BIRDS OF PREY

A Brahminy kite perched on a branch. This is a common raptor found mostly in rural areas. With the encroachment of people on forested areas, there is conflict between people and wildlife. Settlers usually raise chickens in their yard and they become easy prey for raptors like the Brahminy kites (hawk), especially the young chicks. There is a folklore among Filipinos why the Brahminy kites prey on poultry chicks. A long time ago, the boyfriend of the hen was Mr. Brahminy kite and in fact Mr. Brahminy gave an engagement ring to Miss Hen. However, Miss Hen had a change of heart and eloped with Mr. Rooster producing a brood of chicks. When Mr. Brahminy returned, he saw that the ring he gave is no longer in the possession of Miss Hen so he left a bad message before leaving: look for that ring and until you find it, I will prey on all your chicks - this is the reason Miss Hen is always scratching the ground looking for that precious ring...

very low frequency sound in the infra-sound range imperceptible by the human ears but can be detected by the owl. The acoustic apparatus of the owl is also unique because the two external ears are not located on the same plane. One ear is lower than the other ear which is important for using the plain geometric principle of triangulation. The squeak of the mouse reaches the two ears at a different length of a microsecond because of the different positions in the skull of the birds. With its complex brain mechanism, the owl can then pinpoint the exact location of the mouse even in a completely darkened room. The eating habit of owl is also different. Eagles tear and cut the prey into small pieces before swallowing but the owls swallow their prey whole without cutting it into small pieces.

The declaration of a bird as a national bird of a country is a very controversial issue. In the Philippines, the formerly considered national bird was the Philippine Oriole tyler *(Oriolus steerii)*, and not the brown maya. However, the official national bird of the Philippines at present is the Philippine Eagle *(Pithecophaga jefferyi)*. There is a big issue in declaring the Philippine Eagle as our national bird. A group of scientists from the National Geographic Society headed by Dr. Robert S. Kennedy (not the US Senator) embarked on a research expedition to investigate the Philippine Eagle in 1978. After investigating several nesting sites of the Philippine Eagle the scientists found out that the bone remains of most of the prey include only a very small portion from monkeys. Most of the bone remains belong to the flying lemur *(Cynocephalus volans)*. Because of this finding, the group petitioned the then President Ferdinand E. Marcos to issue a presidential decree changing the common name of the Monkey-eating Eagle to a simple name of Philippine Eagle. The former name of the monkey-eating eagle was based on the given scientific name Pithecophaga jefferyi. The name came from the combination of the Latin for monkey (pitheeus) and to eat (phagus, just like phagocytes, macrophage, bacteriophage etc.). Jefferyi is in honor of Jeffery Whitehead who is the father of John Whitehead, a British naturalist who first collected the bird from the island of Samar in 1896. However, changing of the common name can be done immediately but the changing of the scientific name cannot be done immediately because it has to be reviewed and approved by the International Commission on Zoological Nomenclature (ICZN). During the term of President Fidel V. Ramos the Philippine Eagle was officially declared as the national bird of the Philippines in 1995.

The presidential seal of the US President includes the bald eagle. The bald eagle, scientifically named Haliaetus leucocephalus is the national bird of the US. Declaration of the bald eagle as the US national bird won over the golden eagle because it was thought that the bald eagle is indigenous to North America. When you refer to "North" America, it will include Canada where bald eagles are also found. Therefore, the bald eagle is not the sole monopoly of the USA. If the Americans were looking for a real indigenous bird of the US, the strongest candidate is the turkey *(Meleagridis gallopavo)*. Actually, it was Benjamin Franklin who suggested that the turkey be declared as the national bird of US. Well, this is really critical, what US President would like to have the turkey replace the majestic bald eagle in the presidential seal? The turkey receives the limelight only during the American holiday of Thanksgiving, when the US President will give a presidential pardon to a turkey saving the bird from being roasted for dinner. The name turkey is also a very confusing name. When the British first landed in North America, they saw the bird for the first ...

Raptor birds (pointed by the red arrow) are very effective for vermine and snake control. Serpent eagles are very efficient in this kind of biological control. When a sudden increase in rat population was observed in some localities in Bicol, it was suggested to use cobras as biological control. However, this suggestion is accompanied by fear that people might be bitten by venomous snakes. Instead of snakes, raptors would be a more effective and acceptable method of biological control for rodents, especially owls.

BIRDS OF PREY

time. The biologists did not know how to name and classify the bird so they captured some of the birds and sent them to UK so that the Royal Society of London can examine the bird and classify and name it for correct taxonomical grouping. Together with other cargoes, the live bird was shipped to London. However, before landing in London, the ship had to pass by the country of Turkey to unload and load other cargoes. Upon arrival at the port of London, the customs inspector noticed the bird and did not know how to officially record it in the log book and so he asked the quartermaster of the ship what was the last port of call of the ship before docking at London pier. Indeed, the quartermaster answered that the last port of call was Turkey. The customs inspector duly noted that it is a bird from Turkey and from then on the name "turkey" got stuck to the bird. The Royal Society of London also made a blunder and gave it the scientific name Meleagris gallopavo because it thought it is a relative of the guinea fowl *(Numida meleagris)* due to the close physical resemblance of the two birds. The bald eagle, being the national bird of the U.S. is a protected species although it is not in the endangered list. It is against the law to hunt the bird and only the indigenous tribes, the native Americans (formerly called Indians) are allowed to hunt the bird but only for ceremonial reasons of obtaining the feathers to be used in their rituals and ceremonies. A dead bald eagle was once necropsied and found to have lead shots inside the body cavities but no bullet wounds externally. It was found out that the eagle scavenged dead ducks shot by hunters. The eagle ate the duck and indirectly suffered from chronic lead poisoning with the lead shots accidentally eaten also together with the flesh of the duck.

Why is the national bird of the US called "bald" eagle *(Haliaetus leucocephalus)* ? The term bald is the shorter name for white patches called "piebald." The head of adult bald eagle is indeed white which is very appropriate for the second part of its scientific name, "leucocephalus" which is Latin for white head. However, there is a group of raptors that are literally bald, the vultures. In fact, it is not only the head that does not have feathers but the entire neck region is also devoid of feathers. The reason for the absence of feathers on the head and neck region in vultures is nature's way of preventing blood and other body fluids sticking to the bird after scavenging dead animals as part of their normal eating habit. Once the carcass is opened, the vultures will poke their head into the body cavities and pull the viscera out. If they have feathers on the head and neck-surely they will be soiled with blood and other body fluids causing contamination and infection. The vultures have natural immunity built in to their system and can eat animals that died of the most infectious disease like anthrax. In fact, the vultures had been blamed for spreading the disease because after eating a large meal, they tend to →

An injured Philippine Eagle being rehabilitated with a history of bullet wounds. The wing bones were shattered into small pieces and Dr. Roberto P. Puentespina, Jr. had to resort to wing amputation to save the bird. Raptors with severe injury cannot be returned back to the wild and had to be kept in a rehabilitation center because of the injury they suffered, they cannot fend for themselves in the wild.

Philippine macaque *(Macaca fascicularis)* foraging. They are also known as Southeast Asian long-tailed monkey, crab-eating monkey and cynomolgus monkey. Such busy time is the most vulnerable for the species one of the animals in the menu list of the Philippine Eagle.

Ready, get set, go !! Even if the eagle is tethered, it has the instinct of carrying its prey back to its nest where it will be eaten. This behavior is common in eagles with nestling to feed. Philippine eagles build a permanent nest and it is only topped off regularly with sticks, branches, and leaves becoming bigger and bigger ready for the next clutch of eggs.

The male turkey is called a gobbler because of its deep guttural sound produced during display either as an aggressive behaviour or a courting call to mate. The name 'turkey' is confusing because it is not a bird of the country of Turkey but a true indigenous bird of the U.S.A. In fact because of this unique geographical distribution it was proposed to be the national bird of the U.S.A. instead of the bald eagle (Haliaetus leucocephalus). The bald eagle is also found in Canada and is not the sole geographic monopoly of the U.S.A. It was the first time for the English zoologists to see such kind of unique bird and taxonomic classification was given to the Royal Society of London to classify it and give it a name. However, when the live bird was transported to London, the ship carrying the bird had to unload some of its cargo in Turkey first before proceeding to London.

Another group of social birds is the psittacine, here seen feeding from the hands of Dr. Eiichi Hondo. Psittacine birds include the parrots, macaw, parakeets, budgerigars more popularly known as 'love birds,' etc. Dr. Hondo is a professor of Nagoya University specializing in telemetry and remote sensing. His research team had tagged some big fruit bats with radio transmitters and tracked by satellite signals to determine the flight distance and territorial area covered by the species.

BIRDS OF PREY

Another favorite prey of raptor birds is the group of birds known as the galliformes, here represented by the golden pheasant being hand-fed by Dr. Hiroomi Akashi. The pheasant group is a good example of sexually dimorphic birds wherein the male with its colorful plumage can easily be differentiated from the female with its monochromatic plumage. The male pheasant needs this colorful plumage to easily attract females for mating.

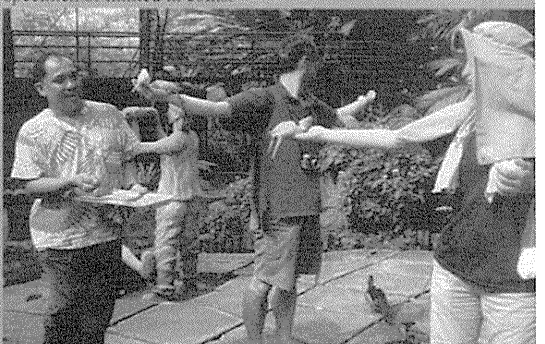
It is easy to train psittacine birds to feed from the hand as demonstrated here by Dr. Joseph S. Masangkay, UPLB College of Veterinary Medicine professor. Raptor birds can also be trained to take pieces of meat from a caretaker. The diet of the bird can be deduced from the shape and size of the beak. The beak of the predator is very different from those of other birds because the raptors specialize in tearing pieces of meat; hence their beaks have sharp scissor-like edges that can easily cut through meat.

In 1985 the Bangko Sentral ng Pilipinas (BSP) minted a series of 50-centavo coins honoring the Philippine Eagle. With the streamlining of the Philippine coins, the 50-centavo coin was removed from circulation.

become thirsty and will fly to the nearest watering hole to quench their thirst. Being birds they have a rapid rate of metabolism and while drinking they also defecate causing the spread of the infection to whatever mammal animal that will drink from the contaminated watering hole.

A very popular member of the vulture group is the California condor *(Gymnogyps californianus)* classified as critically endangered. Because of the possibility of the condor getting instinct in the wild a captive breeding project was started in 1987. The program met an early success and after training the captive-hatched vultures and making sure they can fend for themselves they were released in the wild. After one week, all of the released birds died, they were electrocuted in the high-tension electric wires. This incident was a big disappointment and the program started again from scratch. While waiting for the incubated eggs to hatch a staff member of the program thought of a solution to the problem. It was a simple, less expensive, and effective solution to the problem. It is but natural for newly released birds to meet some accidents in unfamiliar places and objects just like a high-tension electric wire because they were not guided by the mother vultures since they were hatched in captivity and did not know how to recognize such kind of danger. A piece of shiny metal was hanged on the high-tension electric wire that reflects light, moves, and chimes when the wind blows. These shiny metal objects were strategically hanged on the high-voltage wires along the paths of flight of the vultures. This simple and inexpensive method was very effective and no accidental electrocution occurred since then. Similar experience was also observed among eagles released by the Philippine Eagle conservation project in Davao. To avoid such kind of accident, the eagles destined to be released are first trained to recognize electric lines by installing similar-looking electric lines inside the cage. Not too strong current is passed thru the wire to just cause an electric jolt enough to sow fear in the eagle such kind of danger must be avoided.

It is high time that we conserve our wildlife particularly those endangered species like the Philippine Eagle. Conservation is done by the present generation for the future generation to appreciate and enjoy the resources of the country. The Philippine Eagle is a very sensitive creature and once it disappears, it will never be resuscitated back to life and might just be able to be seen by the future generation only in museums as stuffed dead specimen or minted in coins. ■



Dr. Roberto P. Puentespina, Jr. (left) is distributing the feed to the people feeding the birds at Malagos Garden Resort in Davao owned and managed by the Puentespina family. At the center is Dr. Ken Maeda from Yamaguchi University and at right is Dr. Yumi Une, professor from Azabu University. People wishing to feed birds must carefully wash their hands before entering and after coming out of the aviary to prevent possible transmission of diseases. The budgerigars popularly known as love birds because they are monogamous birds kept mated for life are the ones feeding from the hands. Budgies are not sexually dimorphic and the male and female can be difficult to distinguish by an untrained person. Usually male baby budgies have translucent purpley pink ceres whereas females tend to have bluish/purplish ceres with a hint of white around the nostrils. Ceres are the bulging anatomical structures at the base of the beak surrounding the nostrils. However, this is not a general rule because the mutants, especially the male inos (albino, lutino, creamino) will retain their baby-color ceres even up to adulthood.

1. Arayan, L.T., Reyes, A.W.B., Masangkay, J.S., Yoshikawa, Y., Baticados, W.N. and Rovira, H.G. Serological examination of Philippine Bats for Histoplasma capsulatum. Ceylon Journal of Science (Bio. Sci.) 40 (2): 183-186.

2. Reyes, A.W.B., Rovira, H.G., Masangkay, J.S., Ramirez, T.J., Yoshikawa, Y. and Baticados, W.N. 2011. Polymerase Chain Reaction Assay and Conventional Isolation of Salmonella spp. from Philippine Bats. Acta Scientiae Veterinariae, 2011. 39 (1): 947.

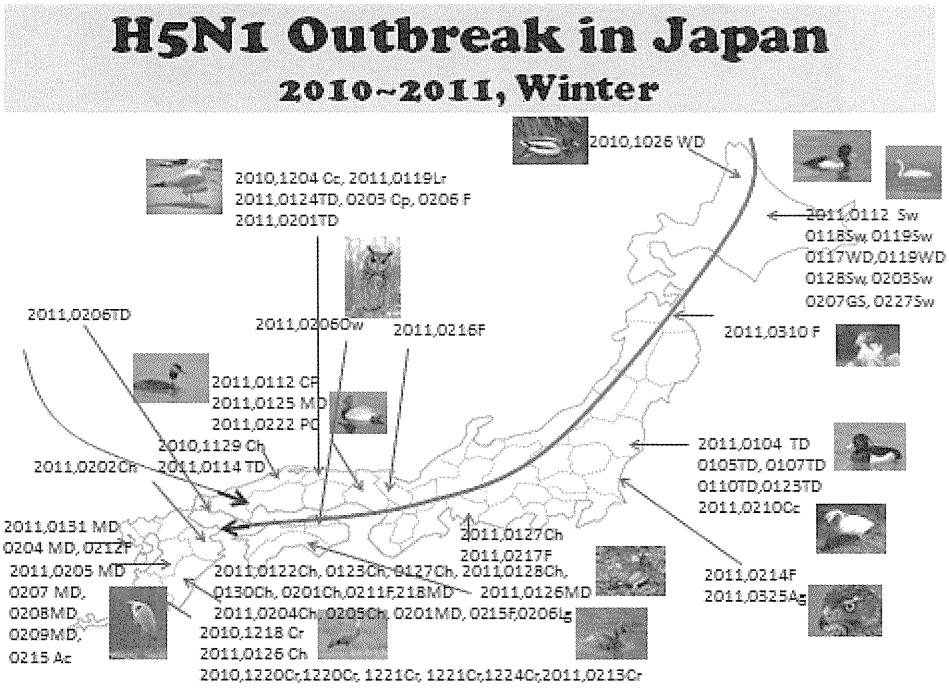
Ⅳ. 業績資料集

動物園の貴重種鳥類を高病原性鳥インフルエンザから守るには

2011 年 10 月 2 日、自由集会　　北里大学獣医学部　吉川泰弘

自由集会開催の趣旨

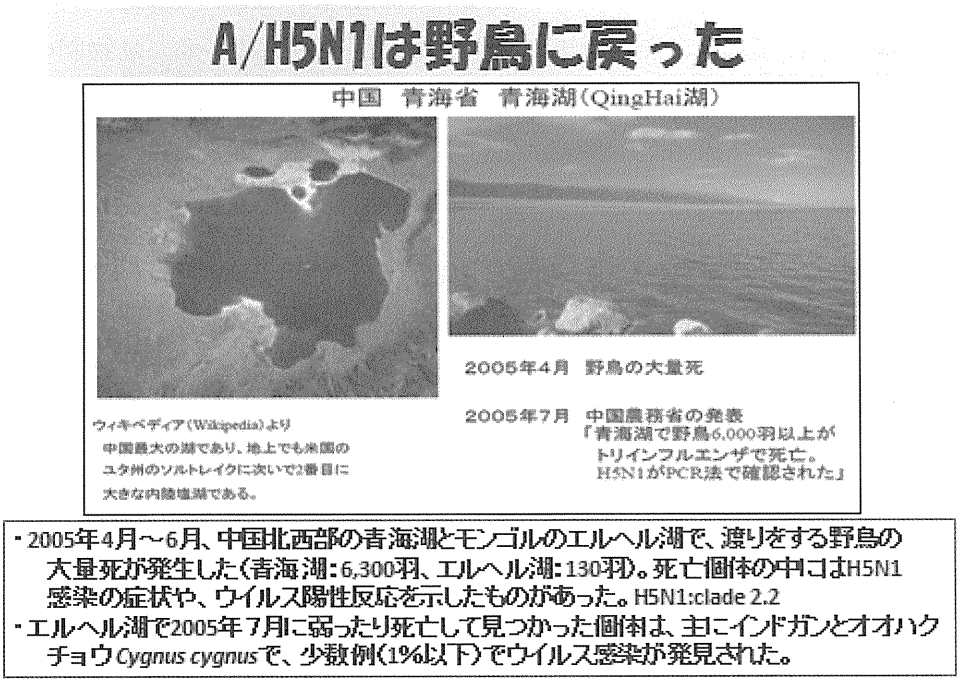
2011 年冬季、日本や韓国における鳥インフルエンザの流行は、それまでの H5N1 高病原性鳥インフルエンザの発生様式とは大きく様相を異にした。わが国では 2004 年の高病原性鳥インフルエンザの発生後、野鳥での陽性例は年間 1～3 例で経過し、家禽での発生も 1～4 件以下であった。しかし、2010 年秋の陽性例から 2011 年春にかけて、野鳥で 15 種 60 例、家禽は 24 件、約 185 万羽の淘汰という異常な頻度になった。野鳥では北からの渡り鳥からはじまり、徐々に定住している猛禽類等に感染が広がっていく傾向がみられた。2012 年以後も、同様の事態が起こる可能性は否定できない。動物園で維持されている貴重種が巻き込まれる可能性も考えられる。わが国の縦割り行政では、動物園の鳥類は家禽の定義には該当しないし、野鳥の範疇にも入らない。現在備蓄用にワクチンが保持されており、また新規のワクチンも開発されている。動物園の鳥類を守るために、不活化ワクチンを接種することの可否を議論し、対策をきめるべき時に来ていると考え、今回の自由集会を発案した。



座長からの問題提起

H5N1 は香港での発生当時から、直接ヒトを巻き込むという特徴を示した。また、東南アジアや中国で撲滅できない間に、中近東、欧州、アフリカに広がり、2005 年には中国の青海湖等で野鳥の大量死が見られ、H5N1 が分離された。これまでの高病原性鳥インフルエンザウイルスと異なり、野鳥に戻っていったことが明らかにされた。2011 年現在、この亜型の流行は終息していない。そして 2011 年冬季には高病原性のままのウイルスを、渡り鳥がもってきたことが明らかとなった。北の極地で H5N1 亜型がどのような振る舞いをしているかは不明である。これからの地道な研究が必要になる。

しかし、極地に戻った高病原性鳥インフルエンザウイルスが、高病原性を維持したまま南に戻ってきたのは、今回が初めてと思われる。新たな危機管理対応を検討する必要があろう。



OIE の野生動物 (wildlife) の定義

OIE の 野生動物疾病ワーキンググループでは、非常にわかりやすい野生動物の定義を用いている。人が介入して動物の遺伝的形質等を改変したか否か、日常的に人のケアを受けているか否かの２ｘ２の４つのカテゴリーに分ける。ヒトにより遺伝型、表現型の

選択を受けた動物で、典型的なヒトのケアを受けるもの(家畜や伴侶動物など)を除き、かつて選択されたが人のケアを離れたもの(野生化した家畜やペット:野イヌ、野ネコ、アライグマ等)、人のケアを受けるが捕獲野生動物であるもの(動物園動物等)、及び純粋に野生で生きている動物は全てwildlifeとして分類される。この意味では動物園の貴重鳥類は立派に野生動物であるが、日本での定義と分類はこの点に関して、非常に判りにくい。どうも飼育動物(伴侶動物、ペット、家畜など)の範疇にはいるが、家禽ではないようである。しかし、烏骨鶏、尾長鶏、小国鶏、蜀鶏(トウマル)、東天紅鶏などは農林規格の在来種で家禽に分類される天然記念物である。

野生動物の定義(OIE/WG)

/ OIEのアドホックグループ(2008, July 2-4, in OIE)は、OIE野生動物WGの提案した野生動物の定義に同意する。
/ 定義は2x2マトリックスにより動物を分類している

	典型的なヒトのケアを受ける	厳密なヒトのケアを受けない
ヒトにより遺伝型、表現型の選択を受けた(品種改良)	家畜(伴侶動物もふくまれる)	野生化した動物(野生生活に戻る)
遺伝型、表現型は自然選択により確立された	捕獲野生動物(動物園動物をふくむ)	野生動物(フリーレンジの野生動物)

HPAIに巻き込まれた公園飼育鳥の運命と動物園飼育鳥

わが国の現行制度では高病原性鳥インフルエンザへの対応として、家禽に関しては農水省が、野鳥に関しては環境省が責任を負う仕組みになっている。しかし、動物園や公園の飼育鳥に関しては、両省のどちらも責任を負う仕組みにはなっていないようである。農水省の例示では家禽における今回の流行は、9県、24農場が巻き込まれ、約185万羽が淘汰された。また、環境省の例示では野鳥15種、60羽 (飼育鳥を除く)がH5N1陽性であったとなっている。

今回のアウトブレイク時には3か所の公園で飼育鳥への感染例が見られた。その顛末は以下のようである。ケース1：2010年12月、富山県高岡市の市営高岡古城公園動物園内に飼育されていたコブハクチョウでH5N1の感染が確認された。同市は、園内のお堀で飼育していた残りのハクチョウなど10羽（死亡例を含む14羽？）を殺処分した。ケース2：2011年2月、兵庫県加東市の状ヶ池公園で飼育していたコブハクチョウ3羽中1羽が死亡（H5N1陽性）。2羽については隔離されたが1羽死亡。ケース3：2011年2月、山口県宇部市の常盤公園で飼育されていたコクチョウ1羽の死骸からHPAI(H5亜型)が検出された。飼育鳥では国内2例目。公園を管理する宇部市は、園内の常盤湖で羽を切って放し飼いにしている水鳥358羽（399羽：ハクチョウ358羽、ガン41羽）を殺処分。ハクチョウは家禽でないため、家畜伝染病予防法に基づく殺処分や周辺養鶏場の移動制限の対象ではなく、国の指針では、殺処分は管理者が自主判断することになっている。宇部市は「被害拡散を食い止めたい」として殺処分に踏み切った。常盤湖では,他に見つかった野生のキンクロハジロ1羽の死骸からH5N1が検出されている。このように飼育鳥の感染症統御は管理者に全責任が負わされている。

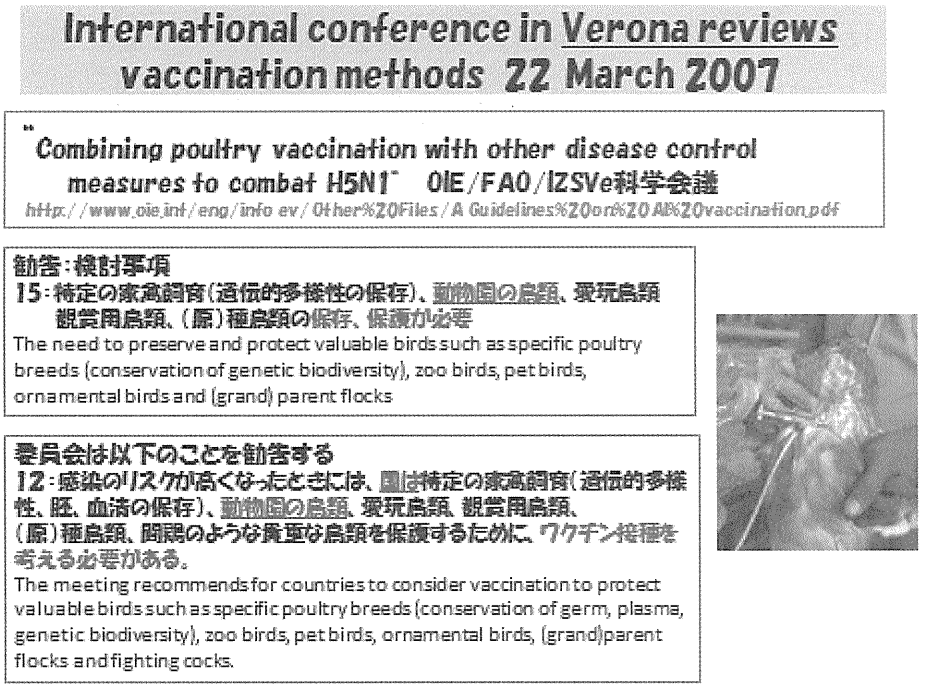
また、2011年1月30日の産経ニュースには、動物園の飼育鳥に関して以下の記述がある。野鳥対策ままならず、動物園は“自主判断”に困惑。野鳥対策として環境省がしているのは、監視強化と感染状況把握のための糞便調査が中心。消毒の徹底や防鳥ネットで防ぐしかなく、日本野鳥の会の金井裕主席研究員は「野鳥に移動制限をかけるわけにはいかないし…」と告白する。まとまった対策が打てない一因に、野鳥は環境省、天然記念物は文部科学省、家禽は農林水産省と役所の所管がわかれていることが挙げられている。特別天然記念物のタンチョウやニホンコウノトリなど多数の希少な鳥類を飼育する上野動物園も、発生後の対応は決まっていない。天然記念物は文科省だが、ワシントン条約に関係する動物は環境省が所管する。動物園は文科省が所管する博物館法の一形態だが、「動物取扱業」の登録をしていれば環境省の所管になる。動物園関係者は「ちゃんと所管してくれるところがどこにもなく、動物園はグレーゾーンだ」と嘆く。日本動物園水族館協会では「希少な動物が感染したときの社会的責任は動物園だけで担えるものではない。社会全体で考える必要がある」（山本茂行会長）と、国に法的な整備を求めている。この課題については広く議論を起こし、適切な法整備と感染症対策措置を取るべきであると考える。また、本学会が、議論を始めるための責務を負うべきであると思う。

4

国際的な対応に関しては？

2007年3月にイタリアのベローナで国際会議が開かれた。OIE（国際動物保健機関）とFAO（国際農業食糧機関）及びIZSVe主催の科学会議である。会議のタイトルは「Combining poultry vaccination with other disease control measures to combat H5N1；H5N1と闘うための他の感染症統御措置と家禽ワクチンの併用」であった。その中で、勧告：検討事項の15項に特定の飼育家禽（遺伝的多様性の保存）、動物園の鳥類、愛玩鳥類、観賞用鳥類、（原）種鳥類の保存、保護が必要と書かれている。さらに、委員会は以下のことを勧告するとして、その12項に感染のリスクが高くなったときには、国は特定の飼育家禽（遺伝的多様性、胚、血清の保存）、動物園の鳥類、愛玩鳥類、観賞用鳥類、（原）種鳥類、闘鶏のような貴重な鳥類を保護するために、ワクチン接種を考える必要があると、明確に国の責務に関して述べている。

今回の様なアウトブレイクが起こる可能性は今後も想定される。このような国際的勧告が出ているのであるから、早急に監督官庁を明確にし、法整備が必要であれば整え、科学的評価に基づく管理措置（リスク回避のためのワクチン接種を含めて）を検討すべきである。



ワクチン接種と課題

諸外国で製造承認されている鳥インフルエンザワクチンの大部分は、ウイルス液をホルマリン等で処理した不活化ワクチンである。低病原性の H5 または H7 亜型のウイルスは鶏間で感染を繰りかえすうちに高病原性化する可能性があるため、弱毒生ワクチンは承認されていない。

他方、組み換えワクチンとしては鶏痘ウイルスに HA 遺伝子を挿入して作製した組換え生ワクチンが開発されメキシコ、グアテマラ、エルサルバドルで応用された。メキシコはニューカッスルウイルスベクターも開発した。中国も組換え生ワクチンを使用している（鶏痘、ニューカッスルウイルスをベクターにしている）。組換えワクチンは生ワクチンであるため、液性免疫と細胞性免疫の両方を誘導することが可能、不活化ワクチンより早期に免疫効果が現れる、組換えワクチンではインフルエンザウイルスの HA 蛋白のみが発現しているため、ワクチン接種による抗体と流行ウイルス感染による抗体との区別が容易等の利点を持つ。他方、ベクターウイルスに対する移行抗体を有するヒナでは効果が低い、鶏痘などベクターとなっているウイルスのワクチン接種鶏に対しては効果が低い等の欠点があること、及び遺伝子組換え生物の使用に関する規制の厳しさ等から実際の使用国は限られている。

さらに、ワクチン接種を巡る問題には、以下の 2 点がある、1、家禽では不活化ワクチン等は、一定期間発症と死亡を防ぐことが可能で、ウイルスの増殖、排泄ウイルス量を減少させる効果はある。しかし、感染及びウイルス排泄を完全に阻止することはできない、あるいは阻止可能期間が比較的短い。従って、家禽ではワクチンのみで流行を阻止することはできず、ワクチンを使用している国の多くは、ウイルスの持続的な流行が起こっている。2、不適切なワクチン使用は、有意に中和しにくいウイルスを選抜することになる。最近の流行株は、これまでの分離株で作成したワクチンに対する抗体ではウイルスを中和しにくくなっている（新しいクレードの多くは、過去のクレードのウイルス株の免疫を回避するような変異になっている）。

H5N1 HPAIの抗原性比較

ウイルス	clade	各高度免疫血清のHI抗体価					
		Tn/SA	Vac-1	Vac-3	Ws/Mon	Ws/HoK	Pf/HK
Tern/South Africa/61(H5N3)	-	640	320	320	1280	40	160
Duck/Hokkaido/Vac-1/04 (re-assort) *A/duck/Mongolia/54/01(H5N2)*	-	160	1280	1280	320	40	80
Duck/Hokkaido/Vac-3/07 (re-assort) *A/duck/Hokkaido/101/04/(H5N3)*	-	320	1280	2560	640	160	160
Chicken/Yamaguchi/7/04	2.5	320	1280	2560	2560	320	640
Whooper swan/Mongolia/3/05	2.2	160	320	5160	2560	320	320
Whooper swan/Hokkaido/1/08	2.3.2	40	40	40	160	1280	160
Peregrine falcon/Hong Kong/810/09	2.3.4	<20	80	160	20	40	2560

-はクレード0~9に分類されない古典的な系統

今後の課題と展望

高病原性鳥インフルエンザのアウトブレイクに対して、動物園の貴重種鳥類や公園の飼育鳥のリスク回避をどのようにするか？座して待つか？一矢報いるか？広範な議論を経て、危機管理（リスク回避）対応措置を取ることが必要である。その際、希少種のプライオリティーはどのような基準にするか？検討しなければならない。

いずれにせよ、無策のままにしておいて、口蹄疫の流行時に起こった黒毛和種の封じ込め域外避難のようなエラーは繰り返してはいけない。国際的な越境感染症の封じ込めには、国際ルールを守る必要がある。国際ルールは、各国がハイリスクの家畜感染症に悩んだ末に決めたルールであり、感染症封じ込めの基本であるからである。

貴重種におけるリスク回避のためのワクチン使用の是非に関しては、リスク評価が必要となる。また有効性に関するデーターの収集や実験的な接種も必要であろう。このような決断を誰が決め、どのようにできるか？その所掌は？法律は？実行性は？と考えると、急がなければならないが、時間のかかる問題であると思われる。継続的に本学会で議論され、適切な解決方法が見つけられることを期待する。

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Research article

Multi-locus sequence analysis reveals host specific association between *Bartonella washoensis* and squirrels

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ABSTRACT

To clarify phylogenetic relationships and genetic diversity among *Bartonella washoensis* strains obtained from squirrels, multi-locus sequence analysis (MLSA) with the 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC*, and *rpoB* genes was applied for 20 strains of *B. washoensis* isolated from five genera of squirrels (*Tamias*, *Tamiasciurus*, *Glaucomys*, *Sciurus*, and *Spermophilus*) within the family Sciuridae. Sequence similarities in the concatenated sequences of *B. washoensis* strains from squirrels of different genera ranged from 94.7% (*Sciurus* vs. *Spermophilus*) to 98.4% (*Tamiasciurus* vs. *Glaucomys*). Phylogenetic trees based on the concatenated sequences revealed that *B. washoensis* strains formed five distinct clades and each clade correlated with the genus of squirrel from which the strains were originally obtained. The discrimination was supported by 100% bootstrap values and posterior probabilities, respectively. These results suggest that *B. washoensis* strains may have co-speciated with their squirrel hosts and provide new insights into the application of the MLSA to identify sources of *B. washoensis* infection with accuracy.

1. Introduction

Bartonella washoensis is Gram-negative, fastidious, facultative intracellular bacteria and was first isolated from a human patient with fever and myocarditis in Washoe County, Nevada in 1995 (Kosoy et al., 2003). Subsequently, the organism was found in California ground squirrels (*Spermophilus beecheyi*) with a high prevalence of infection (>17%) in the same county (Kosoy et al., 2003). The partial DNA sequences of the 16S rRNA, *gltA*, and *groEL* genes of *B. washoensis* strains obtained from ground squirrels were identical to those from the human patient, suggesting that the organism is zoonotic and the ground squirrels are the natural reservoirs of the pathogen in western USA (Kosoy et al., 2003). In the same year, *B. washoensis* was isolated from a dog with mitral valve vegetative endocarditis and the sequences of several genes of the strains were identical to those of *B. washoensis* from the human and the California ground squirrels (Chomel et al., 2003). Thus, *B. washoensis* is suggested to be able to infect several species of mammals.

It has been reported that squirrels in various areas have been infected with *B. washoensis* (Bown et al., 2002; Kosoy et al., 2003; Jardine et al., 2005; Bai et al., 2008; Inoue et al., 2009b). However, the phylogenetic relationships and the genetic diversity of *B. washoensis* strains of among various genera of squirrels have not been investigated to date.

In the present study, we analyzed 20 *B. washoensis* strains from 5 genera of squirrels by using multi-locus sequence analysis (MLSA) of 6 housekeeping genes to

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clarify the relationship between genetic diversity of the isolates and host squirrels.

2. Materials and methods

2.1. B. washoensis strains and growth conditions

A total of 20 *B. washoensis* strains were analyzed in this study. Nineteen strains were isolated from wild squirrels that had been imported as pets into Japan from China or North America between June 2004 and October 2007 (Inoue et al., 2009b). Three strains from three individuals of six squirrel species, i.e. the Richardson's ground squirrel (*Spermophilus richardsonii*), the Columbia ground squirrel (*Spermophilus columbianus*), the Daurian ground squirrel (*Spermophilus dauricus*), the American red squirrel (*Tamiasciurus hudosonicus*), the Southern flying squirrel (*Glaucomys volans*), and the Siberian chipmunk (*Tamias sibiricus*), and a single strain that had been isolated from a Hokkaido squirrel (*Sciurus vulgaris orientis*) were used in this study. The 19 strains were confirmed to form a cluster with *B. washoensis* strain nvh1 from a human patient in the phylogenetic analysis based on a portion of the *gltA* gene (Inoue et al., 2009b). Also included in this study was *B. washoensis* strain Sb944nv, which was isolated from a California ground squirrel (*Spermophilus beecheyi*) in the USA (Kosoy et al., 2003) and had 16S rRNA, *gltA*, and *groEL* gene sequences identical to those from *B. washoensis* strain nvh1 (Table 1).

The cultures were grown on heart infusion agar plates (DIFCO, MI, USA) containing 5% defibrinated rabbit blood. The plates were incubated at 35 °C in a 5% CO_2 atmosphere for 14 days and harvested bacteria were used for DNA extraction.

2.2. DNA extraction and PCR amplification

Genomic DNA was extracted from each strain using an Instagene Matrix (Bio Rad, CA, USA). Partial 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC*, and *rpoB* genes were amplified by PCR. The specific primers used and the PCR conditions for 16S rRNA (Heller et al., 1997), *ftsZ* (Zeaiter et al., 2002b), *gltA* (Norman et al., 1995), *groEL* (Zeaiter et al., 2002a), *ribC* (Inoue et al., 2009a) and *rpoB* (Renesto et al., 2001) amplification were described previously.

2.3. DNA sequencing and phylogenetic analysis

The PCR products were purified using a Spin Column PCR Product Purification Kit (Bio Basic, Ontario, Canada) and were sequenced directly using specific sequencing primers for 16S rRNA (Heller et al., 1997), *ftsZ* (Zeaiter et al., 2002b), *gltA* (Norman et al., 1995), *groEL* (Zeaiter et al., 2002a), *ribC* (Inoue et al., 2009a) and *rpoB* (Renesto et al., 2001). All novel sequences were submitted to GenBank and accession numbers were obtained (Table 1).

The DNA sequence datasets of the six loci (16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC*, and *rpoB*) of all of the *B. washoensis* strains and those of other known rodent-associated *Bartonella* spp., including *B. birtlesii* CIP106294^T (AF204274, AF467762, AF204272, AF355773, AY116632, and AB196425), *B. doshiae* NCTC12862^T (Z31351, AF467754, Z70017, AF014832, AY116627, and AF165991), *B. elizabethae* ATCC49927^T (L01260, AF467760, Z70009, AF014834, AY116633, and AF165992), *B. grahamii* NCTC12860^T (Z31349, AF467753, Z70016, AF014833, AY166583, and AF165993), *B. phoceensis* CIP107707^T (AY515119, AY515135, AY515126, AY515129, AB298328, and AY515132), *B. rattimassiliensis* CIP107705^T (AY515120, AY515133, AY515124, AY515127, AB298327, and AY515130), *B. taylorii* CIP107028^T (Z31350, AF467756, Z70013, AF304017, AY116635, and AF165995), *B. tribocorum* CIP105476^T (AJ003070, AF467759, AJ005494, AF304018, AB292600, and AF165996), *B. vinsonii* subsp. *arupensis* ATCC700727^T (AF214558, AF467758, AF214557, AF304016, AY116631, and AY166582), and *B. vinsonii* subsp. *vinsonii* ATCCVR-152^T (M73230, AF467757, Z70015, AF014835, AY116636, and AF165997), were aligned using Clustal X version 2.0 (Larkin et al., 2007) and were combined. The phylogenetic analyses were performed with the maximum likelihood method (ML), neighbor-joining method (NJ), and Bayesian analyses based on the concatenated sequences of the six loci. *Bartonella bacilliformis* ATCC35685^T (Z11683, AB292602, AB292601, AY664491, AJ236918, and AF165988) was chosen as an out-group reference in each analysis. The ML tree was constructed using PAUP* 4.0β10 (Swofford, 2002). The Tamura and Nei model (Tamura and Nei, 1993) with the assumption that some sites are invariable and others are variable following a discrete gamma distribution (TrN + I + Γ model) was selected for appropriate substitution model by Modeltest 3.7 (Posada and Crandall, 1998) based on hierarchical likelihood ratio tests (hLRT). Heuristic searches were executed using the tree-bisection-reconnection (TBR) branch-swapping algorithm for 10 random additions of taxa. Bootstrap analysis was carried out on 1000 replications of the heuristic search with the TBR branch-swapping algorithm (Felsenstein, 1985). The NJ tree (Saitou and Nei, 1987) was constructed with the Jukes-Cantor parameters model (Jukes and Cantor, 1969) using PAUP* 4.0β10. Bootstrap analysis was carried out on 1000 replications of the dataset. Bayesian inference was conducted using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The General Time Reversible (GTR) model + I + Γ (Yang, 1994) was selected by MrModeltest 2.2 (Nylander, 2004) based on hLRT. Four chains of Markov Chain Monte Carlo algorithms were performed for 3,000,000 generations, sampled every 100 generations, and the first 25% (750,000 generations) of the trees were discarded as burn-in. The remaining generations were used to construct a 50% majority-rule consensus tree and to calculate posterior probabilities for each node in the Bayesian tree. In the ML and NJ trees, we consider nodes with >70% bootstrap as well supported (Hillis and Bull, 1993). In the Bayesian tree, only nodes with >95% posterior probabilities are considered significant.

3. Results

The 20 analyzed *B. washoensis* strains were classified into 8, 15, 16, 18, 18, and 17 genotypes for 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC*, and *rpoB*, respectively (Table 2). The sequence similarities between the examined strains and other known rodent-associated *Bartonella* spp. ranged 98.5–99.2% for 16S

Table 1
Origins of 20 *B. washoensis* strains used for MLSA and the Genbank accession numbers of six housekeeping genes.

Strain	Origin		GenBank accession nos.					
	Host animal	Geographical location	16S rRNA	*ftsZ*	*gltA*	*groEL*	*ribC*	*rpoB*
Sb944nv	*Spermophilus beecheyi*	North America	AB292597	AB292598	AF470616	AF484066	AB292599	AB292596
RJ21-1	*Spermophilus richardsonii*	North America	AB519060	AB519067	AB444959	AB519081	AB519098	AB519115
RJ30-1			Identical to RJ21-1	Identical to RJ21-1	AB444955	AB519082	AB519099	AB519116
RJ33-1			Identical to RJ21-1	Identical to RJ21-1	AB444960	AB519083	AB519100	Identical to RJ30-1
CJ22-1	*Spermophilus columbianus*	North America	Identical to RJ21-1	AB519068	AB444956	AB519084	AB519101	AB519117
CJ23-2			Identical to RJ21-1	AB519069	AB444961	AB519085	AB519102	AB519118
CJ25-1			Identical to RJ21-1	AB519070	AB444957	AB519086	AB519103	AB519119
DR1-1	*Spermophilus dauricus*	China	AB519061	AB519071	AB444962	AB519087	AB519104	AB519120
DR3-1			AB519062	AB519072	Identical to DR1-1	AB519088	AB519105	AB519121
DR10-1			Identical to DR1-1	AB519073	Identical to DR1-1	AB519089	AB519106	Identical to DR3-1
SR22-1	*Tamias sibiricus*	China	AB519063	AB519074	AB444968	AB519090	AB519107	AB519122
SR24-1			AB519064	AB519075	AB444965	AB519091	AB519108	AB519123
SR25-1			AB519065	Identical to SR24-1	AB444964	AB519092	Identical to SR24-1	AB520713
AR2-2	*Tamiasciurus hudsonicus*	USA	Identical to RJ21-1	AB519076	AB444970	AB519093	AB519109	AB519124
AR4-1			Identical to RJ21-1	AB519077	AB444971	AB519094	AB519110	AB519125
AR15-2			Identical to RJ21-1	AB519078	Identical to AR4-1	AB519095	AB519111	AB519126
AM2-1	*Glaucomys volans*	USA	Identical to Sb944nv	AB519079	AB444972	AB519096	AB519112	AB519127
AM5-1			Identical to Sb944nv	Identical to AM2-1	Identical to AM2-1	Identical to AM2-1	AB519113	Identical to AM2-1
AM9-1			Identical to Sb944nv	Identical to AM2-1	AB444973	Identical to AM2-1	Identical to AM5-1	AB519128
ER14-3	*Sciurus vulgaris orientis*	China	AB519066	AB519080	AB444974	AB519097	AB519114	AB519129

Table 2
Sequence similarity of six housekeeping genes and the concatenated sequence between 20 *B. washoensis* strains and known rodent-associated *Bartonella*.

Gene	Length (bp)	No. of genotype	Sequence similarity (%) by:	
			Inter-species of rodent-associated *Bartonella*	Intra-species of *B. washoensis*
16S rRNA	1350	8	98.5–99.2	99.2–100
ftsZ	788	15	88.1–93.3	93.8–100
gltA	328	16	86.3–93.3	91.2–100
groEL	1185	18	86.3–92.9	93.6–100
ribC	618	18	80.4–88.5	88.2–100
rpoB	825	17	87.3–91.2	92.4–100
Concatenated	5092	20	89.8–93.1	94.7–99.9

rRNA, 88.1–93.3% for *ftsZ*, 86.3–93.3% for *gltA*, 86.3–92.9% for *groEL*, 80.4–88.5% for *ribC*, and 87.3–91.2% for *rpoB*, respectively. The sequence similarities within examined strains of *B. washoensis* showed similarities ranging from 99.2–100% for 16S rRNA, 93.8–100% for *ftsZ*, 91.2–100% for *gltA*, 93.6–100% for *groEL*, 88.2–100% for *ribC*, and 92.4–100% for *rpoB*, respectively. The sequence similarities of the concatenated sequence (5092 bp) of the six loci between *B. washoensis* strains and other known rodent-associated *Bartonella* spp. ranged from 89.8% to 93.1% and all 20 strains were individually discriminated in the range from 94.7% to 99.9% similarities (Table 2).

In the phylogenetic tree based on the concatenated sequences, similar tree topologies of 20 *B. washoensis* strains and other known rodent-associated *Bartonella* spp. were obtained by ML, NJ, and Bayesian analyses, respectively. All *B. washoensis* strains formed a large clade separated from other known rodent-associated *Bartonella*

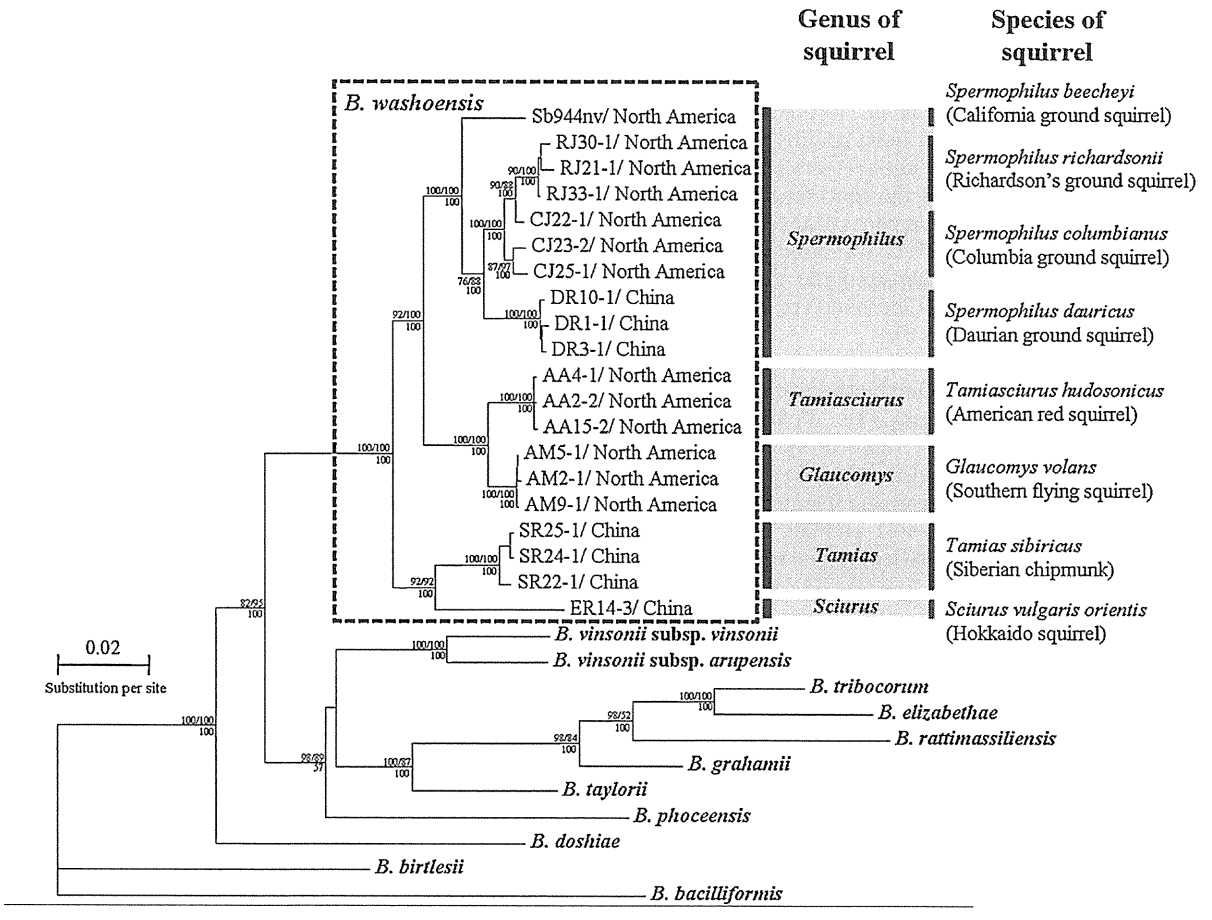

Fig. 1. The ML tree based on the aligned 5092 bp of concatenated sequence of six housekeeping genes, including 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC*, and *rpoB* from 20 *B. washoensis* strains and known rodent-associated *Bartonella* spp. For the *B. washoensis* strains examined in this study, the strain name followed by the country name from which it was derived was indicated. The *Bartonella bacilliformis* strain ATCC35685^T sequences were used as an out-group. Numbers above the branches indicate the bootstrap values (1000 replications) based on ML/NJ analyses and the numbers below branches are Bayesian posterior probabilities.

Table 3
Sequence similarity matrix based on the concatenated sequence of six housekeeping genes among 20 *B. washoensis* strains by squirrel genus.

Genus of squirrel (No. of strains)	Percent sequence similarity (No. of bp differences) with:			
	Spermophilus	*Tamiasciurus*	*Glaucomys*	*Tamias*
Spermophilus ($n = 10$)	97.1–99.8 (11–148)[a]			
Tamiasciurus ($n = 3$)	96.0–96.3 (186–204)	99.9 (6–7)		
Glaucomys ($n = 3$)	96.2–96.6 (175–195)	98.4 (80–83)	99.9 (3–6)	
Tamias ($n = 3$)	95.7–96.1 (199–221)	95.5–95.6 (223–229)	95.8–95.9 (211–214)	99.5–99.9(6–26)
Sciurus ($n = 1$)	94.7–95.1 (248–269)	94.9–95.0 (256–260)	94.9–95.0 (255–258)	96.2 (192–194)

[a] The percentage of sequence similarity in gray cells indicates the intra-species sequence similarity for the genus of squirrel.

spp. and the discrimination was supported with 100% bootstrap values (BV) in ML and NJ analyses and 100% Bayesian posterior probabilities (BPP), respectively (Fig. 1).

In the clade of *B. washoensis*, the strains formed five distinct clades that correlated with the genus of the host squirrel from which the strains were originally obtained, representing clades for *Spermophilus*, *Tamiasciurus*, *Glaucomys*, *Tamias*, and *Sciurus*. These discriminations were also supported by 100% BV in ML and NJ analyses and 100% BPP. Strain ER14-3, which was obtained from *Sciurus vulgaris orientis*, formed a large clade with those from *Tamias sibiricus* with 92% BV in ML and NJ analyses and 100% BPP. Strains from four species of *Spermophilus*, including *Sp. beecheyi*, *Sp. richardsonii*, *Sp. columbianus*, and *Sp. dauricus* consisted of a large clade. Although strain CJ22-1, which was obtained from *Sp. columbianus*, formed a clade with strains from *Sp. richardsonii*, most strains formed sub-clades with the relevant host squirrel species (Fig. 1).

The sequence similarity of the concatenated sequence of *B. washoensis* within the five genera ranged from 97.1–99.8% (*Spermophilus*) to 99.9% (*Glaucomys*) and the similarity of *B. washoensis* between the genera ranged from 94.9–95.0% (*Sciurus* vs. *Glaucomys*) to 98.4% (*Glaucomys* vs. *Tamiasciurus*) (Table 3).

B. washoensis strains except those from the genus *Spermophilus* were also classified by the clade of their host genus and geographic origins, i.e. *Tamiasciurus* and *Glaucomys* squirrels in North America and *Tamias* chipmunks and *Sciurus* squirrels in China. On the other hand, all of the strains derived from the genus *Spermophilus* formed a large clade, although the host animals of the genus *Spermophilus* originated from two different areas, i.e. North America and China (Fig. 1).

4. Discussion

To analyze the relationships between the genetic diversity of *B. washoensis* strains and host squirrels, MLSA with the 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC*, and *rpoB* genes were applied for 20 *B. washoensis* strains, which were isolated from five different genera of squirrels, including *Tamias*, *Tamiasciurus*, *Glaucomys*, *Sciurus*, and *Spermophilus*. These strains formed a large clade with *B. washoensis* strains nvh1 from a human patient in the phylogenetic tree analysis based on the *gltA* gene (Inoue et al., 2009b). However, the strains were not discriminated at the species level of the organisms by the sequence similarity of 16S rRNA, *gltA*, and *ribC* genes, because the similarities between *B. washoensis* strains and other known rodent-associated *Bartonella* spp. showed higher than those within *B. washoensis* strains. On the other hand, the sequence similarities (89.8–93.1%) based on the concatenated sequence of six loci between *B. washoensis* strains and other known rodent-associated *Bartonella* spp. were significantly lower than those (94.7–99.9%) within *B. washoensis* strains examined. In addition, all 20 strains examined were discriminated by the concatenated sequence. These results suggest that multi-gene analyses have higher discrimination power and should be a useful tool for further epidemiological investigation of *B. washoensis*.

In the phylogenetic trees based on the analysis of the concatenated sequence, *B. washoensis* strains formed a large clade apart from any of the other known rodent-associated *Bartonella* spp. Interestingly, *B. washoensis* strains formed five distinct clades by the genus of squirrels, i.e. clades *Spermophilus*, *Tamiasciurus*, *Glaucomys*, *Tamias*, and *Sciurus*, supported by 92–100% BV in ML and NJ and 100% BPP, respectively. The similarities of the concatenated sequences among the genus of squirrels were individually discriminated within the range from 94.9% to 98.4%, suggesting that *B. washoensis* is classified by the genus of the host squirrels. Based on the trees, all strains except those from squirrels of the genus *Spermophilus* were also classified by geographic origin, as the geographic distribution of *Tamiasciurus hudsonicus* and *Glaucomys volans* is limited to North America and that of *Tamias sibiricus* and *Sciurus vulgaris orientis* is in Eurasia. These results suggest that *B. washoensis* may have co-speciated with their host squirrels.

On the other hand, the clade of *Bartonella* strains obtained from *Spermophilus* species formed four sub-clades correspondent to squirrel species, including *Sp. beecheyi*, *Sp. richardsonii*, *Sp. columbianus*, and *Sp. dauricus*. The clades include the strains from two different continents, i.e. North America and Eurasia. This result may indicate that a phylogenetic relationship of *B. washoensis* strains obtained from the genus *Spermophilus* is mainly associated with the genus of squirrel hosts rather than with their geographic origins. Although most *B. washoensis* strains from the squirrels formed sub-clades by the species of squirrel, strain CJ22-1 from *Sp. columbianus* formed a clade with the strains from *Sp. richardsonii*. This result may explain that the habitat of *Sp. columbianus* and *Sp. richardsonii* are overlapped in North America (Nowak, 1999) and may be infected with related strains from common sources by some arthropod vectors.

In conclusion, MLSA based on the concatenated sequences of six genes is a useful tool for the discrimina-

tion of *B. washoensis* at the strain level. The strong host specificity of *B. washoensis* strains could allow to trace the source of *B. washoensis* infection in humans and animals by using MLSA.

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References

Bai, Y., Kosoy, M., Martin, A., Ray, C., Sheff, K., Chalcraft, L., Collinge, S.K., 2008. Characterization of *Bartonella* strains isolated from black-tailed prairie dogs (*Cynomys ludovicianus*). Vector Borne Zoonotic Dis. 8, 1–5.

Bown, K.J., Ellis, B.A., Birtles, R.J., Durden, L.A., Lello, J., Begon, M., Bennett, M., 2002. New world origins for haemoparasites infecting United Kingdom grey squirrels (*Sciurus carolinensis*) as revealed by phylogenetic analysis of bartonella infecting squirrel populations in England and the United States. Epidemiol. Infect. 129, 647–653.

Chomel, B.B., Wey, A.C., Kasten, R.W., 2003. Isolation of *Bartonella washoensis* from a dog with mitral valve endocarditis. J. Clin. Microbiol. 41, 5327–5332.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Heller, R., Artois, M., Xemar, V., De Briel, D., Gehin, H., Jaulhac, B., Monteil, H., Piemont, Y., 1997. Prevalence of *Bartonella henselae* and *Bartonella clarridgeiae* in stray cats. J. Clin. Microbiol. 35, 1327–1331.

Hillis, D.M., Bull, J.J., 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst. Biol. 42, 182–192.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.

Inoue, K., Kabeya, H., Kosoy, M.Y., Bai, Y., Smirnov, G., McColl, D., Artsob, H., Maruyama, S., 2009. Evolutional and geographical relationships of *Bartonella grahamii* isolates from wild rodents by multi-locus sequencing analysis. Microb. Ecol. 57, 534–541.

Inoue, K., Maruyama, S., Kabeya, H., Hagiya, K., Izumi, Y., Une, Y., Yoshikawa, Y., 2009. Exotic small mammals as potential reservoirs of zoonotic *Bartonella* spp. Emerg. Infect. Dis. 15, 526–532.

Jardine, C., Appleyard, G., Kosoy, M.Y., McColl, D., Chirino-Trejo, M., Wobeser, G., Leighton, F.A., 2005. Rodent-associated *Bartonella* in Saskatchewan, Canada. Vector Borne Zoonotic Dis. 5, 402–409.

Jukes, T.H., Cantor, C.R., 1969. Evolution of protein molecules. In: Munro, H.N. (Ed.), Mammalian Protein Metabolism, vol. 3. Academic Press, New York, pp. 21–132.

Kosoy, M., Murray, M., Gilmore Jr., R.D., Bai, Y., Gage, K.L., 2003. *Bartonella* strains from ground squirrels are identical to *Bartonella washoensis* isolated from a human patient. J. Clin. Microbiol. 41, 645–650.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947–2948.

Norman, A.F., Regnery, R., Jameson, P., Greene, C., Krause, D.C., 1995. Differentiation of *Bartonella*-like isolates at the species level by PCR-restriction fragment length polymorphism in the citrate synthase gene. J. Clin. Microbiol. 33, 1797–1803.

Nowak, R.M., 1999. Walker's Mammals of the World, 6th ed., vol. 2. The Johns Hopkins University Press, Baltimore, pp. 1254–1258.

Nylander, J.A.A., 2004. MrModeltest v2. Program Distributed by the Author. Evolutionary Biology Centre, Uppsala University.

Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Renesto, P., Gouvernet, J., Drancourt, M., Roux, V., Raoult, D., 2001. Use of *rpoB* gene analysis for detection and identification of *Bartonella* species. J. Clin. Microbiol. 39, 430–437.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Swofford, D.L., 2002. PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4. 0b10. Sinauer Associates, Sunderland, Massachusetts.

Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10, 512–526.

Yang, Z., 1994. Estimating the pattern of nucleotide substitution. J. Mol. Evol. 39, 105–111.

Zeaiter, Z., Fournier, P.E., Ogata, H., Raoult, D., 2002. Phylogenetic classification of *Bartonella* species by comparing *groEL* sequences. Int. J. Syst. Evol. Microbiol. 52, 165–171.

Zeaiter, Z., Liang, Z., Raoult, D., 2002. Genetic classification and differentiation of *Bartonella* species based on comparison of partial *ftsZ* gene sequences. J. Clin. Microbiol. 40, 3641–3647.

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Polymerase Chain Reaction Assay and Conventional Isolation of *Salmonella* spp. from Philippine Bats

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ABSTRACT

***Background*:** Salmonellae are important food and waterborne pathogens and the leading causes of the most widespread acute gastrointestinal illnesses around the globe. The organism has been detected in a wide range of host species such as mites, insects, crustaceans, mussels, fish, amphibians, reptiles, birds and mammals including wildlife animals. Salmonellae have been isolated in many species of bats in other countries. In the Philippines, there are 70 species of Philippine bats reported of which nine are considered as endemic. Although human salmonellosis (typhoid, paratyphoid and other *Salmonella*-associated infections) was the primary cause of illnesses and death from the 60 reported foodborne outbreaks (1995 to 2004), no case was ever reported involving Philippine bats. Since transmission of *Salmonella* from wildlife to humans is possible, as advocated by previous reports, the present study endeavored to isolate and molecularly detect *Salmonella* spp. from Philippine bats captured from Aklan, Laguna and Quezon City using conventional isolation method and polymerase chain reaction assay respectively.
***Materials, Methods & Results*:** A total of 96 apparently healthy bats were used in the study. Bats were captured using nylon mobile mist nets of 3 m long and 1.5 m high with 35 mm mesh size. Eleven species of bats were collected and identified following the reported key to the identification of Philippine bats. Majority of the collected species were insectivores under family Vespertilionidae while the largest population of the Philippine bats were frugivores belonging to family Pteropodidae. Necropsy was performed and intestines were collected and subjected to conventional culture method and PCR detection for *Salmonella* spp. Two samples (2.08%) were molecularly detected as positive for *Salmonella* spp. bacterial pathogen. The positive samples were obtained from the intestines of the adult female insectivorous bat species, *Miniopterus australis* and *M. schreibersi*, originating from Pangihan cave of Barangay Pablacion, Malay in Aklan. No *Salmonella* spp. was isolated using the conventional method.
***Discussion*:** The study reports the first detection and molecular evidence of *Salmonella* spp. in Philippine bats by PCR using intestinal samples. In addition, the data strongly indicated that PCR detection appears to be more sensitive over the conventional isolation method. The successful detection was attributed to the ability of PCR to sensitively detect atypical *Salmonella* and non-viable *Salmonella* cells. Results in the present study revealed that the Philippine bats, *Miniopterus australis* and *M. schreibersi*, both adult female insect-eating bats captured in Pangihan cave of Barangay Pablacion, Malay, Aklan harbored *Salmonella* in their intestines. Since salmonellae have been detected in a large variety of environment and host species including insects, these bats may have acquired these microorganisms in water and in their diet. This finding shows that Philippine bats may serve as potential reservoir and carrier of *Salmonella* organisms. The data also strongly indicates that bats may actively contribute in the dissemination of salmonellae into the environment through fecal route. This currently makes Philippine bats as a potential threat to livestock and may pose a serious public health concern, since all serotypes of *Salmonella* are considered to be pathogenic to humans.

Keywords: Bats, *Miniopterus australis*, *Miniopterus schreibersi*, *Salmonella*, PCR.

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INTRODUCTION

Salmonellae are important food and waterborne pathogens of the most widespread acute gastrointestinal illnesses worldwide [1,4,28]. The organism is present in the gastrointestinal tract of warm-blooded and cold-blooded animals and hence, excretion in feces results in contamination of water, food and environment [11]. The organism has been detected in a variety of host species such as mites, insects, crustaceans, mussels, fish, amphibians, reptiles, birds and mammals including wildlife animals [2,10,15,17,21]. Many studies have reported bats as natural hosts of many emerging and re-emerging infectious diseases [19].

Salmonellae have been isolated in many species of bats in other countries [2,15]. In the Philippines, there are 70 species of Philippine bats reported of which nine are endemic as listed in the 2000 IUCN Red List of Threatened Species [16]. Since *Salmonella* is zoonotic in nature and previous study has reported that wildlife may serve as a reservoir for *Salmonella* infections [26], the present study endeavored to isolate and molecularly detect *Salmonella* spp. from Philippine bats captured from Aklan, Laguna and Quezon City using conventional isolation and polymerase chain reaction (PCR) assay respectively.

Two PCR positive samples (2.08%) were obtained from the intestines of the adult female insectivorous bat species, *Miniopterus australis* and *M. schreibersi*, collected from Pangihan cave of Barangay Pablacion, Malay in Aklan. No *Salmonella* spp. was isolated using the conventional method. This finding indicates that Philippine bats are potential carrier of *Salmonella* spp. and may play a significant role in the dissemination of these pathogenic organisms in the environment. Furthermore, the study represents the first detection of *Salmonella* spp. in Philippine bats.

MATERIALS AND METHODS

Collection of bats

A total of 96 apparently healthy bats were used in the study. Forty (40) bats were collected at the Pangihan caves in Barangay Pablacion, Malay and Libertad caves in Barangay Libertad, Nabas in Aklan using nylon mobile mist nets of 3 m long and 1.5 m high with 35 mm mesh size. The mist nets were set up on the entrance and inside the caves. Nylon mist nets of 12 m long and 2 m high with 35 mm mesh size were used to capture twenty four (24) and thirty two (32) bats from the University of the Philippines Los Baños (UPLB) Hortorium in Laguna, and UP Diliman Marine Science Institute (MSI) and Protected Areas and Wildlife Bureau (PAWB) in Quezon City respectively. Seven net nights for one night placed along trails on forest gaps and across the river were set up in Laguna while 14 net nights, seven mist nets for two nights placed near swampy areas in Quezon City.

Species identification of bats

Eleven species of bats were collected and identified following the reported key to identification of Philippine bats [9]. Five species of insect-eating bats and one species of fruit-eating bat were captured from Aklan namely, *Miniopterus australis, M. schreibersi, M. tristis*, *Hipposideros diadema, Myotis macrotarsus* and *Ptenochirus jagori* respectively. Three species of fruit-eating bats, *Ptenochirus jagori, Cynopterus brachyotis* and *Eonycteris spelaea,* were collected from Laguna. Two species of insect-eating bats, *Scotophilus kuhlii* and *Pipistrellus javanicus,* and four species of fruit-eating bats, *Ptenochirus jagori, Cynopterus brachyotis, Rousettus amplexicaudatus* and *Eonycteris spelaea* were captured in Quezon City.

Necropsy of bats and sample collection

After the collection, the body weight of each bat was determined and the dosage for anesthetic was computed using a dose of 0.45 mL of 5% zolazepam-tiletamine[1] per 30 g body weight. The anesthetic was given intramuscularly and the bat was euthanized through intracardiac exsanguination. The body parameter measurements of each carcass were recorded to use for identification purposes.

Each bat was then placed on a necropsy board where the skin over the thorax and abdomen was reflected. The thorax was opened and the internal organs were collected by research collaborators from Japan for other investigative works. In the present study, the peritoneum was incised and the intestine was detached from its mesentery. The entire intestinal tract was cut through the rectum, ligated on both ends and placed on a sterile Petri dish with normal saline solution. The carcass was submitted to the UPLB Museum of Natural History for preservation and storage.

Conventional isolation method

The small intestines were minced and transferred to a pre-labeled tube of nutrient broth[2]. The samples were