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Multilocus sequence typing analysis of *Pseudomonas aeruginosa* isolated from pet Chinese stripe-necked turtles (*Ocadia sinensis*)

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Our research sought to characterize the phylogeny of *Pseudomonas aeruginosa* isolated from pet Chinese stripe-necked turtles (*Ocadia sinensis*) to better understand its evolutionary relation to other isolates and increase understanding of a potential zoonotic pathogen transmitted through direct contact with pet turtles. Thirty-one *Pseudomonas aeruginosa* isolates were obtained from both immature and adult turtles sold in pet shops in Korea. To characterize the phylogenic position of Chinese stripe-necked turtle-borne *P. aeruginosa* relative to other strains, multilocus sequence typing (MLST) analysis was performed due to the accessibility and breadth of MLST databases. Seven housekeeping genes (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*) were sequenced and the results were compared with data from the MLST database. The genes were further used for phylogenetic analysis of *P. aeruginosa* using concatenated gene fragments. Both rooted and unrooted phylogenetic trees were generated. Eleven distinct sequence types were present within the isolates among which seven were new. Expanding an unrooted phylogenetic tree to include *P. aeruginosa* MLST sequences isolated from various other geographic locations and sources revealed a divergent cluster containing the majority of isolates obtained from turtles. This suggests that *P. aeruginosa* strains particularly well-adapted for inhabiting turtles occupy a distinct phylogenetic position.

Keywords: MLST, *Pseudomonas aeruginosa*, Chinese stripe-necked turtles (*Ocadia sinensis*), pet turtles, phylogeny

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Pseudomonas aeruginosa is a gammaprotobacterium possessing a high degree of adaptability but thriving in moist or wet conditions [1]. *P. aeruginosa* is also an opportunistic pathogen prevalent in nosocomial infections, particularly among patients with cystic fibrosis [2]. Its adaptability allows it to be disseminated from diverse sources within hospitals, and common-source outbreaks have stemmed from contaminated tap water [3], hand lotion [4], and children's bath toys [5].

The genome size of *P. aeruginosa* ranges from 5.2 to 7.1 Mbp [6]. It consists of a conserved core genome with strain-specific regions of genomic plasticity that allow strains to acquire or shed genomic segments for development of survival traits in a wide range of environment [7]. Furthermore, recent studies show strong indications that *P. aeruginosa* exhibits an epidemic population structure with clinical isolates indistinguishable from environmental isolates due to a lack of specific habitat selection [8]. For example, *P. aeruginosa* isolated clinically and from oil-contaminated soils showed similar pathogenic properties and antibiotic resistance [9], and isolates from a single river in Belgium displayed biodiversity comparable to isolates obtained around the globe [10]. This variability significantly impacts the methodology used to study the evolution and epidemiology of *P. aeruginosa*.

Multilocus sequence typing (MLST) is a strain-typing system that focuses exclusively on conserved housekeeping genes [11,12]. Though pulsed-field gel electrophoresis (PFGE) possesses higher discriminatory power, the lack of a universal standard and portability make MLST more

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ideal for comparative analysis of strain types regardless of region or source. Moreover, in a comparative study of molecular techniques for typing *P. aeruginosa*, MLST had the greatest predictive value (100%) in labeling strains as unique [13]. The standardization of MLST has given rise to databases that enable comparative analysis of allele sequences and identification of unique sequence types.

Chinese stripe-necked turtles (*Ocadia sinensis)* are omnivorous inhabitants of lakes, rivers, and canals [14]. They are native to southern China and parts of Vietnam, but they are commonly imported for sale in Korean markets. Turtles harbor a variety of zoonotic bacteria within their digestive tract [15]. Among these, *Salmonella* spp. in particular have been well-studied as a source of salmonellosis in humans [16,17], but *P. aeruginosa* has also been isolated from sea turtles [18] and desert tortoises [19]. As an opportunistic pathogen in reptiles, it can cause ulcerative stomatitis, pneumonia, dermatitis, and septicemia [20]. As a zoonotic pathogen, *P. aeruginosa* infections can involve any part of the body and cause a range of symptoms from minor gastrointestinal infections resulting in diarrhea, fever, and headache to severe infections including meningitis, bacterial keratitis, endophthalmitis, and necrotizing enterocolitis [21].

Our research sought to characterize the phylogeny of *P. aeruginosa* isolated from pet turtles to better understand its evolutionary relation to other isolates and increase understanding of a potential zoonotic pathogen transmitted through direct contact with pet turtles. To our knowledge, this is the first study to phylogenetically characterize *P. aeruginosa* from pet turtles.

Materials and Methods

Purchase of pet turtles

Forty Chinese stripe-necked turtles were purchased from several pet shops and online markets in Korea. Five turtles were purchased at under four weeks of age and raised in our laboratory for one and a half years. The remaining turtles were under four weeks of age at purchase and studied immediately. All turtles were clinically inspected upon purchase and showed no signs of disease.

Raising condition of pet turtles

A total of three cages were managed with one cage containing the adult turtles and the immature turtles split between the other two. Each cage contained a slope made from soil and pebbles, 2 L of sterilized water, and a canister filter to maintain water quality. The turtles were raised following the general husbandry method [22]. Gammarus dried shrimp with calcium supplements (Amazon Ltd., Seoul, Korea) were fed twice a day while water temperature was kept within 26±2ºC, pH 6.5-8.2, and 12 h of photoperiod each day were maintained during the experiment.

Enrichment and isolation of *P. aeruginosa*

Cloacal swabs were enriched by submerging them in Tryptic Soy Broth (TSB) (MBcell Ltd., Seoul, Korea) and incubating at 37ºC for 24 h. Enriched samples were streaked onto the selective culture medium Cetrimide agar (CN) (MBCell Ltd., Seoul, Korea) and incubated at 37ºC for 24 h. Suspicious colonies were subcultured onto CN agar and incubated once more at 42ºC for 24 h. Isolates that showed growth were presumptively identified as *P. aeruginosa*. To confirm the identity of these isolates, PCR for the *acsA* gene used in the MLST scheme was performed and the fragments were sequenced. A BLAST search was conducted on the resulting sequences to verify their correspondence with previously sequenced *P. aeruginosa* genomes.

Locus selection for MLST analysis

The seven genes *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE* (Table 1) were selected according to the MLST scheme for *P. aeruginosa* created by Curran *et al*. [23]. The loci were originally chosen based upon biological role (e.g. a range of differing central housekeeping roles including mismatch repair, DNA replication, and amino acid biosynthesis), size (>600 bp), location (i.e., a minimum of 6 kbp upstream or downstream from known virulence factors, lysogenic phage, or insertion sequence elements), and suitability for nested primer design and sequence diversity.

Amplification and sequencing of loci

PCR and sequencing was performed by Cosmogenetech, Inc. (Seoul, Korea). The PCR primers described by Curran *et al*. [23] were used, and reaction conditions were as follows: initial denaturation at 96ºC for 1 min, primer annealing at 55ºC, extension at 72ºC for 1 min, followed by a final extension step of 72ºC for 10 min. Each reaction mixture contained 1.0 μL chromosomal DNA (5-20 ng/μL), 1.0 μL forward primer (5 μM), 2.0

μL reverse primer (5 μM), 2.5 μL 10x PCR buffer, 2.0 μL dNTP solution, 5.0 μL tuning buffer, 0.5 μL SP-Taq polymerase, and 12 μL PCR-grade water. The amplification product was purified before use in the sequencing reaction. Big Dye reactions were carried out with a Big Dye Terminator 3.1 Cycle Sequencing Kit and sequencing was performed on an ABI 3730XL genetic analyzer with a 50-cm array. Both forward and reverse sequences were obtained using both the PCR primer and internal sequence MLST primers for each locus.

Sequence data analysis

Forward and reverse sequences were aligned for all seven MLST genes using the Finch TV software program (Geospiza, Inc., Seattle, USA). Positions at which gaps were present in any of the isolates were excluded from comparative analysis. Sequences were confirmed through individual BLAST searches to determine their correspondence with previously sequenced *P. aeruginosa* genomes. The full aligned sequences were then saved in FASTA format. These sequences were aligned using the CLUSTAL W tool present in MEGA 7 [24]. Furthermore, allele sequences for each of the seven genes were downloaded from the MLST database (http://pubmlst.org/paeruginosa) and used to align the internal MLST fragments. These automatically aligned sequences were checked again manually.

Allele and sequence type assignment

Based upon the allele numbering system available in the *P. aeruginosa* MLST database, an allele number was assigned for every gene in each isolate. Alleles that differed by even a single base pair from those present in the database were denominated as "new." Each distinct combination of seven numbers (representing the seven alleles) was assigned a number denoting its sequence type (ST) according to the MLST database. Combinations not present in the database were considered "new" STs.

Phylogenetic analysis

Genetic similarity and evolutionary distance were calculated using the MEGA 7 software program. Dendrograms were generated by Neighbor-Joining (NJ), Maximum Likelihood (ML), Minimum Evolution (ME), and Maximum Parsimony (MP) methods using the Kimura 2-parameter model for distance calculations and 1,000 bootstrap replications [25,26]. Analyses were repeated for both individual gene sequences and the concatenated set of all sequences. Rooted and unrooted NJ trees were created for the concatenated seven-gene sequences, and closely related pseudomonads were used as an out group. The outgroup gene sequences were obtained from the NCBI Gene Bank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html). In addition, an unrooted NJ tree was created for the concatenated seven-gene sequence of the internal MLST fragments of isolates from the present study and previous *P. aeruginosa* isolates obtained from the MLST database. The number of polymorphic nucleotide sites was determined for each internal MLST fragment sequence and the longer PCR sequences.

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study were submitted to the NCBI GenBank. The respective accession numbers for *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE* are KX784267 to KX784297, KX784298 to KX784328, KX784329 to KX784359, KX784360 to KX784390, KX784391 to KX784421, KX784422 to KX784452, and KX784453 to KX784483.

Results

Identification of *P. aeruginosa* isolates

Thirty-one of forty isolates showed positive growth on CN agar at 42ºC. A subsequent BLAST search showed a 99-100% match to *P. aeruginosa* genomes present in GenBank for the *acsA* sequences of these isolates, which confirmed their identity as *P. aeruginosa*.

Allelic profiles

The fragment lengths for MLST sequences (obtained from the MLST primers) and the extended gene sequences (obtained from the PCR primers) along with the number of variable nucleotide sites are shown in Table 1. Each allelic sequence was checked against the MLST database. All sequences were present in the database with the exception of one *nuoD* sequence. Eleven distinct STs were found amongst the isolates with seven of these constituting new STs. The allelic profiles and STs are shown in Table 2 with the isolates labeled according to their age (adult or immature) and the number designated to the turtle from which they were isolated.

Among the 11 distinct STs, four types (110, 266, 492, 1209) had been previously isolated and uploaded to the MLST database. The isolate Immature-09 corresponded

Table 1. Functions, lengths, and number of variable sites among the isolates for the PCR and MLST fragments

Gene	Function	PCR Fragment		MLST Fragment	
		Length (bp)	Variable Sites	Length (bp)	Variable Sites
acsA	Acetyl coenzyme A synthetase	811	15	390	11
aroE	Shikimate dehydrogenase	825	23	495	16
guaA	GMP synthase	914	20	372	10
mutL	DNA mismatch repair protein	905	16	441	7
nuoD	NADH dehydrogenase I chains C and D	1164	18	366	6
*ppsA**	Phosphoenolpyruvate synthase	387	7	369	7
trpE	Anthralite synthetase component I	783	17	441	12
Total		5789	116	2874	69

*The PCR primer failed to adequately replicate a sufficient quantity of the PCR fragment of the *ppsA* gene. Therefore, the MLST primer was used and the resulting sequence length is almost identical to that of the MLST fragment.

Table 2.MLST allelic profiles and strain types for 31 *P. aeruginosa* isolates[a]

Turtle isolated from	Allelic type							Sequence type
	acsA	aroE	guaA	mutL	nuoD	ppsA	trpE	
Adult-01	11	5	4	11	3	15	19	New (1)
Adult-03	16	5	11	72	44	7	52	266
Adult-04	11	5	4	11	3	15	19	New (1)
Adult-05	11	5	4	11	3	15	19	New (1)
Immature-02	40	5	30	61	1	7	14	492
Immature-04	121	4	26	11	4	38	9	1209
Immature-08	121	4	26	11	4	38	9	1209
Immature-09	15	5	1	3	2	12	7	110
Immature-11	28	3	5	4	4	7	3	New (2)
Immature-12	121	4	26	11	4	38	9	1209
Immature-13	121	4	26	11	4	38	9	1209
Immature-14	40	5	30	61	1	7	14	492
Immature-15	121	4	26	11	4	38	9	1209
Immature-16	5	50	65	31	New	6	26	New (3)
Immature-19	28	14	17	7	3	7	9	New (4)
Immature-20	28	14	17	7	3	7	9	New (4)
Immature-21	40	5	30	61	1	7	14	492
Immature-22	28	3	5	4	4	7	3	New (2)
Immature-23	121	4	26	11	4	38	9	1209
Immature-24	121	4	26	11	4	38	9	1209
Immature-25	121	4	26	11	4	38	9	1209
Immature-26	1	5	11	11	2	15	10	New (5)
Immature-27	5	50	65	31	1	6	26	New (6)
Immature-28	28	14	17	7	3	7	9	New (4)
Immature-29	1	5	11	11	2	15	10	New (5)
Immature-30	28	14	17	7	3	7	9	New (4)
Immature-31	121	4	26	11	4	38	9	1209
Immature-32	121	4	26	11	4	38	9	1209
Immature-33	121	4	26	11	4	38	10	New (7)
Immature-34	121	4	26	11	4	38	9	1209
Immature-35	5	50	65	31	1	6	26	New (6)

[a]The numbers correspond to the allele numbers assigned in the MSLT database. Any allele not contained within the database was designated "new". Using the seven allelic types for each gene, the sequence type was determined by referencing the MLST database. Known sequence types were given the corresponding number from the database while sequence types not available within the database were designated "new" and assigned a number in order of appearance when the turtles from which they were isolated were listed alphanumerically. The total number of distinct allelic types were: 8 for *acsA*, 5 for *aroE*, 8 for *guaA*, 7 for *mutL*, 5 for *nuoD*, 5 for *ppsA*, and 8 for *trpE*. One new allelic type was found for *nuoD* and 7 new sequence types were noted.

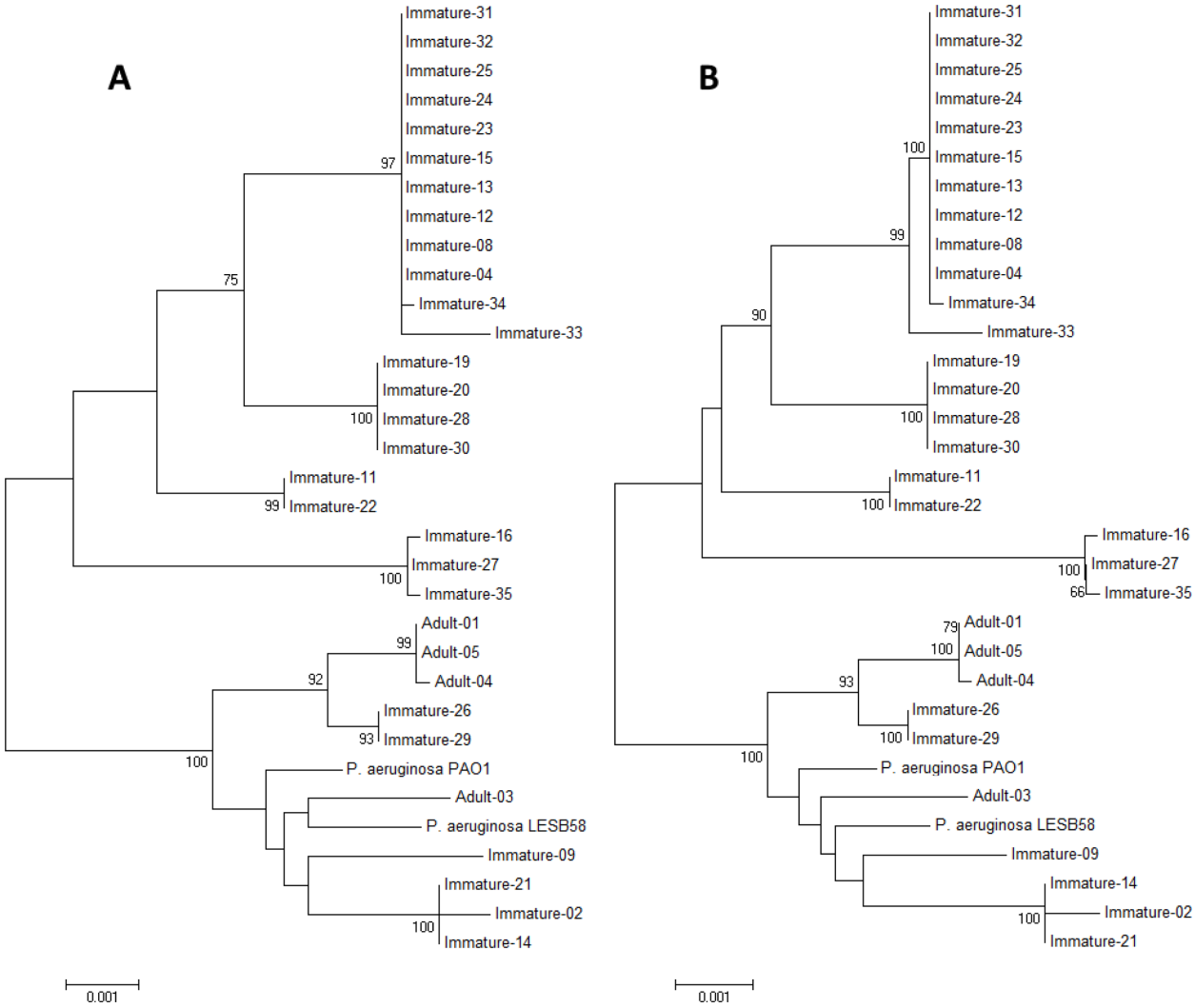

Figure 1. Unrooted phylogenetic trees using the concatenated sequences of extended PCR fragments from seven housekeeping genes (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*) of *P. aeruginosa* using **A.** the ML and **B.** the NJ method with the Kimura 2-parameter model for distance calculations. The total length of the concatenated sequences was 5,784bp. Bootstrap percentages received from 1,000 replications were used and values over 50% are shown in the trees. The scale bars (0.001) indicate the number of nucleotide substitutions per site.

to ST 110, which had been isolated from an upper respiratory infection in a cystic fibrosis patient in Australia. Isolate Adult-03 matched ST 266, which was found in water, sputum, and a urinary tract infection (UTI) in Australia. Three isolates from immature turtles matched ST 492 deposited from a sputum sample in the Netherlands and eleven isolates from immature turtles matched ST 1209 from a sputum sample in the Czech Republic. The high number of isolates matching ST 1209 indicate potential transfer of strains between turtles from the same pet shop.

Phylogenetic analysis

To analyze the phylogenetic relationships between the isolates, the PCR sequences for each gene and the concatenated sequences of all genes were used to construct phylogenetic trees. The NJ, MP, ME, and ML methods were separately used to construct these trees with distance calculated by the Kimura 2-parameter model. Thirty-six strains were used including all isolates, two reference strains (*P. aeruginosa* PAO1 and LESB58) and three outgroup strains (*P. protegens* Pf-5, *P. fluorescens* SBW25, and *P. syringae pv tomato* DC3000). Figure 1 shows the unrooted tree generated through the NJ and ML methods without the outgroup strains. As Figure 1 shows, different tree-generating methods produced similar results; therefore, trees created with other methods were not included in this publication. Figure 2 shows the

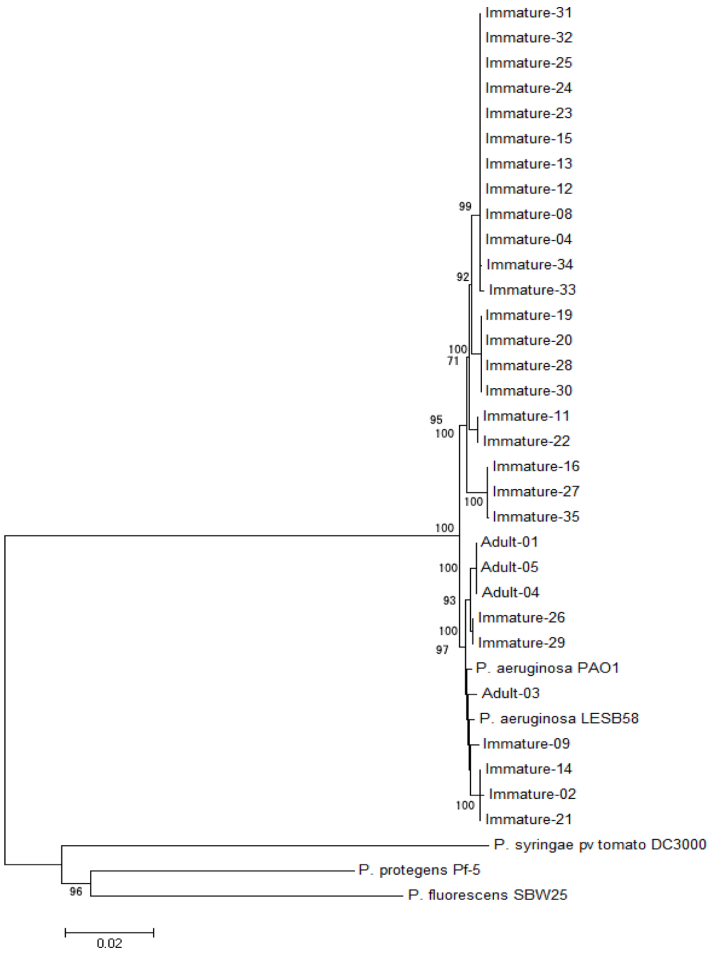

Figure 2. Rooted phylogenetic tree using the concatenated sequences of extended PCR fragments from seven housekeeping genes (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*) of *P. aeruginosa* using the NJ method with the Kimura 2-parameter model for distance calculations. The total length of the concatenated sequences was 5,784bp. Bootstrap percentages retrieved from 1,000 replications were used and values over 50% are shown in the tree. The scale bar (0.02) indicates the number of nucleotide substitutions per site. Data for reference and outgroup strains were collected from the NCBI GenBank database.

rooted NJ tree created by including the outgroup strains. To compare the sequences of isolates from the present study with other MLST-analyzed strains of *P. aeruginosa*, the MLST sequences of twenty-five randomly chosen isolates from various geographic regions and sources were downloaded from the MLST database. Figure 3 shows a radiation tree comparing our isolates with these randomly chosen isolates and the reference strains. Due to the presence of multiple isolates with identical STs, nodes containing multiple isolates were abbreviated into a label containing the ST and corresponding number of isolates.

Discussion

P. aeruginosa has demonstrated ready adaptability to and survival in natural environments, animals, and humans. As exotic pet ownership becomes increasingly popular, awareness about the associated public health concerns should grow as well. Reptiles in particular are a known reservoir for zoonotic pathogens and are therefore considered a high-risk pet group [27]. Moreover, genetic analysis of *P. aeruginosa* in pet turtles provides important information about its geographic distribution and adaptations for survival in turtles. We focused our study on solely Chinese stripe-necked turtles because they are a popular pet species in Korea and, as this is a preliminary study on MLST analysis of *P. aeruginosa* in turtles, using multiple species of turtles could introduce a confounding variable into our analysis if strains differ between turtle species.

The present study used MLST analysis on 31 strains

Figure 3. Radiation tree using the concatenated sequences of internal MLST fragments from seven housekeeping genes (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*) of 57 strains of *P. aeruginosa* (31 isolates from this study, 25 from the MLST database, and PAO1 from the NCBI GenBank database).The NJ method was used with the Kimura 2-parameter model for distance calculations. The total length of the concatenated sequences was 2,8744bp. Nodes were supported by 1,000 bootstrap replications. The large cluster containing exclusively isolates from this study was demarcated as it contained the majority of isolates from immature turtles. The scale bar (0.001) indicates the number of nucleotide substitutions per site.

and discovered 11 distinct sequence types. In addition, a radiation tree constructed from the concatenated sequences of internal MLST fragments of both our isolates and previous MLST isolates showed a cluster formed exclusively by isolates from the present study. Previous studies have focused on MLST analysis of clinical isolates [28,29] or marine isolates [30], yet to our knowledge this is the first to focus on *P. aeruginosa* obtained from turtles.

We chose MLST for this study to easily compare our data with those of other strains and for its ability to accurately identify unique strains of *P. aeruginosa* [13]. Phylogenetic analysis of the resulting data also enables clear representation of the relative intraspecies evolutionary position of turtle-inhabiting strains. Furthermore, the MLST database referenced (http://pubmlst.org/paeruginosa) contained 5,653 *P. aeruginosa* isolates with 2,341 unique STs; however, none of these isolates were obtained from

turtles.

Phylogenetically, the cluster demarcated in Figure 3 that formed when our concatenated sequences were compared to other MLST sequences from various geographic regions and sources also developed in Figure 1 in the upper half of the trees. Their distinct phylogenetic position away from both other MLST sequences and the reference strains suggests that this cluster indicates strains particularly well-adapted to inhabiting Chinese stripe-necked turtles. Therefore, this cluster indicates a connection between the suitability of certain *P. aeruginosa* strains for inhabiting a particular environment and their phylogenetic position. Similarly, a study by Khan *et al*. [30] studied marine *P. aeruginosa* strains and concluded that isolates from the ocean were phylogenetically distinct from isolates in other marine environments. They also proposed a connection between geographical location and phylogenic position that shows the adaptations a strain undergoes in particular environments. On the other hand, a study conducted by Pirnay *et al*. [10] found a comparable degree of *P. aeruginosa* biodiversity within the Woluwe River in Belgium and isolates obtained on a global scale. They proposed that this implied *P. aeruginosa* was either an autochthonous member of the river's bacterial community which proceeded to homogeneously spread throughout the world or the dense human population surrounding the river influenced the development of highly diverse strains.

These studies clearly promoted the concept that clinical *P. aeruginosa* strains are merely environmental strains whose adaptations allowed them to successfully infiltrate humans. STs obtained in the present study also matched STs in the MLST database isolated from clinical samples, which further reinforces this concept. Consequently, pet turtle owners in countries lacking exotic pet market regulations such as Korea should be informed of the associated risk of zoonotic infection from *P. aeruginosa*.

In conclusion, MLST analysis of 31 *P. aeruginosa* isolates obtained from Chinese stripe-necked turtles sold as pets revealed 11 distinct sequence types. Among these, seven were designated as "new," and the others matched previously isolated clinical strains. Phylogenetic analysis revealed a distinct cluster formation amongst the majority of the isolates that may suggest an evolutionary adaptation for inhabiting Chinese stripe-necked turtles. Further studies should expand upon this and also focus on characterization of the isolated strains, particularly their potential for pathogenicity and antimicrobial resistance as these are important public health considerations.

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Conflict of interests The authors declare that there is no financial conflict of interests to publish these results.

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