virus host group. Mexico H5N2 and Gs/GD lineage viruses clades 2.3.4.4 (2.3.4.4a-2.3.4.4h) are marked on the tree. Scale bar represents 0.02 substitutions per nucleotide site. TW, Taiwan; Dk, duck; Sb, spoonbill; JPWe, Japanese white eye.



Supplementary figure S2.

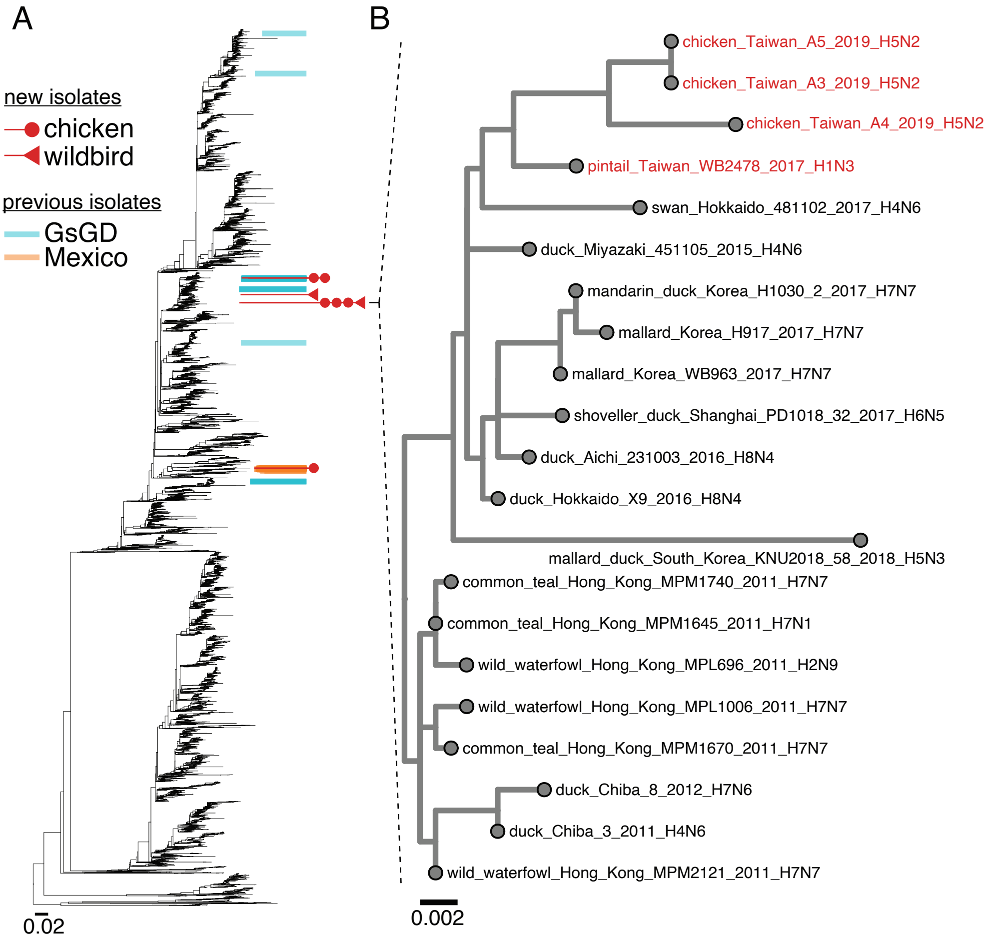
Maximum likelihood (ML) tree and root-to-tip regression of Gs/GD clade 2.3.4.4c viruses. (A) The ML tree was reconstructed by the same alignment as Figure 1 (n=122) with tips colored according to collection location. Bootstrap supporting values >70% are indicated at nodes by black circles. Genotypes (numbered 1-8) are appended to tip labels of viruses isolated in Taiwan. (B) Linear regression of collection dates against genetic distances to root of the ML tree shown in (A). Dots are colored according to the same scheme as (A). Scale bar represents 0.005 substitutions per nucleotide site.



Supplementary figure S3.

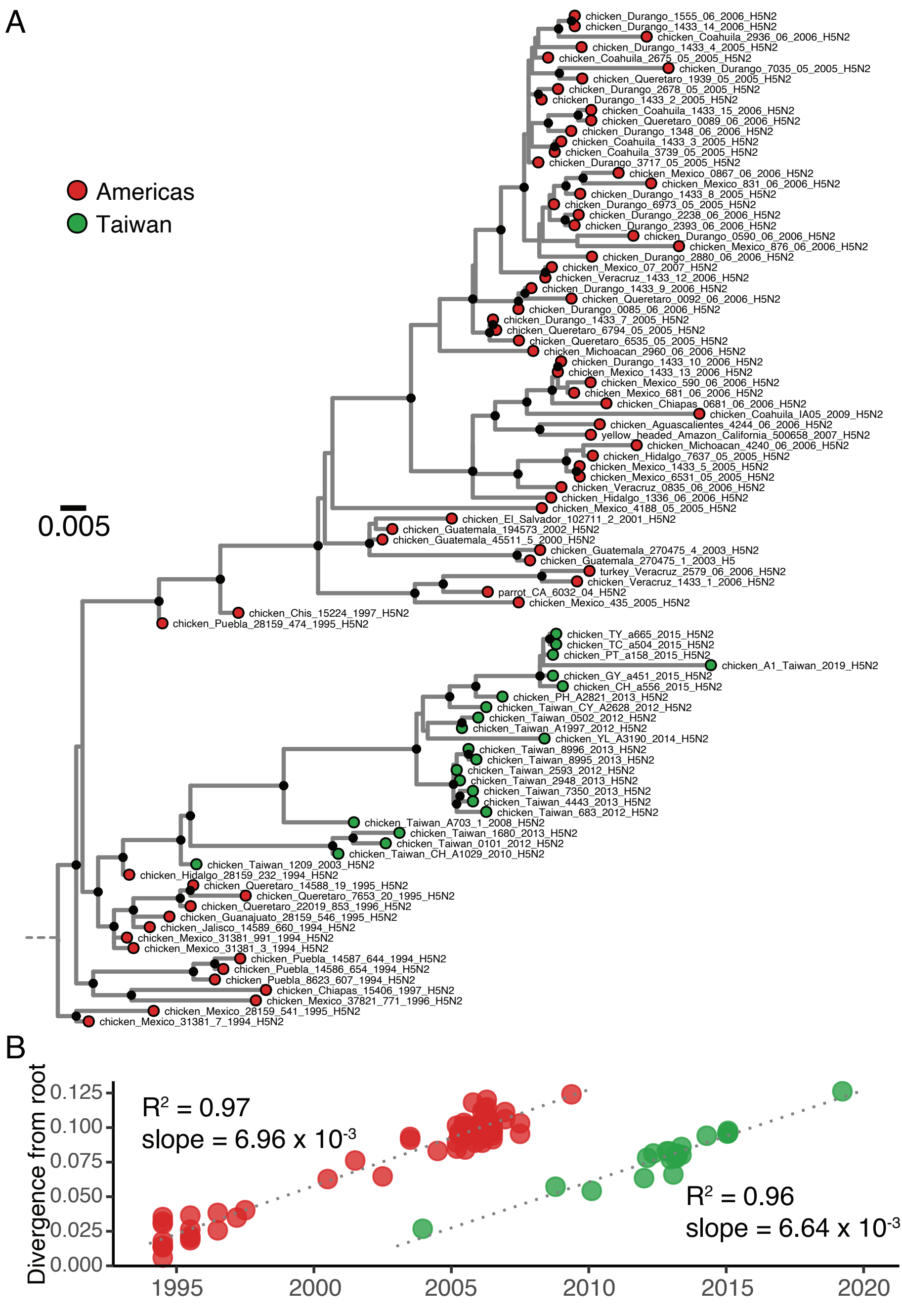
Phylogeny of internal genes of H5N2 viruses isolated in this study.

Maximum likelihood (ML) trees were reconstructed by PB2, PB1, PA, MP and NS genes of H5 viruses identified in Taiwan and relevant reference strains. Viruses isolated in this study are labeled in red, whereas tip labels in blue and brown represent previously identified Taiwanese Gs/GD and Mexico-like H5 viruses, respectively. Genotypes in the clade 2.3.4.4c (numbered 1-8) and major lineages are also indicated. Bootstrap supporting values >70% are highlighted at nodes by black circles. Long branches at the tree root were omitted and shown as shorter dashed branches. Scale bars represent 0.001 substitutions per nucleotide site. Korea, South Korea; TW, Taiwan; Ck, chicken; Dk, duck; Gs, goose; Pt, pintail; BHG, bar headed goose; teal, common teal; GWteal, green winged teal; GFc, gyrfalcon; HMn, hill myna, JPWe, Japanese white eye; Mall, mallard; Sb, spoonbill; WSw, whooper swans.



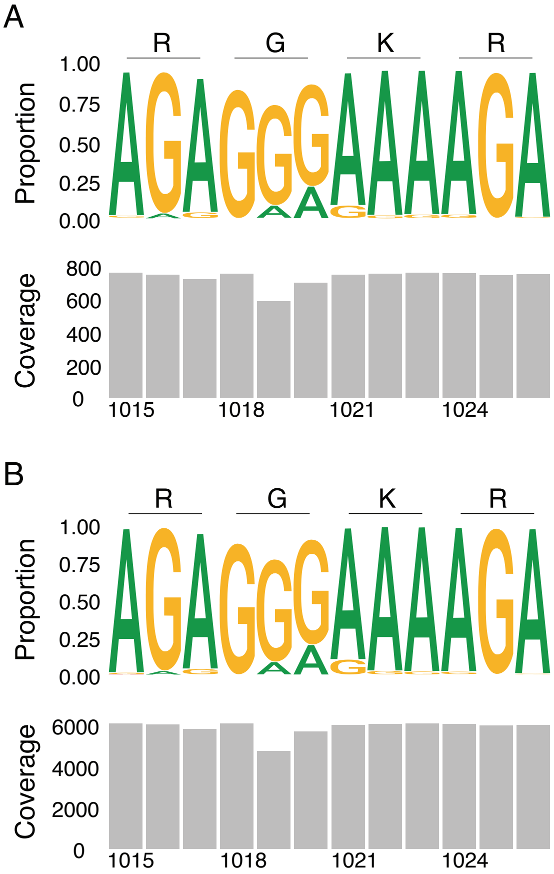
Supplementary figure S4.

Maximum likelihood (ML) tree of NP genes of avian influenza viruses. (A) Previously identified Gs/GD and Mexico lineage H5 viruses from Taiwan are highlighted with blue and orange bars, respectively, next to the tree. Viruses isolated in this study are indicated by red lines with circles (chicken) or triangles (wild bird) head. (B) A Zoomed-in version of the ML tree showing NP genes of genotype 8 clade 2.3.4.4c viruses are most phylogenetically related to the NP gene of A/pintail/Taiwan/WB2478/2017. Viruses found in this study are labeled in red. Scale bars represent 0.02 (A) and 0.002 (B) substitutions per nucleotide site.



Supplementary figure S5.

Maximum likelihood (ML) tree and root-to-tip regression of Mexico lineage H5N2 viruses. (A) The ML tree was reconstructed using Taiwanese isolates (n=23, identical to the sequences used in Figure 3) combined with viruses from the ancestral lineage isolated in Mexico, Guatemala, El Salvador and USA (n=74). Bootstrap supporting values > 70% are indicated at nodes by black circles. (B) Linear regression was built separately for viruses isolated in Taiwan and viruses in North Americas by plotting collection dates against genetic distances to root of the ML tree (A). Tip labels (A) and the dots in (B) share the same color scheme. Scale bar represents 0.005 substitutions per nucleotide site.



Supplementary figure S6.

HA cleavage site of the Mexican-like H5N2 virus isolated in this study. Sequence logos and depth of coverage (number of reads mapped to the reference) were derived from two independently prepared libraries using A1 sample, where A/chicken/Taiwan/A1/2019 (H5N2) was identified (Technical Appendix Table 2). 12 nucleotide positions containing residues -4 to -1 at the cleavage site are shown (nucleotide positions 1015-1026), with putative amino acids indicated at the top.