Bacteria and costs of hybridization in flycatchers

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TABLE OF CONTENTS

ABSTRACT………………………………………………………………………………...…… **3**

INTRODUCTION…………………………………………………………………...……….. **4**

MATERIALS AND METHODS…………………………………………...………..….. **6**

RESULTS………………………………………………………………………….……..….. **10**

DISCUSSION…………………………………………………………………..……… **12**

ACKNOWLEDGEMENT …………...……………………………………….……….… **13**

REFERENCES……………………………………………………………………………. **14**

ABSTRACT

The process of hybridization, which means mating across species boarders resulting in creation of hybrid offspring, is a fascinating problem to explore and understand. A better understanding of causes and consequences of hybridization is one way of pinpointing the mechanisms of speciation. One of most interesting issues touched in this domain considers hybrid's resistance to parasites and its comparison with resistance of parental species. I attempted to survey the intestinal bacterial communities in feces of collared and pied flycatcher, as well as their hybrids using the t-RFLP method. Furthermore, the occurrence of parasitic bacteria would be investigated and correlated with different life-history traits. However, among 30 feces samples only from 3 (10%) I was able to obtain PCR product despite various techniques and facilitators applied. As feces are well known PCR-inhibitor I distinguish other domains which should be improved to obtain better results in future experiments. They are: the samples' way of storage, initial material mass as well as DNA yield evaluation method. Although this study did not lead to any novel insights into the role of bacteria in the life of the two species of flycatchers and their hybrids – it has lead to a major step forward in the development of a suitable protocol for achieving this goal.

INTRODUCTION

Since decades scientists have been investigating the issues concerning the origins of species (*Darwin 1859*). The process of hybridization, which means mating across species boarders resulting in creation of hybrid offspring, is a fascinating problem to explore and understand. A better understanding of causes and consequences of hybridization is one way of pinpointing the mechanisms of speciation. Not least intriguing remain the question of relations between hosts and their parasites, impact of the latter on host fitness and life history traits.

The parasitism can be described as the ecological relationship between two different organisms - the parasite and the host. The parasites are physiologically or metabolically dependent on their hosts, but their reproductive potential is much higher. They might be protozoan blood parasites for instance, as well as intestinal parasitic bacteria. Parasites can affect hosts in different ways – influencing their behavior, mating and reproductive success, or even they may cause death. During generations of mutual interactions and arms race hosts managed to get immune to some of parasites having been open to get infected by the others.

Astonishingly, a few studies bring up a question dealing with parasitism and animal hybridization (*Moulia 1999*). One of most interesting issues touched in this domain considers hybrid's resistance to parasites and its comparison with resistance of parental species.

In next paragraphs I will explain what is the relation between hybridization and different evolutionary histories of species, including speciation. I will also present Flycatcher study system as a perfect one to investigate such issues.

Hybridization and Speciation

Hybridization is the process of mating across species boarders resulting in creation of hybrid offspring. One can expect that there should be strong selection against interspecific mating, as hybrids are considered to be less fit than any of parental species. However, at least 25% of plant species and 10% of animal species, mostly the youngest species, are involved in hybridization and potential introgression with other species (*Mallet 2005*). There are several reasons why hybridization occurs, among others lack of conspecific mates and errors in species recognition (*Wiley 2006*), as well as it may sometimes bring direct or indirect benefits to one or both parental species (*Veen et al. 2001, Wiley et al. 2007*)

More significantly, the hybridization might be crucial to speciation – that is the process of emergence of new species. It may influence the creation of new taxa in at least two ways: 1) newly formed hybrids may become true species if they have high fitness at least in some kinds of habitats and they achieve reproductive isolation from both of their parental species; 2) If hybrids have lower fitness than its parental species it can result in reinforcement of reproductive isolation between them and strong selection against hybrids will occur (*Hegarty & Hiscock 2005*). Thus, the relative fitness of hybrids may have strong effects on evolutionary patterns. However, estimating fitness of hybrid in generally difficult in wild populations. The flycatcher study system is a rare example where it is possible to investigate long-term effects of hybridization and this system hence seems to be a perfect tool to carry out such research.

Flycatcher System

Flycatchers are small passerines birds who breed in Europe and who are migratory, wintering in sub Sahara Africa. Collared (*Ficedula albicolis*) and pied flycatchers (*F.*

hypoleuca) hybridize throughout central and eastern Europe, and on the Swedish islands of Gotland and Öland. The hybridization results in hybrid individuals among which females are infertile, whereas males have decreased fertility (*Veen et al. 2001*). Pied flycatchers are the rarer species on both islands, representing 4% and 18% of breeding flycatchers on Gotland and Öland, respectively (*Willey et al. 2005*). Collared males can be easily distinguish from Pied's by well visible collar on their neck (see Figure 1.). Hybrid males in most cases have broken collar, intermediate phenotype between two species. Females of both species are gray-brown, but can be distinguished by subtle differences in plumage (*Svensson & Grant 1999*). Flycatchers have adapted to breed in nest boxes, which had been installed on both islands. Breeding season starts at the end of April and it lasts until the beginning of July.

Figure 1. **Left**: Adult male Collared Flycatcher (*Ficedula albicolis*), **Middle**: Adult male Hybrid, **Right**: Adult male Pied Flycatcher (*F. hypoleuca*) *Photos kindly provided by Thor Veen*

Are hybrids more or less resistant to parasites?

We can consider alternative hypothesis predicting the status of hybrid resistance to natural parasites (*Fritz et al. 1994, Moulia 1999*):

1) *The Additive Hypothesis*, hybrid resistance does not differ from the mean resistance of parents
2) *The Dominance Hypothesis*, hybrid resistance is similar to one of parental species
3) *The Hybrid Susceptibility Hypothesis*, hybrids are less resistant to parasites than their parents
4) *The Hybrid Resistance Hypothesis*, hybrids are more resistant than their parents.

Field study experiments, as well as common garden trials and laboratory research show different and diverse results, in various plant and animal taxa. In animals, we can find cases supporting The Additive (*Trouvé et al. 1998*, F1 generation hybrids), The Dominance (*Jackson & Tinsley 2003*), The Hybrid Susceptibility (*Parris 2004, Wolinska et al. 2004*), as well as The Hybrid Resistance Hypothesis (*Joly et al. 2007*). Some reports show that hybrids are as susceptible as parental species in peculiar parasite infection whereas being infected by other species, they are less resistant (*Derothe et al. 1999, 2001*).

Theoretically, one can argue for hybrid susceptibility, as they often possess intermediate and rarely unique (i.e. morphological) features (*Fritz et al. 1999*). That means they could share parasites of both parental species (*Whitham 1989*). Alternatively, because of their

uniqueness hybrids can be suitable for parasites, which do not occur in either parental species (*Mattson et al. 1996*). Moreover, genes in parents are the ones that within generations have co-adapted to specific parasites - in different species and different environments they underwent differential selection. Once hybridization occurs, it may lead to genetic breakdown, especially in advanced generations of hybrids (*Trouvé et al. 1998, Moulia 1999*).

On the other hand, since hybridization may lead to genetic breakdown in phylogenetically distant species, it can result in enhanced resistance through a heterosis in case of closely related taxa (*Joly et al. 2007*). Similarly, hybrid uniqueness (physiological, ethological, etc.) may also play into hybrid's hand by the fact that hybrids may be unsuitable to develop any parasites. However, Fritz and co-workers (*1999*) show that susceptibility of hybrids prevails.

One can ask if the resistance against individual parasites should be regarded as stable and invariable feature, or rather as temporal and unstable, according to Red Queen Hypothesis and parasite driven host frequency-dependent selection (*Wolinska et al. 2006, 2007; Lively & Dybdahl 2000*).

Feces as PCR inhibitor

Feces is the material that is hard to extract DNA from and even if successful, precipitated DNA contains inhibitory substances, such as bile acids, complex polysaccharides and/or phytic acid (*Kreader 1996, Monteiro et al. 1997, Thorton & Passen 2004*). To prevent them from affecting the PCR reaction, a special pre-PCR processing is needed (*Rådström et al. 2004, Idaghdour & Broderick 2003, Lou et al. 1996, Boström et al. 2004, Clement & Kitts 2000*). Also, it is recommended to use additional PCR-facilitators, such as Bovine Serum Albuminum (BSA), T4 gene 32 Protein (gp32) (*Kreader 1996, Al-Soud & Rådström 2000, Wilson 1997*) or phytase (*Thorton & Passen, 2004*). BSA may be able to scavenge a variety of substances and thereby prevent their binding and inactivation of *Taq* DNA polymerase. On the other hand gp32, a single-stranded DNA-binding protein, may facilitate PCR by binding with denatured strands of DNA to retard reannealing and perhaps by stimulating the DNA polymerase (*Kreader 1996*). As some reports show, even a type of used polymerase may influence PCR result in presence of inhibitors (*Al-Soud & Rådström, 1998*).

If PCR inhibition occurs, it is often recommended to dilute the DNA sample in order to eliminate inhibition. It will result in diluting both the template DNA and the inhibitor. Therefore, PCR products should be obtained from template DNA, even if it requires an increase of the number of cycles (*McPherson & Møller 1990)*.

The main goal of my project is to evaluate molecular procedures in order to determine a survey of the intestinal bacterial communities in bird feces. Investigation is based on sympatric species of flycatchers: collared and pied flycatcher, as well as their hybrids. Furthermore, achieved knowledge would be used to investigate and correlate the occurrence of parasitic bacteria with different life-history traits.

MATERIALS AND METHODS

Samples collecting

Samples used in this project were collected during breeding season 2007 on Öland. Birds were captured and kept in clean paper bags until they defecate. After releasing birds, feces samples were collected to 2ml tubes and kept in -20°C until transportation to Uppsala. Subsequently they were kept in - 80°C until extraction.

DNA extraction

To extract DNA from bird feces I applied several of different kits and protocols using 2-6 variants each, 1 – 3 samples for a trial. Each tube contained 10 – 40 µg of fecal material. For survey of followed protocols – see Table 1. Mixed protocol using 5 Prime kit included prolonged digestion with Lysosyme and Proteinase K, as well as precipitation suing 70% ethanol. Contamination of extraction reagents was monitored using negative controls. To see if extraction was successful, positive controls were applied using material from cultivated bacteria or bacteria from sediments. In applied protocols concentrations were as follow: Mussel Glycogen 2mg/ml; Protein Degrader 5mg/ml; Proteinase K 20mg/ml; RNase A 2mg/ml. All the other solutions were provided in kits by distributor. Results were revealed on 1% agarose gel with addition of 2µl of ethidium bromide (0,4% vol/vol).

PCR optimization

The total volume of the PCR mixtures was 20µl. It always contained Finnzymes 1x Optimized DyNAzyme® buffer (10nM Tris-HCl, 1.5mM $MgCl_2$, 50mM KCl, 0.1% Triton X-100), each of the deoxyribonucleoside triphosphates at a concentration of 0.2mM, forward universal primer 27F (AGRRTTTGATYBTGGYTCAG) and reverse universal primer 519R (5'-GTATTACCGCGGCTGCTG-3') each at a concentration of 100nM and 0.25U Taq Polymerase. Numerous trials were applied using different DNA concentrations and PCR facilitators (see Table 2.). The reaction mixtures after Initial Denaturation for 3 minutes in 94°C were subjected to 25-35 amplification cycles consisting of heat denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and DNA extension at 72°C for 45 s. Finally, the mixtures were maintained at 72°C for 7 min for the final extension of DNA. The number of cycles differed. In dependence on amount of template DNA and dilution rate it was 20 – 35 cycles. Final results were revealed on 1% agarose gel with addition of 2µl of ethidium bromide (0.4% vol/vol). 4 samples from which it was unsuccessful to obtain any visible bands on agarose gel neither after extraction nor after PCR (up to 0.4% vol/vol template DNA dilution) were ran in RT-PCR.

Real-time Polymerase Chain Reaction

Real time polymerase chain reaction (RT – PCR) is also called quantitative real time PCR (qRT-PCR) or kinetic PCR. It is a modification of PCR which simultaneously quantifies and amplifies a specific part of a given template DNA. Its mechanism bases on the kinship of fluorescent dye to double – stranded DNA (dsDNA), which fluoresce once binded to it. It helps to determine whether a specific nucleic acid sequence is present in the sample and how many its copies there are after each full cycle. That is its *real time* aspect. It is a useful

technique for the investigation of gene expression, viral load, pathogen detection and numerous other applications.

In **RT-PCR** the total volume of the PCR mixtures was 20µl. It contained 10µl of DyNAmo™ Green qPCR master-mix, each of the deoxyribonucleoside triphosphates at a concentration of 0.2mM, forward universal primer 27F (AGRRTTTGATYBTGGYTCAG) and reverse universal primer 519R (5'-GTATTACCGCGGCTGCTG-3') at a concentration of 250nM each, 200ng of BSA and template DNA (25% vol/vol). The reaction mixtures after Initial Denaturation for 2 minutes in 95°C were subjected to 35 amplification cycles consisting of heat denaturation at 95°C for 30 s, primer annealing at 50°C for 30 s, and DNA extension at 72°C for 30 s. Finally, the mixtures were maintained at 72°C for 7 min for the final extension of DNA.

Table 1. The survey of applied methods in DNA extraction of fecal samples of flycatchers (collected in field season 2007)

1. **Mo Bio, PowerSoil™ DNA Kit**
    - With/without bead-bitting

2. **Qiagen, DNeasy ® Blood & Tissue Kit**
    a) Pretreatment for Gram- Negative Bacteria
        - With/without bead-bitting

    b) Pretreatment for Gram – Positive Bacteria
        - With/without bead-bitting

3. **Invitrogen, Easy-DNA™ Kit for Genomic isolation**
    a) Protocol 3# - Small Amounts of Cells, Tissues, or Plant Leaves
        - With/without Mussel Glycogen (20µg/ml)
        - With/without Protein Degrader (100µg)
        - With double precipitation step, with Mussel Glycogen (20µg/ml)

    b) Protocol 4# - Large Samples or Tissue, Cells, or Blood
        - With/without Mussel Glycogen (20µg/ml)

    c) Protocol 8# - Isolation of DNA from Mouse Tails
        - With/without Protein Degrader (100µg)

4. **5 Prime, ArchivePure DNA Cell/Tissue Kit**
    a) Mixed protocol

Table 2. Trial variations in PCR optimization of fecal samples of flycatchers.

# of trial	DNA vol/vol concentration (%)														BSA [ng/µl]		Gp 32 [ng/µl]
	40	30	20	10	5	2	1	0.4	0.2	0.1	0.07	0.05	0.04	0.03	250	400	100
1	*	*	*	*													
2		*	*	*	*												*
3		*	*	*	*										*		
4					*	*	*	*	*							*	
5								*	*	*	*	*	*	*		*	

RESULTS

Best results in extraction were obtained with Invitrogen® and 5 Prime kits. Yet, no extraction protocol provided enough DNA to obtain a well visible band on agarose gel using low-mass ladder (min. approx. 3ng/µl). One sample showed on the gel degraded DNA. Surprisingly, among 30 samples only from 3 (10%) I was able to obtain PCR product. The best results were achieved in case of 25- and 50 times DNA dilutions (see Fig. 1. and Fig. 3.). Any attempts to optimize extraction protocol or fixed DNA dilution to obtain predictable PCR results failed. Different extraction methods and DNA dilutions brought different results and DNA yields (Fig. 1, Fig. 2., Fig. 3.). RT-PCR analysis was applied to samples which did not give any DNA yield in any dilution. It showed that failure in achieving satisfactory DNA yield was due to too small amount of template DNA.

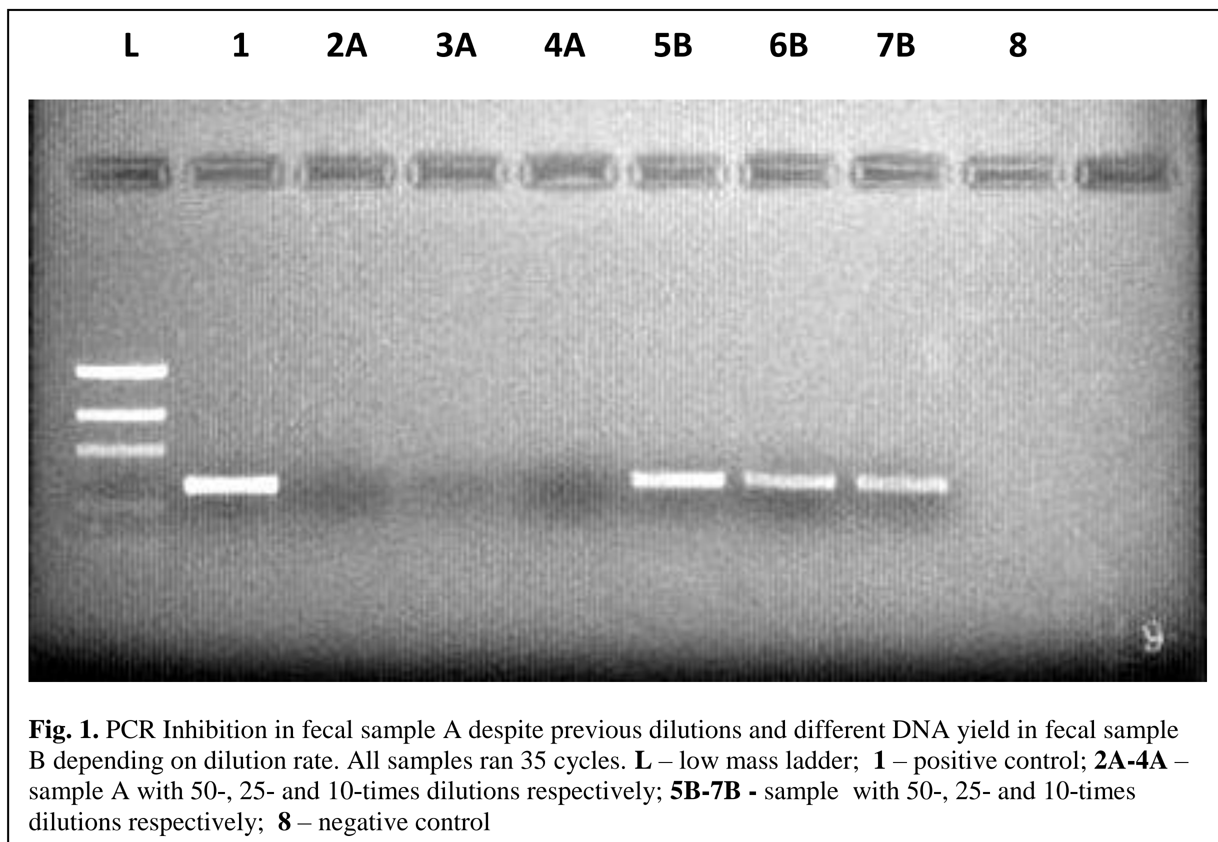

Fig. 1. PCR Inhibition in fecal sample A despite previous dilutions and different DNA yield in fecal sample B depending on dilution rate. All samples ran 35 cycles. **L** – low mass ladder; **1** – positive control; **2A-4A** – sample A with 50-, 25- and 10-times dilutions respectively; **5B-7B -** sample with 50-, 25- and 10-times dilutions respectively; **8** – negative control

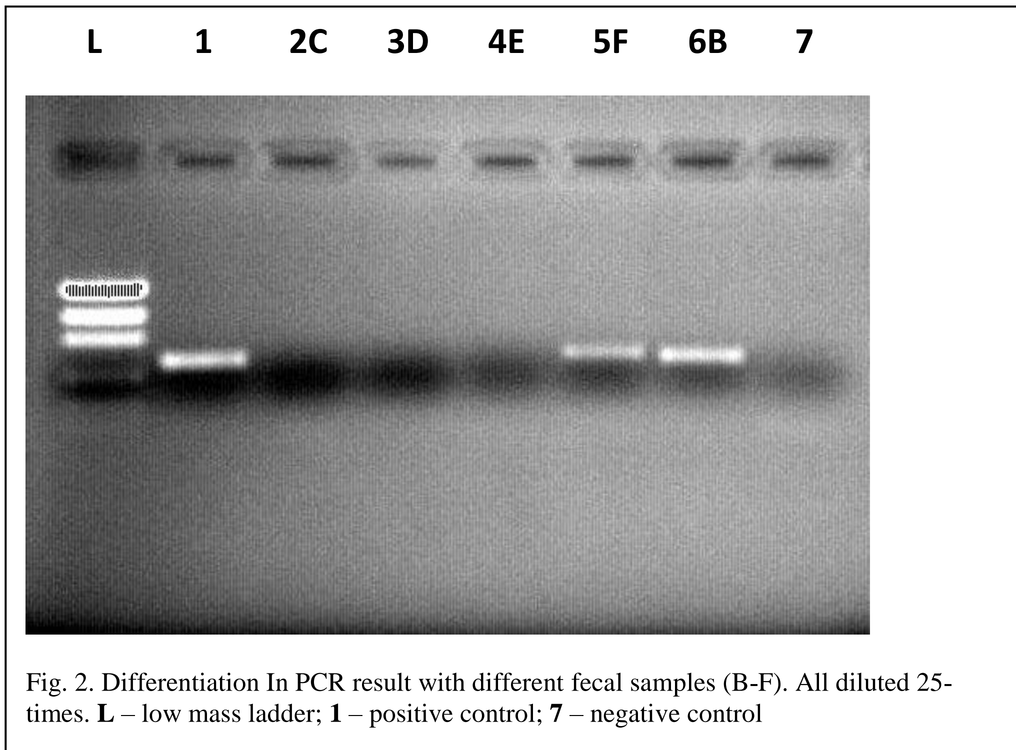

Fig. 2. Differentiation In PCR result with different fecal samples (B-F). All diluted 25-times. **L** – low mass ladder; **1** – positive control; **7** – negative control

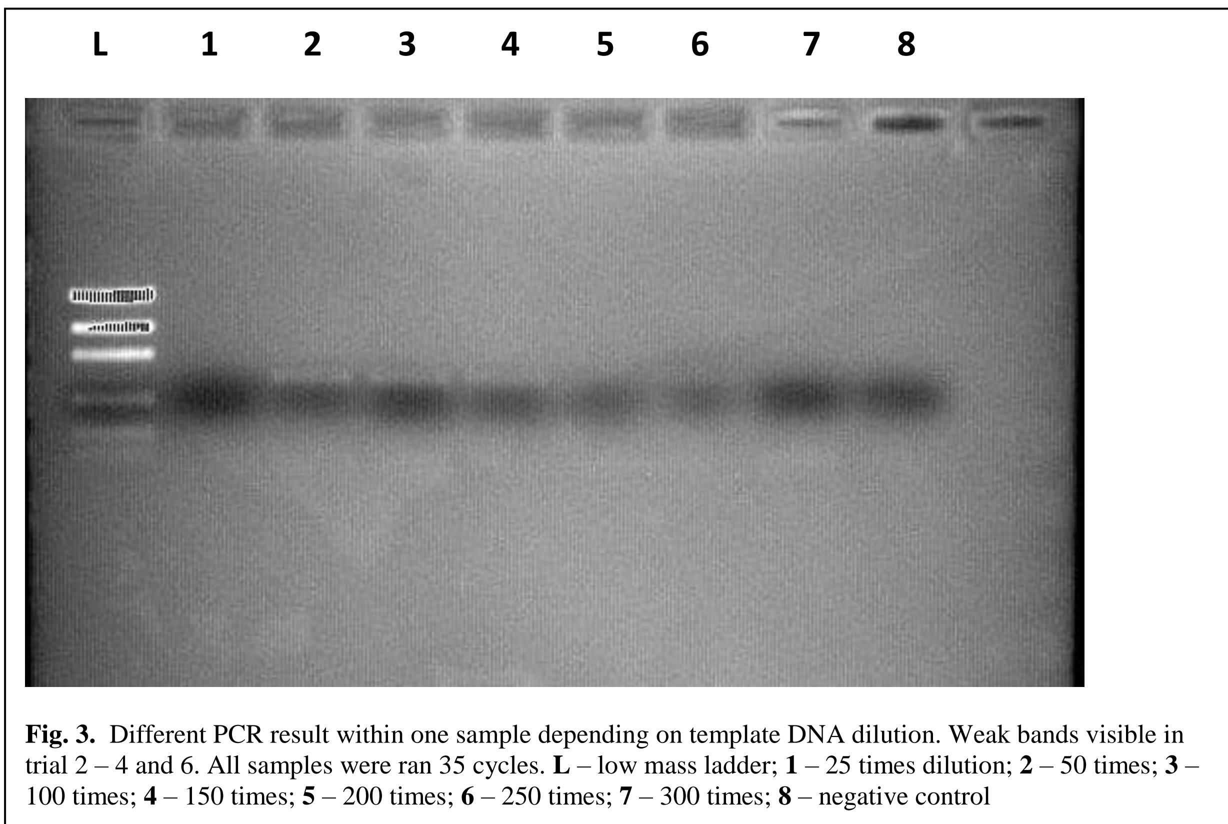

Fig. 3. Different PCR result within one sample depending on template DNA dilution. Weak bands visible in trial 2 – 4 and 6. All samples were ran 35 cycles. **L** – low mass ladder; **1** – 25 times dilution; **2** – 50 times; **3** – 100 times; **4** – 150 times; **5** – 200 times; **6** – 250 times; **7** – 300 times; **8** – negative control

DISCUSSION

Over the last decades parasites were in the scientists' limelight. Researchers revealed their impact on longevity (*Morand & Harvey, 2000*), fecundity (*Obrebski 1975*) and other life history traits as well as on the origin of sex (*Hamilton et al. 1990*) or maintenance of bright ornaments in birds (*Hamilton & Zuk, 1982*). In all these investigations bacteria taxa were decidedly overlooked. One reason for this omission may be that Hamilton and Zuk *(1982)* argued that suitable parasites for testing their theory are those that 1) debilitate their host instead of killing it or allowing total recovery; and 2) that cause a disease, which can be acute and can result in heavy juvenile mortality, but persists in chronic form in survivors. The aim of these requirements is to target parasites that have a visible effect of infection, which can be judged by mates and therefore selected.

It is true that bacterial infections may cause an acute phase and that it might be sometimes deadly to juveniles or at least the lack of symbiotic species may result in lower probability of juvenile survival (*Moreno et al. 2003*). In most cases though, bacterial infection after its acute phase brings total recovery or in extreme case, causes death. It results from the fact, that coevolution between bacteria and its host closes within the resistance-virulence cycle. The most virulent strains cause the highest mortality, but since density of the host population and possibility to infect more individuals decrease, mutations causing lower virulence become promoted and selected. (*Bush et al. 2001*) At the same time host individuals susceptible to most deadly strains either perish or obtain lower fitness. Thus, benefit of resistance against bacterial infection does not have to be subjected to sexual selection but rather to natural selection, as these features might be highly adaptive. I suggest that two parental species and their hybrids might either share the same or similar opportunistic strains and have different resistance against them or they can bear distinct pathogenic strains with diverse load which could result in fitness differences. Such differences in fitness may, in turn, have a significant effect on process of speciation.

Studies on bacteria and its link to fitness traits in animals, including birds, have been appallingly neglected, therefore I recommend to highlight this issue, as we dispose the proper equipment and molecular methods.

Such as attempt has been done during this project, but I met with numerous obstacles. Fecal specimen is commonly known PCR-inhibitor. To level its inhibitory effect it is necessary to dilute template DNA with inhibitor. To obtain satisfactory DNA yield after extraction, special storage manner of initial material and extraction method have to be applied. Even though I used extraction kits, which seemed to be outshined compared to other methods (see *Scupham et al. 2007* for comparison), from a small number of samples I obtained enough DNA to achieve satisfactory PCR-product yield. It could be caused by several of reasons.

The way of storage. Feces are very degrading environment for such a fragile molecule as DNA is. It contains acids and other substances, such as DNases which may destabilize it (*Regnaut et al. 2006*). It was showed that the best result in extracting DNA from fecal samples are achieved in case of fresh specimens (*Idaghdour et al. 2003*), as well as that the time in the field, temperature and dew point impacts DNA amplification to the most degree (*Murphy et al. 2007*). Different researchers compared different storage methods of fecal samples and their investigations showed that the most efficient results in DNA amplification were obtained after keeping fresh material in 90% ethanol in room temperature, but no longer than one week (*Murphy et al. 2002*). During my project after collection of fecal samples they were kept in room temperature even for hours before being frozen. After that it took up to

three months before extraction protocol was applied. For future research ethanol-storage method will be employed as well as a shorter time between collection and extraction.

Initial material mass. In most extraction methods from fecal samples 100-200mg of initial material is recommended (*Lou et al. 1996, Scupham et al. 2007*). In this project only 2 samples exceeded initial mass of 100mg. Average mass of most samples was about 15mg. The low initial mass could affect subsequent results. Therefore in future research more fecal samples will be collected and applied in extraction protocol.

DNA yield evaluation. To evaluate DNA yield after both extraction and PCR, gel electrophoresis with ethidium bromide was used. It is not only time taking method but more important, it is not sensitive enough to indicate low DNA yield (below 3ng/µl). For future research other methods, like spectrophotometry or RT-PCR only should be applied to interpret all results in a proper way. Thus, although this study did not lead to any novel insights into the role of bacteria in the life of the two species of flycatchers and their hybrids – it has lead to a major step forward in the development of a suitable protocol for achieving this goal.

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REFERENCES

Abu Al-Soud, W. & Rådström P. (1998) Capacity of nine thermostable DNA polymerases to mediate DNA amplification in the presence of PCR-inhibiting samples. *Appl. Environ. Microbiol.* **64,** 3748-3753

Abu Al-Soud, W. & Rådström, P. (2000) Effects of amplification facilitators on diagnostic PCR in the presence of blood, feces, and meat. *J. Clin. Microbiol.* **38**, 4463–4470.

Boström K. H., Simu K., Hagström Å., Riemann L. (2004) Optimization of DNA extraction for quantitative marine bacterioplankton community analysis. *Limnol. Oceanogr.: Methods* **2**, 365–373

Bush A. O., Fernández J. C., Esch G. W., Seed R. J. (2001) Parasitism. The diversity and ecology of animal parasites, *Cambridge university Press*

Clement, B., & Kitts, C.L. (2000). Isolating PCR quality DNA from human feces with a soil DNA kit. *Biotechniques* 28: 640-646

Darwin C. (1859) On the Origin of Species by Means of Natural Selection, *Broadview Press*, reprint of 6th edition, 2003

Derothe J-M., Loubes C., Perriat-Sanguinet M., Orth A., Moulia C. (1999) Experimental trypanosomiasis of natural hybrids between house mouse subspecies. *Int J Parasitol* 29:1011–1016

Derothe J-M., Le Brun N., Loubes C., Perriat-Sanguinet M., Moulia C. (2001) Susceptibility of natural hybrids between house mouse subspecies to Sarcocystis muris. *Int J Parasitol* 31:15–19

Fritz, R. S., Moulia, C. & Newcombe, G. (1999). Resistance of hybrid plants and animals to herbivores, pathogens, and parasites. Annual Review of Ecology and Systematics 30, 565–591. \

Fritz R. S., Nichols-Orians C. M., Brunsfeld S. J. (1994) Interspecific hybridization of plants and resistance to herbivores: hypotheses, genetics, and variable responses in a diverse community. *Oecologia*

Hamilton W. D. & Zuk M. (1982) Heritable True Fitness and Bright Birds: A Role for Parasites?, *Science*, **218**, 384-386

Hamilton W. D., Axelrod R., Reiko T. (1990) Sexual reproduction as an adaptation to resist parasites (A Review), *Proc. Nail. Acad. Sci.* **87**, 3566-3573

Hegarty M. J. & Hiscock S. J. (2005) Hybrid speciation in plants: new insights from molecular studies, *New Phytologist*, **165**, 411-423

Idaghdour Y., Broderick D., Korrida A. (2003) Faeces as a source of DNA for molecular studies in a threatened population of great bustards *Conservation Genetics* 4: 789–792

Jackson, J. A. & Tinsley, R.C. (2003) Parasite infectivity to hybridising host species: a link between hybrid resistance and allopolyploid speciation? *Int J Parasitol*, **33**, 137–144.

Joly P., Guesdon V., Fromont E., Plenet S., Grolet O., Guegan J. F., Hurtrez-Bousses S., Thomas F., Renaud F. (2007) Heterozygosity and parasite intensity: lung parasites in the water frog hybridization complex *Parasitology* Published online 02 Oct 2007

Kreader, C. A. (1996) Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. *Appl. Environ. Microbiol*. **62,** 1102–1106.

Lively C. M. & Dybdahl M. F. (2000) Parasite adaptation to locally common host genotypes, *Nature,* **Jun 8;** 679-681

Lou, Q., Chong S. K. F., Fitzegerald J. F., Siders J. A., Allen S. D., Lee Ch. H. (1996) Rapid and Effective Method for Preparation of Fecal Specimens for PCR Assays. *J. Clin. Microbiol*, **1**, 281–283

Mallet, J. (2005) Hybridization as an invasion of the genome. *Trends in Ecology and Evolution*, **20**, 229–237.

Mattson W. J., Haack R. K., Birr B. A. (1996) F1 hybrid spruces inherit the phytophagous insects of their parents. In *Dynamics of Forest Herbivory: Quest for Pattern and Principle* ed. Mattson W., Niemela P., Rousi M., **183**, 142–49

McPherson M. J. & Møller S. G. (2000) PCR, *BIOS Scientific Publishers Limited*, Oxford

Monteiro, L., Bonnemaison D., Verkis, A., Petry K. G., Bonnet, J., Vidal, R., Carbita, J., Mégrand F. (1997) Complex Polysaccharides as PCR Inhibitors in Feces: *Helicobacter pylori* Model. *J. Clin. Microbiol*, **4**, 995–998

Morand S. & Harvey P. H. (2000) Mammalian metabolism, longevity and parasite species, *Proc. Roy. Soc.,* **267**, 1999-2003

Moreno J., Briones V., Merino S., Ballesteros C., Sanz J. J., Tomás G. (2003) Beneficial effects of cloacal bacteria on growth and fledging size in nestling Pied Flycatchers (*Ficedula hypoleuca*) in Spain, *The Auk*, **120**, 784-780

Moulia, C. (1999) Parasitism of plant and animal hybrids: Are facts and fates the same? *Ecology*, **80**, 392–406

Murphy M. A., Kendall K. C., Robinson A., Waits L. P. (2007) The time impact and field conditions on brown bear (Ursus arctos) faecal DNA amplification, *Conserv. Genet.* **8**, 1219-1224

Murphy M. A., Waits L. P., Kendall K. C., Wasser S. K., Higbee J. A., Bogden R. (2002) An evaluation of long-term preservation methods for brown bear (Ursus arctos) faecal DNA samples, *Conserv. Genet.* **3**, 435-440

Obrebski S. (1975) Parasite reproductive strategy and evolution of castration of hosts by parasites, *Science*, **188**, 1314-1316

Parris, M. J. (2004) Hybrid response to pathogen infection in interspecific crosses between two amphibian species (Anura: Ranidae). *Evolutionary Ecology Research*, **6**, 457– 471.

Rådström, P., Knutsson R., Wolffs P., Lövenklev M., Löfström C. (2004) Pre-PCR processing . Strategies to generate PCR-compatible samples. *Molecular Biotechnology,* **2**, 133-146

Regnaut S., Lucas F. S., Fumagalli L. (2006) DNA degradation in avian faecal samples and feasibility of non-invasive genetic studies of threatened capercaillie populations, *Conserv. Genet.* **7**, 449-453

Scupham A. J., Jones J. A., Weasley I. V. (2007) Comparison of DNA extraction methods for analysis of turkey cecal microbiota, *J. Appl. Microbiol.*, **102**, 401-409

Svensson L., Grant P. J. (1999) Bird Guide, HarperCollins*Publishers*, London

Thorton, C. G. & Passen, S. (2004) Inhibition of PCR amplification by phytic acid, and treatment of bovine fecal specimens with phytase to reduce inhibition. *Journal of Microbiological Methods* **59** 43– 52

Trouvé S., Renaud, F., Durand, P., Jourdane, J. (1998) Experimental evidence of hybrid breakdown between genetically distinct populations of *Echinostoma caproni. Parasitology* **117**, 133-135.

Whitham T. G. (1989) Plant hybrid zones as sinks for pests. *Science* **244**, 1490–93

Wiley C. (2006) Speciation – What can be learned from a Flycatcher Hybrid Zone? *Acta Universitatis Upsaliensis*, Uppsala

Wiley C., Fogelberg N., Sætre S. A., Veen T., Svedin N., Kehlenbeck J., Vogel Qvarström A., (2007) Direct benefits and costs for hybridizing *Ficedula* Flycatchers, *J. Evol. Biol.*, **20**, 854-864

Wilson, I. G. (1997) Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol*. **63:**3741–3751.

Wolinska, J., Keller, B., Bittner, K., Lass, S., Spaak, P. (2004) Do parasites lower *Daphnia* hybrid fitness? *Limnology and Oceanography*, **49**, 1401–1407.

Wolinska, J., Bittner, K., Ebert, D., Spaak, P. (2006) The coexistence of hybrid and parental *Daphnia* – the role of parasites. *Proceedings of the Royal Society of London Series B-Biology Sciences*, **273**, 1977–1983

Wolinska, J., Keller, B., Marina, M., Spaak, P (2007) Parasite survey of a *Daphnia* hybrid complex: host-specificity and environment determine infection. *Journal of Animal Ecology,* **76**, 191–200
Veen T., Borge T., Griffith S. C., Sætre G., Bures S., Gustafsson L., Sheldon B. C. (2001) Hybridization and adaptive mate choice in flycatchers, *Nature*, **411**, 45-50