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Research Article

Identification of Rodent Species That Infest Poultry Houses in Mafikeng, North West Province, South Africa

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Rodents cause serious adverse effects on farm production due to destruction of food, contamination of feed, and circulation of diseases. The extent of damage or the diseases spread will depend on the type of rodents that invade the farm. This study was conducted in order to find out the species of rodents that infest poultry farms around Mafikeng, North West Province of South Africa. The study was part of a broader project that was investigating *Salmonella* vectors in the poultry farms around the province. The study trapped 154 rodents from selected farms and used the *Cytochrome oxidase subunit 1 (COI)* and the *Cytochrome b* (*Cyt-b*) barcoding genes for species identification. Two rodent pest species, namely, *Rattus rattus* (the black rat) and *Rattus tanezumi* (the Asian Rat/Asian House Rat) were identified. A total of 99 (64.3%) were identified as *Rattus rattus* and 55 (35.7%) were *Rattus tanezumi*. Between the two target genes, *Cyt-b* gene was only able to identify 40 (25.97%) of the total samples while *COI* was more efficient and amplified all the samples and thus was a better target gene for this kind of identification. The two rat species identified are known vectors of serious diseases; thus their presence should be regarded as an indication of high risk for diseases. Despite having been detected in the country before, finding *R. tanezumi* as the second largest rat species in the area was unexpected since this species is known to be indigenous to Asia.

1. Introduction

Rodents are relatively small mammals belonging to the order Rodentia that includes porcupines, rats, mice, squirrels and marmots [1]. They are famously known to cause huge losses to stored food, crops, and property and also to transmit many pathogens that cause diseases of humans and animals [2]. The house mouse (*Mus musculus*), roof rats (*Rattus rattus*), and the brown rat (*R. norvegicus*) are the three main species of rodents usually found worldwide [3]. The genus *Rattus* is one of the most common rodents found in poultry houses worldwide.

The genus *Rattus* consists mainly of Black rat (*R. rattus*), Norway rat (*R. norvegicus*), Asian rat (*R. tanezumi*), and *R. mulium*. The genus has some of the most adaptable rodents in the world and most of them have their origins in Asia where they migrated from to other parts of the world following the development of agriculture which provided food and shelter for their survival. Their intricate association with farms makes them very important vectors of pathogens some of which are zoonotic. For instance, the brown rat is famous as a carrier of gastrointestinal helminths and mites responsible for Plague, the black rat is a carrier of trematode species, cestode species, and *Salmonella* spp., and the Asian rat is a source of gastrointestinal helminths [4–7].

Identifying the rodent species in a farm set-up is, therefore, important in determining the specific rat species' risk to disease as well as other adverse effects in a farm.

Figure 1: Map of Africa showing the Mafikeng sampling area in the North West Province of South Africa.

Unfortunately, rodents are not very easy to distinguish by the routine methods available that use physical attributes and so molecular identification has been offering the best option for identification. Molecular identification can be achieved by a number of methods but DNA barcoding, which is a taxonomic method that uses a short genetic marker in an organism's DNA to identify it to a particular species, has been found easy and particularly effective for this purpose [8]. The target gene used for barcoding is the *COI* gene which is a very common gene among species and has been fairly conserved over generations [9, 10]. Another gene commonly used is the *Cytochrome b* gene which is also a very good discriminatory gene for species identification [8, 11]. These two genes were, therefore, used in this study to identify rodents in poultry houses from selected farms around Mafikeng, North West Province of South Africa.

2. Materials and Methods

2.1. Study Area. The study was carried out in Mafikeng, the North West Province of South Africa. The North West Province is referred to as one of the biggest agricultural production areas in South Africa, with some of the largest cattle herds in the country found at Stellaland (Vryburg) and mixed crop farming land. The province is also the second largest chicken producer in South Africa at 21.3% after Western Cape with 21.9% (SAPA, 2014). The province has four districts, namely, Bojanala Platinum, Ngaka Modiri Molema, Dr Ruth Segomotsi Mompati, and Dr Kenneth Kaunda. This study was conducted around Mafikeng in The Ngaka Modiri Molema district (Figure 1). The city lies between 25 and 28°C South of the Equator and 22 and 28°C longitude east of the Greenwich meridian. It shares an international border with the Republic of Botswana in the North and is 260 km West of Johannesburg. Mafikeng is built on the open veld at an elevation of 1500 m along the banks of the Upper Molopo River. Climatic conditions of the province differ significantly from West to East. The Western region receives less than 300 mm of rain per annum, the central region around 550 mm per annum, while the Eastern and South Eastern regions receive over 600 mm per annum [12].

2.2. Collection of Samples. A list of poultry farms in the Mafikeng area was compiled using the Department of Agriculture records. A few farms in the north, south, east, and west were randomly selected, the farmers were approached, and those that agreed were included in the study. Rodents were captured using Sherman rat traps [13] baited with peanut butter plus cheese and placed where the rats regularly visit. The traps were checked each morning during three consecutive days. The target number of rats was between 150 and 200 based on previous studies [2, 5]. Live rats were euthanized humanely using chloroform inhalation [14]. Their surface was disinfected with 70% ethyl alcohol before dissection. Dissection of the abdominal cavity was done using a surgical blade, a pair of forceps, and kidneys were harvested and placed in 4°C until processing. Extra care was taken in order to avoid cross-contamination by using new disposable utensils like scalpels, forceps, petri-dishes, and gloves for each sample. After collecting the samples, carcasses were

placed in carcass containers located within designated carcass refrigerators/freezers in the post mortem room and then incinerated.

2.3. DNA Extraction. DNA was extracted from tissues (kidney) using a QIAamp DNA Blood and Tissue Kit [Qiagen, Hilden, Germany (No. 69504)]. The procedure was performed according to protocols provided by the manufacturers. The DNA extracted was stored at −80°C until analysis by PCR.

2.4. Evaluation of the Quantity and Quality of Isolated DNA. The amount of DNA extracted from the samples was determined by spectrophotometry with a NanoDrop ND-1000 system (NanoDrop Technologies, Inc., Wilmington, DE, USA). The purity of DNA was determined spectrophotometrically from the ratio of absorbance at 260 and 280 nm (A_{260}/A_{280}). A ratio of between 1.7 and 2 indicates an excellent quality of DNA.

2.5. PCR for Amplification of CO1 and Cyt-b Genes of Captured Rats. For rat species identification, PCR was used targeting the *CO1* and *Cyt-b* genes of the rats following published protocols [8, 15]. The final reaction mixture was 25 μl and consisted of 2 μl of template DNA, 8.5 μl double distilled water, 2X Dream *Taq* Green PCR Master Mix (2X Dream *Taq* Green buffer, 4 mM $MgCl_2$, 0.4 mM) of each dNTP and 1 unit/μl of thermo stable *Taq* polymerase (Thermo Scientific, USA), the primer mix contained 10 μM of each oligonucleotide primer.

To amplify the 750 bp product of *COI,* the primers used were BatL5310 (5′-CCT ACT CRG CCA TTT TAC CTA TG-3′) and R6036R (5′-ACT TCT GGG TGT CCA AAG AAT CA-3′). The PCR conditions were one initial denaturation step of 94°C for 2 min, subjected to 35 cycles, denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and the first extension at 72°C for 1 min and a final extension step of 72°C for 5 min with the holding temperature at 4°C [15].

To amplify the 762 bp product of *Cyt-b* gene, the primers used were RGu2L (CAG CAT TTA ACT GTG ACT AAT GAC) and RCb9H (TAC ACC TAG GAG GTC TTT AAT TG), with the following PCR conditions used: 94°C for 3 min, 35 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, followed by a final extension at 72°C for 5 min, and cooling down to 4°C and storage [16].

The PCR amplicons in both PCR reactions were analysed by electrophoresis in 1% (w/v) agarose gel containing ethidium bromide (10 μg ml^{-1}) then viewed under UV light at 420 nm wavelength. A ChemiDoc Imaging System (Bio-Rad ChemiDocTM MP Imaging System, UK) was used to capture the image using Gene Snap (version 6.00.22) software.

2.6. Sequencing. Seventeen micro liters of all positive PCR products were sent for sequencing at Inqaba Biotechnical Industries (Pty) Ltd in Pretoria, South Africa. The acquired sequence was aligned against GenBank data base using Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST) from the National Center for Biotechnology Information (NCBI) to identify sequences with high similarity (38). One direction sequencing was done.

2.7. Phylogenetic Analysis. Gene sequences obtained from all positively tested amplicons were edited using BioEdit [17] to remove any degenerate base pairs and then saved as FASTA format. To confirm sequences obtained from *CO1* and *Cyt-b* analysis, the nucleotide basic local alignment search tool (BLASTn) was used. Only gene sequences with 97% to 100% similarity match score were considered as significant.

The phylogenetic tree was constructed to illustrate the evolutionary relationships among *Rattus* spp. Multiple alignments of the sequences were carried out by MAFFT program 6.864 against corresponding nucleotide sequences retrieved from Gen-Bank. Evolutionary distance matrices were generated [18]. The aligned *Cyt-b* sequences were used to construct a phylogenetic tree as implemented in the MEGA 7 package and the neighbor-joining (NJ) and distance matrix methods were used [18]. A bootstrap confidence analysis was performed with 1000 replicates. A putative chimeric sequence was identified using the Chimera Buster 1.0 software. Manipulation and tree editing was carried out using Tree View [17].

For *CO1* analysis, multiple and pairwise alignments were done by ClustalW on Mega 7 [19]. Subsequently, the evolutionary history was inferred based on the Hasegawa-Kishino-Yano model [20] with 1000 bootstrap support values. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 69.02% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Ethics Committee Approval. Prior to the commencement of the study, the research proposal was approved based on Animal Research Ethics Committee (NWU-00274-18-A5) guidelines by North West University Research Ethics Regulatory Committee (NWU-RERC).

3. Results

3.1. Rodent's Identification. A total of 154 rat samples were captured and examined using *Cyt-b* and *COI* genes to identify the rodent species. Of the 154 rodents, 99 (64.3%) were identified by the *COI* gene primers as *Rattus rattus* and the rest 55 (35.7%) were *Rattus tanezumi* (Table 1). Figure 2 shows how the fragments appeared on a gel after amplification with of the *COI* gene primers.

Using the *Cyt-b* gene primer only 40 samples were amplified from a total of 154 as follows: *Rattus rattus* 26 (16.9%) and *Rattus tanezumi* 14 (9.1%) (Table 1). Many samples did not show any amplification products for *Cyt-b* as shown in Figure 3. For the samples which were not amplified, the PCR was unsuccessful in three repetitions.

Table 1: Number of rodents from different farms that were identified using both *CO1* and *Cyt-b* gene.

Farm	No. of rats	Species	*Cytochrome oxidase 1*	*Cytochrome b*
A	25	(i) *Rattus rattus*	19	14
		(ii) *Rattus tanezumi*	6	2
B	3	(i) *Rattus rattus*	3	–
		(ii) *Rattus tanezumi*	–	–
C	21	(i) *Rattus rattus*	15	4
		(ii) *Rattus tanezumi*	6	–
D	17	(i) *Rattus rattus*	10	1
		(ii) *Rattus tanezumi*	7	2
E	68	(i) *Rattus rattus*	46	10
		(ii) *Rattus tanezumi*	22	5
H	20	(i) *Rattus tanezumi*	14	1
		(ii) *Rattus rattus*	6	1
Total	*154*		*154*	*40*

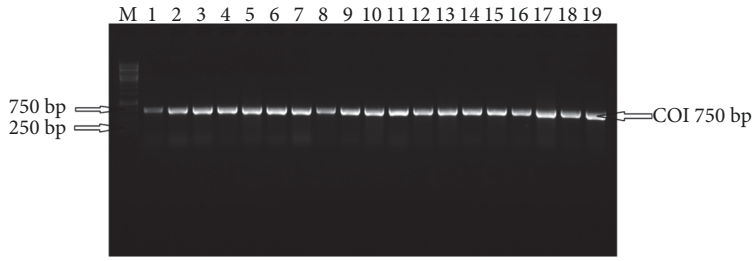

Figure 2: PCR amplification of *COI* gene. Lane M: molecular weight marker (1kb). Lanes 1-19 are *COI* gene fragments from DNA extracted from Rodents.

3.2. Phylogeny of R. rattus and R. tanezumi. *Phylogeny of R. rattus and R. tanezumi* for both *Cytochrome Oxidase I* (*COI*) and *Cytochrome b (Cyt-b)* genes was inferred using sequences derived from this study as well as those downloaded from GenBank from different countries around the world. In both tree topologies monophyly between *R. rattus* and *R. tanezumi* was well supported with high bootstrap support values. Figure 4 represents the tree topology inferred for *Cyt-b* gene. *Hylomyscus simus* was used as an out-group and three major clades were derived from the analysis. The clades consisted of the *Rattus rattus* clade, *R. tanezumi* clade, and *R. norvegicus* clade, respectively. All three derived clades had well supported bootstrap values.

For the *COI* tree topology, *Hylomyscus simus* and *Micromy enthrotis* were used as out-groups (Figure 5). In this phylogenetic tree, a monophyletic clade between *R. rattus* and *R. tanezumi* was again supported with high bootstrap support values. The observations made from the two phylogenetic trees do confirm that the samples collected in this study are indeed *R. rattus* and *R. tanezumi,* respectively.

3.3. Accession Numbers Obtained from GenBank. The representative sequences obtained in this study have been deposited to the GenBank database with assigned accession numbers ranging from MK645246 to MK645295.

4. Discussion

The primary objective of the study was to document rodent species that invade poultry houses in the North-West Province in particular and in South Africa in general. This information is important for economic as well as disease risk assessment [21, 22]. We were therefore able to show that of the 154 rodents, 99 (64.3%) were *R. rattus* and the other 55 (35.7%) were *R. tanezumi*. The *R. rattus* (the Black rat) was the most dominant species and it has been known to be in South Africa for many years [23, 24]. It is an important rat species because it is the most damaging invasive rodent in the world [1]. Furthermore, the rat has also been known as a vector of diseases infectious to humans [24]. The rat is a carrier of trematode species, cestode species, and nematode species [5]. A study by Reusken *et al.* [4] also implicated these rats as vectors spreading *Coxiella burnetii*. It may also carry important protozoa which are mainly dangerous for immune compromised patients [1]. Bacterial pathogens like *Salmonella* that are important to both humans and livestock have also been isolated from *R. rattus* in many countries, i.e., Japan, [25], Reunion Island [26], Pakistan [27], and Canada [14]. In the phylogenetic tree it clustered well with the species found in India, Jordan, Tanzania, and other South African studies. The rat's occurrence, especially in livestock farms, is therefore a significant health risk factor.

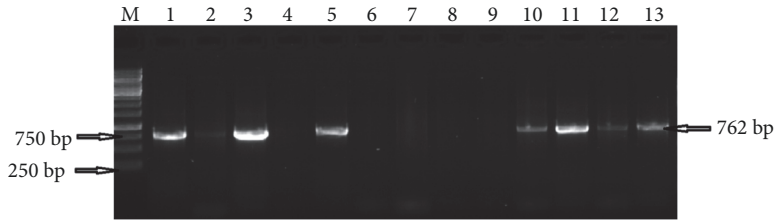

Figure 3: Lane M: molecular weight marker (1kb). Lanes 1, 2, 3, 5, 10, 11, 12, 13: amplified genes for *Cyt-b*. Lanes 4, 6, 7, 8, 9 are samples that were not amplified.

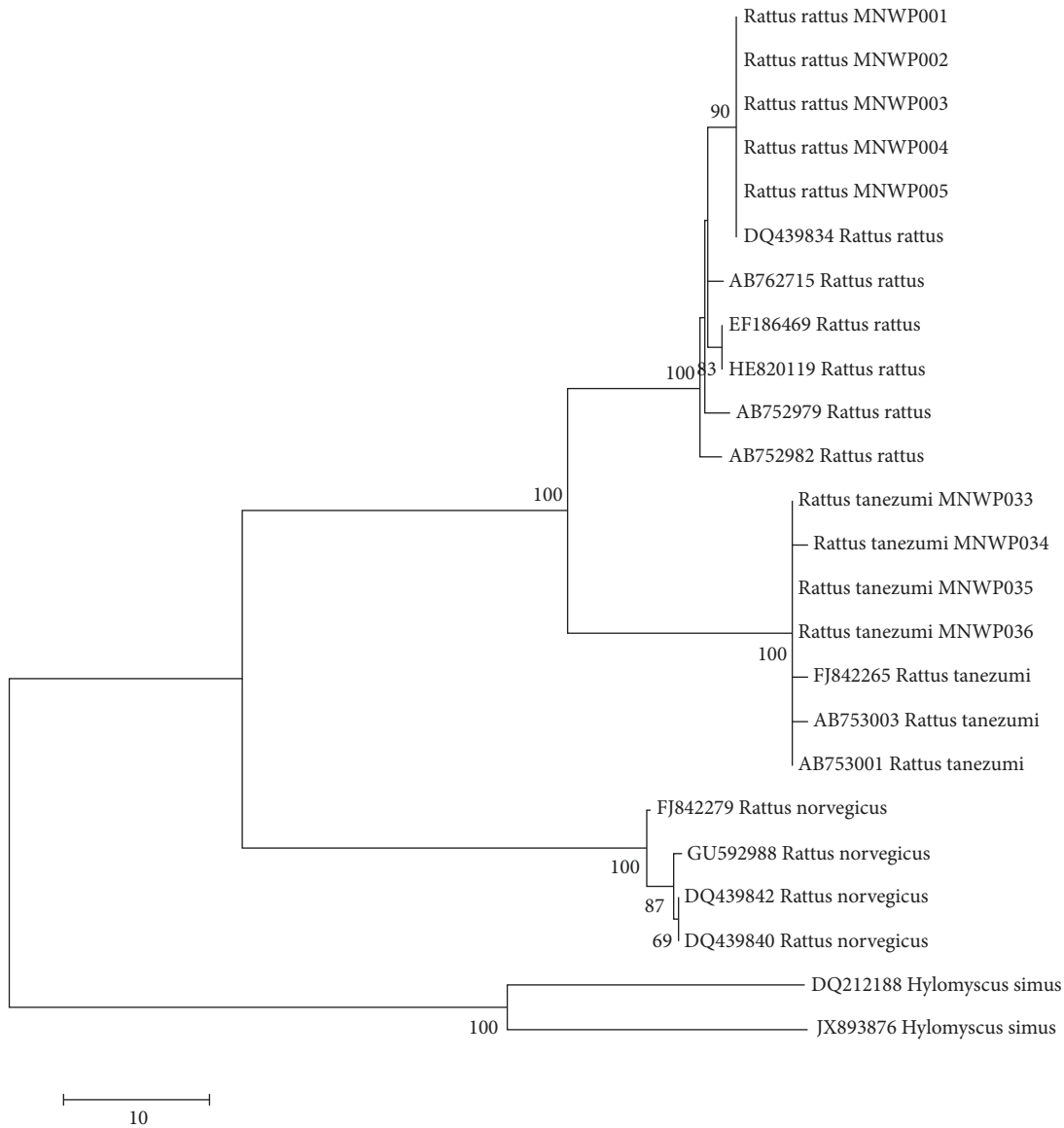

Figure 4: Neighbour-joining tree from the *Cyt-b* gene. The analysis involved 24 nucleotide sequences whereby 9 sequences were derived from this study and the remaining from the NCBI database with 1000 replicates. All positions containing gaps and missing data were eliminated. There were a total of 608 positions in the final dataset.

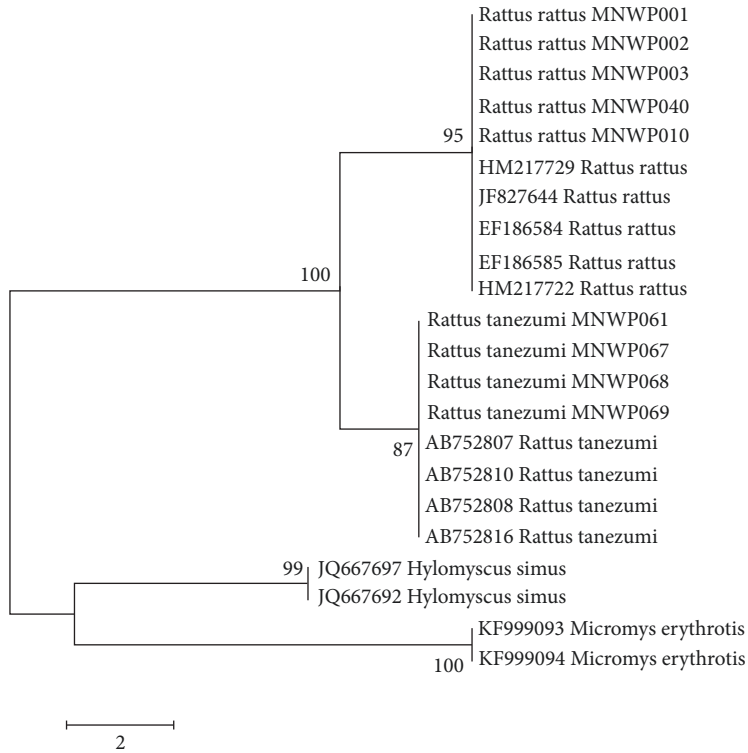

Figure 5: Phylogenetic tree based on distance matrix analysis of *COI* gene sequences from *R. rattus* and *R. tanezumi* based on the Hasegawa-Kishino-Yano model with 1,000 bootstrap support values. The analysis involved 22 nucleotide sequences whereby 9 sequences were derived from this study and the remaining from the NCBI database. All positions containing gaps and missing data were eliminated. There were a total of 598 positions in the final dataset.

The other *Rattus* species found in this study, *R. tanezumi*, has also been detected in South Africa before [6, 28]. *R. tanezumi* was first identified in Limpopo Province [24]; however to the best of our knowledge, this is the first report of its detection in the North West Province of South Africa. What is also significant was the finding that it is the second most dominant species in the poultry farms meaning it is getting more prolific and invasive all over the country. This rat species is predominantly found in Asia and wherever it is discovered it will always have its origins from Asia hence the name the Asian House rat. It is a carrier of hantaviruses [29] and it has also been found to carry important mites as well as helminths [6, 7]. Apart from diseases, the rat plays an important role by causing serious damage to field crops, destroying food stores, and also causing infrastructural damage [21, 30, 31]. It is obvious, therefore, that its increasing presence in South Africa brings with it these negative traits in the farm and human environment.

Of the two mitochondrial DNA genes used for this study, *cyt-b* and *COI,* the *Cyt-b* could only amplify 40 (25.97%) samples out of 154. However, *COI* gene amplified all the samples thus demonstrating that *COI* is a better gene for this purpose. The effectiveness of *COI* gene in species classification has also been reported before in wildlife [9], nematodes [32], reedbuck [10], birds [33], rodents [15], and lepidoptera [34]. The *COI* genes have been the most frequent methods used for species identification in animal biological studies due to its high degree of phylogenetic species differentiation as compared to other *mtDNA* genes [33]. Our study confirmed these findings and recommends its superior usage over *Cyt-b*.

5. Conclusion

The study established that the two rodent species found in poultry houses around Mafikeng, North West province of South Africa were *R. tanezumi* and *R. rattus*. The finding that *R. tanezumi* is the second most prominent rat species in these farms was unexpected because the rat species was first reported in South Africa not so long ago and that the species is not indigenous to Africa but Asia. It is thus important to expect it to increase and become a prominent species in the years to come. The study also confirmed that *cytochrome oxidase I* (*COI*) genes serve as a reliable and more precise target for identification of these rodent species.

Data Availability

The sequences data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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