The White Stork:
STUDIES IN BIOLOGY, ECOLOGY AND CONSERVATION
Eds. L. Jerzak, J. Shephard, J.I. Aquirre, J. Shamoun-Baranes, P. Tryjanowski
Zielona Góra 2016

Gottfried Wilharm[1], Evelyn Skiebe[1],
Mariusz Kasprzak[2], Marcin Bocheński[2], Leszek Jerzak[2]

How microbiology can contribute to our understanding of White Stork biology

Abstract

We have recently identified several novel bacterial species when sampling White Stork nestlings highlighting the attractiveness of White Stork for microbiologists in general. As a major finding we identified a novel *Corynebacterium* species, now described as *Corynebacterium pelargi* sp. nov., which was isolated from almost half of the samples, indicating a commensal or symbiotic status of this species with a long-standing history of coevolution with White Storks. Here, we discuss how this finding and microbiology in general might be exploited to contribute to our understanding of White Stork biology.

Keywords: Microbiology – White Stork – *Ciconia ciconia* – *Corynebacterium pelargi* sp. nov. – co-evolution – phylogeography

Introduction

White Stork (*Ciconia ciconia*) has attracted interest by infection biologists as a synanthropic species profiting from human settlements. From an epidemiologist's perspective the close contact with human housing and cultivated land offers manifold possible transmission routes for infectious agents. Not only humans, but also farm animals and pets could be implicated. What is more, as migratory birds travelling between Europe and Africa, storks could potentially mediate transcontinental transfer of pathogens. Herpesvirus, Newcastle disease virus (Kaleta et al. 1980; Kaleta and Kummerfeld 1983; Kaleta and Kummerfeld 1986; Kaleta and Kummerfeld 2012), West Nile virus (Malkinson et al. 2002), and rarely avian influenza A virus (Muller et al. 2009) have all been isolated from White Stork. The prevalence of antibodies against the food-borne pathogen *Listeria moncytogenes* was found to be above 50% in White Stork chicks (Andrzejewska et al. 2004). Conversely, pathogens and microbiota associated with human civilization might constitute a transmission hazard for storks and are there-

1 Robert Koch Institute, Wernigerode Branch, Burgstr. 37, D-38855 Wernigerode, Germany. Address correspondence to: Gottfried Wilharm, Robert Koch-Institut, Bereich Wernigerode, Burgstr. 37, D-38855 Wernigerode, Germany, e-mail: wilharmg@rki.de, phone: +49 3943 679 282, fax: +49 3943 679 207.

2 Faculty of Biological Sciences, University of Zielona Góra, Prof. Z. Szafrana Street 1, 65-561 Zielona Góra, Poland.

fore of interest in the context of environmental monitoring. To exemplify the latter, antibiotic-resistant salmonellae recently isolated from White Stork presumably have been transferred from human and/or livestock to stork indicating the dissemination of antibiotic resistance genes and antibiotic resistant bacteria into the environment after their selection through human behaviour (Palomo et al. 2013). Furthermore, the prevalence and antibiotic resistance of enteropathogenic *Campylobacter* spp. has been determined in White Stork chicks in Western Poland (Szczepańska et al. 2015). Also *Proteus mirabilis* isolates recovered from White Stork have been characterised regarding susceptibility to antibiotics (Kwiecińska-Piróg et al. 2010). Finally, infection biology is interested in the pathogens storks suffer from, the knowledge of which is important for our understanding of the critical determinants of stork population dynamics. For instance, it is known that White Stork chicks suffer from pneumonia caused by various fungal pathogens (Olias et al. 2010). Also enterotoxaemia caused by the ubiquitously found bacterium *Clostridium perfringens* has been reported (Boujon et al. 2005).

Our original interest lay in determining the prevalence of the Gram-negative opportunistic pathogen *Acinetobacter baumannii* within migratory birds. The results of these studies will be reported elsewhere. However, as a "byproduct" of this project we discovered a number of previously unknown bacterial species associated with White Stork samples. As novel species, we already described *Psychrobacter ciconiae* (Kämpfer et al. 2015d), *Gemmobacter intermedius* (Kämpfer et al. 2015c), *Corynebacterium trachiae* (Kämpfer et al. 2015b) and *Corynebacterium pelargi* (Kämpfer et al. 2015a). In the context of the novel *Corynebacterium spp.* identified, it is interesting to note that another *Corynebacterium* species, termed *C. ciconiae* (Fernandez-Garayzabal et al. 2004), has been previously isolated from the black stork *Ciconia nigra*. To date, no isolates belonging to the species *C. ciconiae* have been reported from sources other than *C. nigra* suggesting host-specificity of these bacteria. Likewise, despite an increasing number of extensive metagenome studies from various habitats worldwide, there are no database entries (http://www.ncbi.nlm.nih.gov/genbank/) related to the novel species *C. pelargi* and *C. trachiae* other than from White Stork isolates. Here, our purpose is to discuss how we can exploit the suggested coevolution between bacteria and birds to add to our understanding of stork biology.

Results

In the course of a study dealing with the identification of natural reservoirs of the nosocomial pathogen *Acinetobacter baumannii* we took choana samples from nestling of White Stork (*Ciconia ciconia*) in Poland in the year 2013. We sampled 54 adolescent storks in western Poland in the voivodship Lubusz, some of them repeatedly, resulting in a total of 121 samples. The samples were plated on blood agar plates without

selective additives and incubated for 24 hours at 37°C. Colonies representing different morphotypes were selected and subjected to partial 16S rRNA sequencing (Weisburg et al. 1991), typically determining about 1400 base pairs of the 16S rRNA gene.

Overall, representatives of the *Enterobacteriaceae* were isolated from the choana of every individual stork. Most frequently isolated species belonged to *Proteus* spp., in line with a previous report (Kwiecińska-Piróg et al. 2010), but also *Enterobacter* spp., *Citrobacter* spp., and *E. coli* were highly abundant. However, many isolates could not be even tentatively assigned to the genus level within the *Enterobacteriaceae*. It is known that 16S rRNA gene sequence data are insufficient to discriminate species within this family and that sequencing of other housekeeping genes is required to judge on the species level with some certainty (Roggenkamp 2007). Above, this indicates that a lot of novel *Enterobacteriaceae* species can be isolated from White Stork. Since apart from *Enterobacteriaceae*, *Enterococcus* spp. are highly abundant in the choana samples this indicates a permanent faecal contamination resulting from storks' feed. This part of the microbiota is thus considered transient rather than resident. The detailed outcome of these studies will be published elsewhere.

In the following we confine to the outcome that we identified two novel *Corynebacterium* species from the choana samples. Isolate Corynebacterium 280/10, later proposed to represent a novel species with the name *Corynebacterium trachiae* sp. nov. (Kämpfer et al. 2015b), was a singleton. In addition, we isolated representatives of a novel *Corynebacterium* sp., now designated *Corynebacterium pelargi* sp. nov. (Kämpfer et al. 2015a) from 56 samples (46% of all samples). However, the actual colonization rate among the storks is probably much higher. The highly abundant *Proteus* spp. are capable of swarming across large areas of agar media within a few hours thus covering and/or outcompeting the growth of other colonies. Hence, in future studies the use of swarming inhibitors (Williams 1973; Wang et al. 2006) should be considered to get a more representative picture.

The phylogenetic position of the novel *Corynebacterium* spp. is illustrated in Figure 1 based on partial 16S rRNA gene sequences. Interestingly, the closest relative of *C. trachiae* sp. nov. is *Corynebacterium ciconiae* isolated from black stork (Fernandez-Garayzabal et al. 2004). By contrast, *Corynebacterium pelargi* sp. nov. is only distantly related to these species. Corynebacteria are Gram-positive bacteria which belong to the phylum *Actinobacteria*. This ancient phylum also includes mycobacteria, propionibacteria and streptomycetes. The genus *Corynebacterium* presently comprises more than 80 published species and includes pathogenic bacteria such as *C. diphteriae* (Ventura et al. 2007). The pathogenetic potential of the new *Corynebacterium* spp. is currently unknown. Given the high abundance of *C. pelargi* sp. nov., however, a commensal/symbiotic relationship with White Stork is likely and requires additional verification. In support of the latter, random analysis among choana samples recovered from White

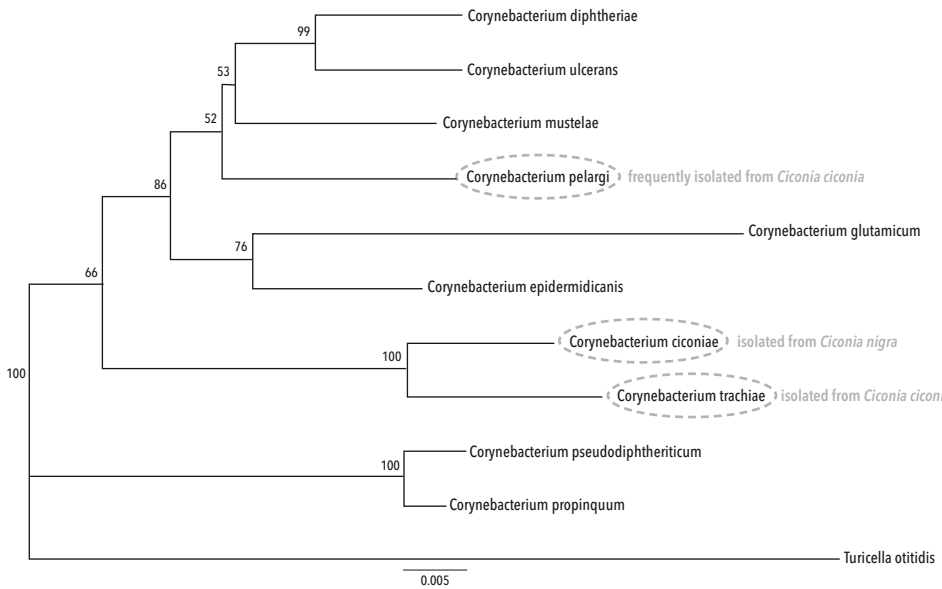

Figure 1. Neighbour-joining tree based on partial 16S rRNA gene sequences of selected *Corynebacterium* spp. and *Turicella otitidis* as an outgroup; bootstrap support values after 100 resamplings are given. Bar indicates 0.005 substitutions per nucleotide position. *Corynebacterium* spp. associated with White and Black Stork, respectively, as indicated by grey dotted ovals

Stork nestling in the year 2014 in the same region again revealed the widespread occurrence of isolates belonging to *C. pelargi* sp. nov. based on 16S rRNA sequencing (> 99% identity to the type strain 136/3^{T}).

Discussion

Taken together, our findings suggest a co-evolutionary relationship between White Stork and *C. pelargi* sp. nov. Here we discuss the application of this finding to our understanding of White Stork biology and pose a number of research challenges that require attention. In particular, we ask the scientific community to verify the phylogeographic potential of a co-evolutionary relationship and its potential to study the synanthropic relationship between White Storks and human civilisation as well as to clarify the apparent divergence between black and White Stork in this regard.

Recently, Shephard et al. (2013) presented a genetic analysis of the population structure in the European White Stork based on partial sequences of the mitochondrial DNA (mtDNA) and microsatellite genotyping. The objective of these phylogeographic studies was to clarify the contention that eastern and western migration flyways were genetically distinct originally but had been perturbated by translocation in the course

of reintroduction activities. The studied material included historical as well as contemporary samples from all over Europe as well as from Israel, Algeria, East and South Africa. Interestingly, the outcome was that of an effectively panmictic population of the European White Stork as the data overall lacked structure on the spatial or temporal scale. In other words, even before artificial translocation of individuals there was significant permeability between flyways. However, some spatial clustering of the mtDNA data might become resolvable in the future.

Can microbiology contribute to such phylogeographic questions? The prime example to illustrate this potential is represented by studies on the Gram-negative bacterium *Helicobacter pylori* which colonises the stomach of about half of the human population worldwide. These bacteria are transmitted primarily within the family which has resulted in phylogeographic marks within their genomes. The genetic geography of *H. pylori* nicely coincides with that of human populations giving the ability to trace ancient human migration, in some cases at higher resolution than traditional human genetic measures. Overall, it was demonstrated that an ancient interrelationship between *H. pylori* and humans has lasted for at least 100,000 years; that is before human migration out of Africa. Moreover, a second "out of Africa" migration of humans could be evidenced by studying this coevolution (Covacci et al. 1999; Moodley et al. 2012).

Similarly, the human microbiome in saliva has also been investigated to study human migration events and functions as 'proof of concept' that geographic signatures of bacterial housekeeping genes can be used to explain population connectivity (Henne et al. 2014).

Taken together, it is tempting to speculate that a phylogeographic analysis of the genome structure of *Corynebacterium pelargi* sp. nov. isolates from different stork populations can teach us about the relationship among the European distribution of White Storks.

Campylobacter spp. are widespread among avian and mammalian hosts suggesting low host specificity. Interestingly, however, *Campylobacter jejuni* populations from different wild bird species were found to be distinct from each other and the host species was identified as the major determinant of the genotype of *C. jejuni* (Griekspoor et al. 2013). Thus, *Campylobacter* spp. could become useful to trace back the evolution and divergence of avian species. This could become an interesting complement to the growing number of bird genomes available (Zhang et al. 2014). By contrast, *C. jejuni* is not suitable for phylogeographic studies as the geographic origin of *C. jejuni* isolates was found to have little impact on their genotypes (Griekspoor et al. 2013). Thus, even though it is possible that *Corynebacterium* spp. likewise prove uninformative for phylogeographic studies, they might still be useful to study the divergence of stork species. Of course, such potential is not restricted to bacteria. Indigenous herpesviruses have been identified in both white and black stork and found to be serologically identical but clearly distinct from any other avian herpesvirus (Kaleta et al. 1980, 2012). Their

genetic characterisation could be an interesting complement and could help in genetic time scaling.

Behaviour of White Stork likely changed with the Neolithic when humans introduced farming and permanent settlement. It is unknown at what time the White Stork became the synanthropic species it is nowadays. It is known, however, that the Neolithic revolution had a great impact on the evolution of many bacterial species, notably commensals and pathogens of human and livestock but also other bacteria specialised in human-associated niches, e.g. pathogens of domesticated plants and microorganisms involved in food production (Mira et al. 2006). From whole genome sequencing data on various bacteria it can be deduced that species with a specialised niche (e.g. restriction to a single host organism) show characteristic genomic changes in comparison to related species not similarly adapted. In particular, a process of reductive evolution through elimination of genes can be observed. Gene inactivation can be mediated by transposable insertion sequences (IS elements) and the expansion of such mobile genetic elements is often observed in genomes of bacteria with a supposedly recent niche adaptation (Mira et al. 2006). Many bacterial pathogens adapted to humans, livestock and crops show signs of recent genome changes in the form of gene inactivation and expansion of IS elements. As an example, the causative agent of whooping cough in human, *Bordetella pertussis*, shows an extraordinary expansion of IS elements whereas the close relative *Bordetella bronchiseptica*, exhibiting a much broader host range, does not. Collectively, marked changes in bacterial genomes have been attributed to niche adaptations in the context of the Neolithic revolution (Mira et al. 2006). This illustrates the remarkable speed of bacterial adaptation to human cultural evolution and the potential of using bacterial coevolution to trace history of their hosts. It is tempting to speculate that the concentration of storks within villages also imposed changes within their microbiotia which might be traceable via next generation sequencing approaches. The revolution in high-throughput DNA sequencing technologies within the last few years make bacterial genomes accessible at low costs providing a plethora of applications (McAdam et al. 2014). Sequencing of *C. pelargi* sp. nov. and *C. trachiae* sp. nov. associated with White Storks should be prioritised, especially since whole genome shotgun sequence data on *Corynebacterium ciconiae* type strain DSM 44920^{T} isolated from black stork have recently become available via GenBank (accession no. NZ_AQUW00000000.1). A genome comparison will not only tell us a lot about the relationship and evolutionary divergence of the bacterial species but also about their hosts. The lack of synanthropic behavior in the black stork make it a very interesting complement to the White Stork regarding its microbiota and relationship with human civilisation. Possibly, signs of the Neolithic revolution will be found in the genomes of bacteria adapted to *C. ciconia* but not in that bacteria adapted to *C. nigra*.

Conclusions and Perspectives

It is our hope to have illustrated the fascinating potential of studying microbe-stork coevolution. In the case of *Corynebacterium pelargi* sp. nov., at present, we cannot exclude with certainty food, parasites or other environmental factors as primary source of these bacteria. Thus, as the next essential step, stable colonisation of White Stork individuals should be confirmed as well as the passing on from parents to descendants as the primary route of transmission. Sequencing of a few housekeeping genes of *C. pelargi* sp. nov. such as *rpoB*, *hsp60* should suffice to assess this, using isolates with a defined familial relationship of their respective hosts.

It is explicitly stated here that the microbiologists among us authors (GW & ES) will not be able to continue with this project since it is too far from our duties at the Robert Koch Institute, dedicated to public health protection. Our aim is thus to stimulate the community to proceed. We shall be happy to provide every material we collected to support you.

Acknowledgements

We would like to thank our colleagues at the central DNA sequencing lab (ZBS 1) of the Robert Koch Institute for excellent technical assistance.

References

Andrzejewska I., Dolata P.T., Jerzak L., Ptaszyk J., Tryjanowski P. & Zduniak P. 2004. Prevalence of agglutinating antibodies against *Listeria monocytogenes* in chicks of the White Stork (*Ciconia ciconia* Linnaeus, 1758) in Poland. European Journal of Wildlife Research 50: 218-220.

Boujon P., Henzi M., Penseyres J.H. & Belloy L. 2005. Enterotoxaemia involving beta2-toxigenic *Clostridium perfringens* in a White Stork (*Ciconia ciconia*). Vet. Rec. 156: 746-747.

Covacci A., Telford J.L., Del Giudice G., Parsonnet J. & Rappuoli R. 1999. *Helicobacter pylori* virulence and genetic geography. Science 284: 1328-1333.

Fernandez-Garayzabal J.F., Vela A.I., Egido R., Hutson R.A., Lanzarot M.P., Fernandez-Garcia M. & Collins M.D. 2004. *Corynebacterium ciconiae* sp. nov., isolated from the trachea of black storks (*Ciconia nigra*). Int. J. Syst. Evol. Microbiol. 54: 2191-2195.

Griekspoor P., Colles F.M., Mccarthy N.D., Hansbro P.M., Ashhurst-Smith C., Olsen B., Hasselquist D., Maiden, M.C. & Waldenstrom, J. 2013. Marked host specificity and lack of phylogeographic population structure of *Campylobacter jejuni* in wild birds. Mol. Ecol. 22: 1463-1472.

Henne K., Li J., Stoneking M., Kessler O., Schilling H., Sonanini A., Conrads G. & Horz H.P. 2014. Global analysis of saliva as a source of bacterial genes for insights into human population structure and migration studies. BMC Evol. Biol. 14: 190.

Kaleta E.F. & Kummerfeld N. 1983. Herpesviruses and Newcastle disease viruses in White Storks (*Ciconia ciconia*). Avian Pathol. 12: 347-352.

Kaleta E.F. & Kummerfeld N. 1986. Persistent viraemia of a cell-associated herpesvirus in White Storks (*Ciconia ciconia*). Avian Pathol. 15: 447-453.

Kaleta E.F. & Kummerfeld N. 2012. Isolation of herpesvirus and Newcastle disease virus from White Storks (*Ciconia ciconia*) maintained at four rehabilitation centres in northern Germany during 1983 to 2001 and failure to detect antibodies against avian influenza A viruses of subtypes H5 and H7 in these birds. Avian Pathol. 41: 383-389.

Kaleta E.F., Mikami T., Marschall H.J., Heffels U., Heidenreich M. & Stiburek B. 1980. A new herpesvirus isolated from black storks (*Ciconia nigra*). Avian Pathol. 9: 301-310.

Kämpfer P., Jerzak L., Bochenski M., Kasprzak M., Wilharm G., Golke J., Busse H.J. & Glaeser S.P. 2015a. *Corynebacterium pelargi* sp. nov., isolated from the trachea of White Stork nestlings. Int. J. Syst. Evol. Microbiol. 65: 1415-1420.

Kämpfer P., Jerzak L., Wilharm G., Golke J., Busse H.J. & Glaeser S.P. 2015b. Description of *Corynebacterium trachiae* sp. nov., isolated from a White Stork (*Ciconia ciconia*). Int. J. Syst. Evol. Microbiol. 65: 784-788.

Kämpfer P., Jerzak L., Wilharm G., Golke J., Busse H.J. & Glaeser S.P. 2015c. *Gemmobacter intermedius* sp. nov., isolated from a White Stork (*Ciconia ciconia*). Int. J. Syst. Evol. Microbiol. 65: 778-783.

Kämpfer P., Jerzak L., Wilharm G., Golke J., Busse H.J. & Glaeser S.P. 2015d. *Psychrobacter ciconiae* sp. nov., isolated from White Storks (*Ciconia ciconia*). Int. J. Syst. Evol. Microbiol. 65: 772-777.

Kwiecińska-Piróg J., Bogiel T., Gospodarek E., Kasprzak M., Jerzak L. & Kamiński P. 2010. [The susceptibility of *Proteus mirabilis* strains isolated from White Stork (*Ciconia ciconia*)]. Medycyna doświadczalna i mikrobiologia 63: 139-144.

Malkinson M., Banet C., Weisman Y., Pokamunski S., King R., Drouet M.T. & Deubel V. 2002. Introduction of West Nile virus in the Middle East by migrating White Storks. Emerg. Infect. Dis. 8: 392-397.

Mcadam P.R., Richardson E.J. & Fitzgerald J.R. 2014. High-throughput sequencing for the study of bacterial pathogen biology. Curr. Opin. Microbiol. 19: 106-113.

Mira A., Pushker R. & Rodriguez-Valera F. 2006. The Neolithic revolution of bacterial genomes. Trends Microbiol. 14: 200-206.

Moodley Y., Linz B., Bond R.P., Nieuwoudt M., Soodyall H., Schlebusch C.M., Bernhoft S., Hale J., Suerbaum S., Mugisha L., Van Der Merwe S.W. & Achtman M. 2012. Age of the association between *Helicobacter pylori* and man. *PLoS Pathog* 8, e1002693.

Muller T., Hlinak A., Freuling C., Muhle R.U., Engelhardt A., Globig A., Schulze C., Starick E., Eggers U., Sass B., Wallschlager D., Teifke J., Harder T. & Conraths F.J. 2009. Virological monitoring of White Storks (*Ciconia ciconia*) for avian influenza. Avian Dis. 53: 578-584.

Olias P., Gruber A.D., Winfried B., Hafez H.M. & Lierz M. 2010. Fungal pneumonia as a major cause of mortality in White Stork (*Ciconia ciconia*) chicks. Avian Dis. 54: 94-98.

Palomo G., Campos M.J., Ugarte M., Porrero M.C., Alonso J.M., Borge C., Vadillo S., Dominguez L., Quesada A. & Piriz S. 2013. Dissemination of antimicrobial-resistant clones of *Salmonella enterica* among domestic animals, wild animals, and humans. Foodborne Pathog. Dis. 10: 171-176.

Roggenkamp A. 2007. Phylogenetic analysis of enteric species of the family *Enterobacteriaceae* using the oriC-locus. Syst. Appl. Microbiol. 30: 180-188.

Shephard J.M., Ogden R., Tryjanowski P., Olsson O. & Galbusera P. (2013). Is population structure in the European White Stork determined by flyway permeability rather than translocation history? Ecol. Evol. 3: 4881-4895.

Szczepańska B., Kamiński P., Andrzejewska M., Śpica D., Kartanas E., Ulrich W., Jerzak L., Kasprzak M., Bocheński M. & Klawe J.J. 2015. Prevalence, Virulence, and Antimicrobial Resistance of *Campylobacter jejuni* and *Campylobacter coli* in White Stork *Ciconia ciconia* in Poland. Foodborne pathogens and disease 12: 24-31.

Ventura M., Canchaya C., Tauch A., Chandra G., Fitzgerald G.F., Chater K.F. & Van Sinderen D. 2007. Genomics of *Actinobacteria*: tracing the evolutionary history of an ancient phylum. Microbiol. Mol. Biol. Rev. 71: 495-548.

Wang W.B., Lai H.C., Hsueh P.R., Chiou R.Y., Lin S.B. & Liaw S.J. 2006. Inhibition of swarming and virulence factor expression in *Proteus mirabilis* by resveratrol. J. Med. Microbiol. 55: 1313-1321.

Weisburg W.G., Barns S.M., Pelletier D.A. & Lane D.J. 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173: 697-703.

Williams F.D. 1973. Abolition of swarming of *Proteus* by p-nitrophenyl glycerin: application to blood agar media. Appl. Microbiol. 25: 751-754.

Zhang G., Li C., Li Q., Li B., Larkin D.M., Lee C., Storz J.F., Antunes A., Greenwold M.J. & Meredith R.W. (2014). Comparative genomics reveals insights into avian genome evolution and adaptation. Science 346: 1311-1320.

Oficyna Wydawnicza