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# Detection of *Yersinia pseudotuberculosis* in Apollo Butterfly (*Parnassius apollo*, Lepidoptera: Papilionidae) Individuals from a Small, Isolated, Mountain Population

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## Abstract

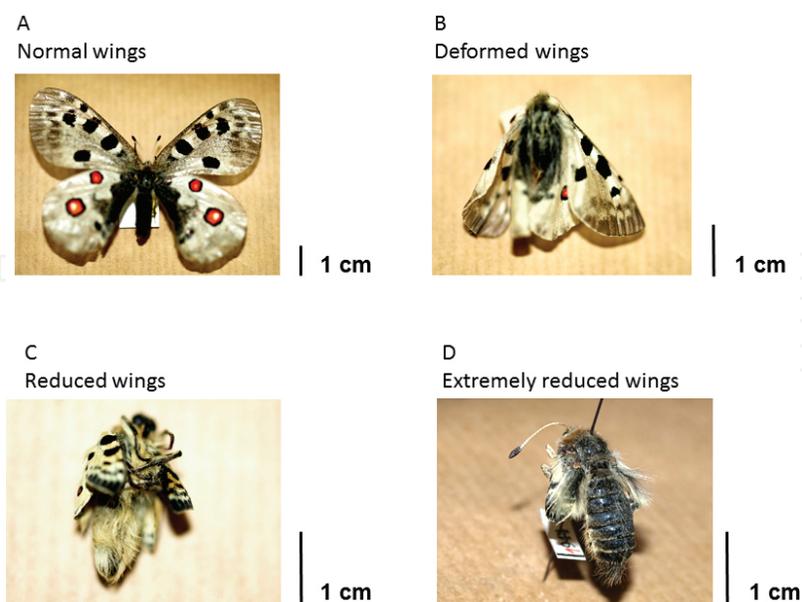
*Yersinia pseudotuberculosis* is a bacterium pathogenic to humans and other mammals; however, its insecticidal activity has also been documented in laboratory studies. A small population of Apollo butterfly (*Parnassius apollo*), reconstituted from less than 30 individuals in 1990s, occurs in Pieniny National Park (Poland). In this report, we demonstrate that a DNA fragment specific to *Y. pseudotuberculosis* could be detected in 40% of biological samples isolated from insects belonging to the Apollo butterfly population. Although *Y. pseudotuberculosis* DNA occurred in both normal and malformed insects, the difference between the fractions of infected individuals was statistically significant ( $p=0.044$  in the Fisher's exact test). No such DNA could be detected in analogous samples from other butterflies (*Pieris napi*, *Pieris rapae*, and *Zerynthia polyxena*) occurring in separate habitats (either a meadow near the city of Cracow, Poland, or in a mountain region of Greece). It is suggested that infection with *Y. pseudotuberculosis* might weaken the general condition of the *P. apollo* population from Pieniny and contribute to the appearance of developmental abnormalities of the butterflies. Thus, it appears that *Y. pseudotuberculosis* infections of insects may be of biological significance in natural environment.

**Keywords:** Apollo butterfly, deformed wings, reduced wings, *Yersinia pseudotuberculosis*, isolated butterfly population

## 1. Introduction

*Yersinia pseudotuberculosis* is a bacterium pathogenic to humans and other mammals. However, it also reveals insecticidal activity due to the production of specific toxins [1, 2]. Infections with this bacterium cause a serious, often deadly, disease of various insects, including species belonging to Lepidoptera [3, 4]. Nevertheless, reports indicating occurrence of *Y. pseudotuberculosis* in insects and its pathogenicity to these hosts are based on laboratory, rather than environmental, studies.

*Parnassius apollo* (Lepidoptera: Papilionidae), known as Apollo butterfly, is a rare, seriously endangered species. It is often considered as near threatened [5], despite the fact that its population in Europe was relatively large for some 100 years ago [6]. While reason(s) for *P. apollo* extinction are debatable, and only partially explained [7], various programs for saving and reconstitution of this butterfly have been established. In Pieniny National Park (Poland), the population of Apollo butterfly declined to less than 30 individuals at the beginning of the last decade of twentieth century [8]. Nevertheless, a specific program allowed to enlarge this population significantly [9]. On the other hand, surprisingly frequent appearance of malformed butterflies has been noted [10]. Such insects occurred in the natural environment of Pieniny National Park, but this phenomenon was more pronounced in the reared population, kept in seminatural conditions in order to increase the number of *P. apollo* individuals (most probably, malformed insect died and/or were eaten in the natural environment). The most striking malformed phenotypes include deformation and reduction of wings [10]. Examples of malformed individuals, in comparison with the normal one, are depicted in **Figure 1**.



**Figure 1.** Examples of *P. apollo* individuals with different patterns of wings: normal (A, wings characteristic for healthy butterflies), deformed (B, wings of the size similar to normal, but with changed shape and arrangement), reduced (C, wings smaller than normal, sometimes with different morphology), and extremely reduced (D, very small wings, resembling buds rather than mature organs, sometimes almost invisible). Photographs made by the authors.

Until recently, the cause of the malformations in *P. apollo* from Pieniny was unknown. However, when genetic materials from normal and malformed insects were compared, some significant differences could be identified. In butterflies with deformed or reduced wings, mutations in the *wingless* gene, coding for a protein involved in wing development, were found to be common [11]. Deficiency in laccases, enzymes which are involved in detoxification of some compounds found in normal diet of caterpillars, was significantly more frequent in malformed than in healthy butterflies [12]. Moreover, many individuals with deformed or reduced wings did not contain *Wolbachia*, a prokaryotic symbiont that can modulate some important physiological processes in insects [13]. These results indicate that there are genetic, biochemical, and microbiological reasons for malformations of wings in the isolated population of *P. apollo*. On the other hand, statistical analyses indicated that none of the mentioned reasons can be considered a sole cause of the developmental changes [11–13]. Therefore, further studies on this phenomenon appear to be warranted. In this report, we present evidence that a considerable fraction of the population of Apollo butterfly from Pieniny is infected with *Yersinia pseudotuberculosis*.

## 2. Materials and methods

### 2.1. Insects

Insects used in this work were either withdrawn from a meadow near the city of Cracow, Poland (individuals of *P. napi*, *P. rapae*), taken from a mountain region in Greece and obtained from a private collection of butterflies (individuals of *Z. polyxena*) or obtained from the collection of dried insects of Pieniny National Park (individuals of *P. apollo*). The permission for the use of this material has been obtained from the Director of Pieniny National Park (permission no. PB-5232-24/07, topic ID: p0748). For DNA isolation, a material from 3 specimens of *P. napi*, 4 of *P. rapae*, and 2 of *Z. polyxena*, and 15 of *P. apollo* was used. Among *P. apollo* individuals, 12 had normal wings and 3 had malformed wings.

### 2.2. DNA isolation and amplification

A material extracted from legs of investigated insects was used for DNA studies. This material was subjected to wash using deionized water before the procedure to avoid environmental contamination. The procedure was conducted by employing the Sherlock AX Purification Kit (A&A Biotechnology), according to the manufacturer's instruction. Following PCR-mediated amplification of specific DNA fragments (using primers listed in **Table 1**), they were separated by agarose gel electrophoresis and analyzed as described previously [14].

### 2.3. DNA cloning and sequencing

Selected products of DNA amplification were cloned into a plasmid vector by using the TOPO TA Cloning Kit Dual Promoter (with pCR II-TOPO vector) with One Shot TOPO10F' Chemically Competent *Escherichia coli* (Invitrogen). DNA sequencing was conducted com-

mercially in the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, Institute of Biochemistry and Biophysics of the Polish Academy of Sciences (Warsaw, Poland).

Gene	Primers (forward and reverse)	References
<i>dpp</i>	5' AGA GAA CGT GGC GAG ACA CTG 5' GAG GAA AGT TGC GTA GGA ACG	[24]
<i>hh</i>	5' AAG GAA AAA CTG AAT ACG CTG GC 5' CGA GAC GCC CCA ACT TTC C	[24]
<i>ptc</i>	5' CTC CGA AGA AGG TCT GCC GCA AG 5' AAT TCG TGC TCG TCG TAT TTT C	[24]
<i>inv</i>	5' TAA GGG TAC TAT CGC GGC GGA 5' CGT GAA ATT AAC CGT CAC ACT	[15]

**Table 1.** Specific primers used in PCR.

## 2.4. Statistical analysis

Since only a low number of samples could be analyzed (due to restrictions caused by regulations of *P. apollo* protection and protective rules of the collection of Pieniny National Park), the statistical analysis was performed by using the Fisher's exact test. Statistical significance was assumed when  $p < 0.05$ .

## 3. Results

In the course of our studies on the reasons of deformation and reduction of wings in the population of *P. apollo* from Pieniny National Park, we tested various genes involved in the development of differentiation of various insect organs. Since Apollo butterfly genome has not been sequenced yet, in order to amplify some genes, primers were designed on the basis of DNA sequences from other insects. Although this strategy was often successful [11, 12], specific DNA fragments were unambiguously identified (e.g., those amplified with primers for *dpp*, *hh*, and *ptc* genes, listed in **Table 1**; this was also a positive control for the quality of DNA samples) in some cases and no amplification products of desired genes could be obtained. Instead, in a few cases, PCR-derived DNA fragments of unexpected lengths appeared and were particularly abundant. An example was ~160-bp PCR product, amplified with the use of primers (5'-TCG GAA AAA TTG TGG ATC GAG G and 5'-AAA TCC GAA GCC GAT GTT GTC) initially devoted for amplification of the *wg* gene fragment (with expected length of 220 bp, assuming a sequence homology of the *wg* gene from *P. apollo* to that from other insects). This ~160-bp DNA fragment was cloned in a plasmid vector and sequenced (the actual length of the insert was 158 bp). The BLASTx-mediated search indicated a homology to two proteins of *Y. pseudotuberculosis*, an RND family efflux transporter and hemolysin secretion protein D.

These results suggested a possibility of the presence of this bacterium in a biological material withdrawn from bodies of investigated insects. Therefore, we aimed to test this hypothesis.

Using primers specifically designed to identify *Y. pseudotuberculosis* (reported previously [15]), it was possible to detect the presence of this bacterium in samples from normal and malformed *P. apollo* individuals. Among 15 samples tested, the *Y. pseudotuberculosis*-specific PCR product was detected in 6 (**Table 2**). Three of them were from normal individuals, and three of them were from insects with deformed or reduced wings. Statistical analysis indicated that the malformed butterflies were significantly more often infected than normal individuals ( $p = 0.044$  in the Fisher's exact test). In control experiments, no *Y. pseudotuberculosis*-specific DNA could be detected in samples from *P. napi*, *P. rapae*, and *Z. polyxena* (**Table 2**). These control samples came from insects withdrawn from habitats located outside of Pieniny National Park, that is, either a meadow near the city of Cracow (Poland) or a mountain region in Greece.

Species and characteristics	Number of individuals used for DNA isolation		
	All tested	With <i>inv</i> specific PCR product	Without <i>inv</i> specific PCR product
<i>P. napi</i> (normal)	3	0	3
<i>P. rapae</i> (normal)	4	0	4
<i>Z. polyxena</i> (normal)	2	0	2
<i>P. apollo</i> (normal) <sup>a</sup>	12	3	9
<i>P. apollo</i> (with malformed wings) <sup>a</sup>	3	3	0

<sup>a</sup>The  $p$  value, in the Fisher's exact test, for normal individuals vs. malformed insects was 0.044.

**Table 2.** Results of PCR-mediated DNA amplification with the use of indicated templates and primers specific to the *inv* gene of *Y. pseudotuberculosis*.

## 4. Discussion

Pathogenicity of *Y. pseudotuberculosis* to insects was demonstrated previously under laboratory conditions [3, 4]. Its detection in samples from *P. apollo* individuals coming from Pieniny National Park indicates that this bacterium can infect butterflies in natural habitats and may suggest that the investigated Apollo butterfly population is endangered by insecticidal activity. Although the extinction of this population due to *Y. pseudotuberculosis* infection is rather unlikely, the presence of this pathogen may significantly weaken the insects. One might suggest that *Y. pseudotuberculosis* infections could contribute to developmental changes observed in these insects. Although statistically significant difference was found between the frequency of infected normal and malformed Apollo butterflies, detection of *Y. pseudotuberculosis* in samples from healthy individuals demonstrated that the infection occurs in the entire population. Perhaps, butterflies weakened by other factors, such as deficiency of laccase or the

absence of *Wolbachia*, might be more susceptible and more sensitive to *Y. pseudotuberculosis* infection.

The presence of *Y. pseudotuberculosis* in butterflies from Pieniny National Park, and its absence in samples from other butterflies withdrawn from other habitats (either in Poland or in Greece), might seem surprising. However, this bacterium has also been described as a pathogen of sheep around the world [16–19]. There is a broad area of a sheep pasture ecosystem in Pieniny, where sheep grazing is particularly extensive [20]. Importantly, it occurs even at upper mountain parts. Therefore, sheep can be considered as a source of *Y. pseudotuberculosis* in this region. Bacteria may be excreted with feces of sheep, causing contamination of local plants [21, 22], and then, they can be spread through various animals, becoming potential infectious agents for insects in Pieniny National Park. One might suppose that infections of *P. apollo* by *Y. pseudotuberculosis* could contribute to developmental abnormalities of butterflies, due to weakening of the insects and causing physiological disturbance, especially in combination with genetic, biochemical, and symbiosis problems which the population in Pieniny suffers from (and which were described previously [11–13]). Interestingly, insecticidal activity of cell extracts from *Yersinia enterocolitica*, a species closely related to *Y. pseudotuberculosis* and producing the same kinds of toxins, was demonstrated to be present only when bacteria were cultured at low temperature (10°C), in contrast to higher temperature (30°C) [23]. Because the population of *P. apollo* in Pieniny exists in the mountain region, where temperatures are commonly around 10°C from late spring to early fall, a deleterious effect of *Y. pseudotuberculosis* infection on this population seems likely.

The question appears what might be effects of infections of Apollo butterflies with *Y. pseudotuberculosis*? In fact, in our work, focused on the biological material from a collection, we could only detect the presence of this bacterium in samples of insect bodies. To determine how severe such infections could be, laboratory studies, with experimental administration of bacteria to insects' bodies would be necessary. Then, symptoms of the infection might be observed and investigated, with assessment of their severity. Moreover, it would be particularly interesting to test whether *Y. pseudotuberculosis* infection affects the development of Apollo butterfly. Again, experimental studies with the use of *P. apollo*, including larvae and imago forms, would be necessary. The problem is that Apollo butterfly is a rare species (particularly subspecies *frankenbergeri*, occurring in Pieniny), protected by law. Thus, no individuals can be withdrawn from their natural habitat to conduct biological experiments. The only possibility would be to use insects from a culture; however, to our knowledge, no such culture is currently available.

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