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# CONTENTS

Soňa Vařachová, Bikram Shrestha: Climate-smart conservation, a new way to tackle the global species conservation crisis .....	67
Spyros Tsiftsis: The role of Natura 2000 network in protecting the orchid flora of East Macedonia (NE Greece) .....	71
Richard Svidenský, Andrea Kučerová, Hana Čížková: Causes of the dieback of littoral stands in an overpopulated water bird reserve: Role of eutrophication, fish and geese .....	79
Zdenka Křenová, Zdeňka Chocholoušková, Vladimír Zýval: Salt no longer travels through the Bohemian Forest along the Golden Trail, but halophytic neophytes do .....	91
Lassaad Mdellel, Ahmed Abdelli, Khaled Omar, Waleed El-Bassam, Mazen Al-Khateeb: Effect of aqueous extracts of three different plants on <i>Myzus persicae</i> Sulzer (Hemiptera: Aphididae) infesting pepper plants under laboratory conditions .....	101
Jitka Kufnerová: A novel approach of using shed skins of the green tree python, <i>Morelia viridis</i> , for forensic purposes .....	107
Daniel Vaněk, Edvard Ehler, Lenka Vaňková: Technical note: Development of DNA quantitation and STR typing systems for <i>Panthera tigris</i> species determination and individual identification in forensic casework .....	113



# CLIMATE-SMART CONSERVATION, A NEW WAY TO TACKLE THE GLOBAL SPECIES CONSERVATION CRISIS

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## ABSTRACT

Climate change is something no one can ignore. While some people are still questioning the source of this issue, many are already working on solutions for the world's species, for which climate change might mean another step toward extinction. We are presenting here the basic idea of an innovative conservation approach, climate-smart conservation, which has a potential to mitigate the impacts of climate change and therefore protect some vulnerable species from demise. Next to its key characteristics we present examples of already ongoing practices involving climate-smart conservation and possible use of this approach in conservation of the snow leopard.

**Keywords:** biodiversity; climate change; climate-smart conservation; conservation; snow leopard

## Introduction

Climate change represents a phenomenon of our time, and together with its possible impacts it has become a widely discussed topic (Bílá and Kindlmann 2019). Although there are some people still questioning or even denying the amount of human impact on this global matter, only humans are capable of tackling this major issue. Unfortunately, impacts of climate change drastically affect a variety of species of both flora and fauna, and therefore they are negatively contributing to an already massive biodiversity loss. There are many countries and societies realizing the current biodiversity crisis and they are also trying to face this problem. However, their conservation plans are often built on traditional approaches and are lacking innovation for a fast adaptation when facing unpredictable effects of climate change. The key to this can be a modification of such approaches, as for example an adaptive management based on an idea of an implement-monitor-evaluate-adjust cycle, which allows a later reaction to an at a first sight unexpected scenario (Hansen et al. 2010). Climate-smart conservation offers such innovation model through its focus on flexibility and consideration of a wide scale of scenarios (Stein et al. 2014), which together with prevision (Hansen et al. 2010) are necessary to overcome the possible changes in different regions coming with climate change. Its strategy can also be implemented into already planned, or even ongoing projects, which saves not only time but also money (Stein et al. 2014). This makes climate-smart conservation budget friendly and therefore more accessible for even smaller projects with less finances.

## Climate-Smart Conservation

Climate-smart conservation is a new concept of an adaptive management, which seems to be able to over-

come static habits in conservation. Its main idea is being explained by a couple of definitions throughout the literature and conservation organisations, and each of them usually presents its own key elements or characteristics to better define the main idea of this adaptation strategy. However, the main idea remains the same. The most complex description of this adaptation strategy was presented by Stein et al. (2014), who describe climate-smart conservation as:

“The intentional and deliberate consideration of climate change in natural resource management, realized through adopting forward-looking goals and explicitly linking strategies to key climate impacts and vulnerabilities.”

Apart from this definition, Stein et al. (2014) also set their own key characteristics, closer identifying proper practice. First of them is *Linking actions to Climate Impacts*. Here Stein et al. (2014) explain that action plans and strategies should target existing threats alongside with the impacts of climate change. In case of including climate-smart conservation into already ongoing projects, an understanding, or a hypothesis of climate-related vulnerabilities reduction, or – on the other hand – a knowledge of any climate-related opportunities is necessary. The second, *Embracing of Forward-Looking Goals*, then explains that while facing the climate change, we can no longer rely on the traditional natural resources management approach, as it is often counting with static information about the environment, such as weather, habitats or species (Stein et al. 2014). New goals in conservation should focus on future conditions and they should consider possible need of strategies transitions. Another key element of climate-smart conservation described by Stein et al. (2014) is *Consideration of Broader Landscape Context*. This characteristic describes two important points in this climate change adaptation strategy: consideration of changes in distribution of species due to their adapta-

tion to new conditions coming alongside climate change, and collaboration among institutions, communities or even landowners to support a common goal and avoid any conflicts in conservation actions and objectives. The key characteristic *Adopting Strategies Robust to Uncertainty* then explains that due to the uncertainties not only in the situations coming alongside the climate change, but also in the human reactions to it, new conservation strategies should benefit several future scenarios (Stein et al. 2014). Another vital element is then *Employment of Agile and Informed Management*, as it is required to deal with rapid changes of conditions not only related to the climate and the environment, but also to socioeconomics (Stein et al. 2014). Surely, *Minimizing of Carbon Footprint* is also important point, as strategies dealing with the climate change impacts should not contribute to this issue. Therefore, climate-smart conservation projects should direct their plans to minimize greenhouse gas emissions and the use of energy. The climate-smart projects should also *Take into Account the Climate Influence on Project Success*, as they should avoid investing into plans vulnerable to the climate change. Such vulnerability can appear in many sectors, including socioeconomics or ecology (Stein et al. 2014). Already during planning of the adaptation strategy, *Safeguarding of People and Nature* should be considered as well, as focus directed not only on the ecosystem, but also on local communities contributes to a support by locals, which leads to a better success of the entire plan. Finally, climate-smart conservation should always focus on *Avoiding of Maladaptation*, as no strategy should compromise other climate change focused plans or disturb higher conservation targets (Stein et al. 2014).

The WWF (2021), which also works with the climate-smart conservation idea, comes with much simpler key elements:

- a) Understanding the implications of climate change, including how human responses might lead to changes in other conventional threats.
- b) Developing and implementing no-regret actions that address current threats, do not erode options for responding to future climate change, and avoid contributing to greenhouse gas emissions.
- c) Taking an integrated approach to adaptation, contributing to nature conservation and fair, equitable and sustainable development.
- d) Active learning to build capacity and work collaboratively to plan and respond to increasing change and uncertainty.
- e) Bringing about changes in policy that create an enabling environment across scales (local to international) for adaptive governance.

However, many of their features are included in the Stein et al. (2014) key characteristics as well. WWF (2021) also offers its own description, which again is much more simplified: “Climate-smart conservation considers how climate and non-climate related pressures affect species, ecosystems and people.”

Hansen et al. (2010) then comes with their own key elements (tenets) for the climate-smart conservation strategy. They consider important to include *Adequate and appropriate space*, which can later serve either as climate refugia with less severe climatic changes, as corridors to allow movement of species, or as network for allowing population connectivity. According to Hansen et al. (2010), it is also important to *Reduce Non-climate stresses* as “already stressed ecosystems and organisms are less resilient to climate-change effects”. Another important tenet is then to *Adopt Adaptive Management*, which requires systematic evaluation of implemented strategies and in case appropriate adjustment. As Hansen et al. (2010) mention, “adaptation is a bicycle we must build while we ride it”. The last tenet mentioned by Hansen et al. (2010) is to *Reduce the Rate and Extent of Climate Change*, which they emphasize is the key to conservation success as extension of climate change and its impacts can increase the adaptation costs but on the other hand decrease the chances of success. While all three sources present their key characteristics in a different way, they all share the same idea.

## Climate-Smart Conservation in the World

To properly understand the right meaning of climate-smart conservation, it is better to present it in real examples. The first example of climate-smart conservation project is creation of setbacks with the Bruun Model for Beach Recession to help to predict natural shifts of beaches, and therefore preserve nesting beaches of hawksbill turtles (*Eretmochelys imbricata*) in Barbados (Fish et al. 2008). Hansen et al. (2010) describe, how sea level rising caused by climate change threatens nesting areas of sea turtles. Together with their sex determination being dependent on temperature during incubation, possible demise of sea turtle species is tightly connected to the climate change (Janzen 1994; Hansen et al. 2010). However, early determination of adequate spaces behind current nesting beaches – setbacks, which will be protected from human development and included into local conservation plans, can contribute to natural shift of nesting beaches inland, which even has a potential to be more sustainable than armouring, both ecologically and economically (Hansen et al. 2010). Another positive aspect of inland shifting of nesting beaches is that incubation temperature can be lowered by vegetation shade, naturally occurring in the setbacks, and therefore preserve sex ratio balance (Janzen 1994; Hansen et al. 2010).

Another example set by Hansen et al. (2010) comes from the Sundarbans National Park (West Bengal, India), where conflicts between Bengal tigers (*Panthera tigris tigris*) and humans are likely to escalate. The reason for this is a threat to local mangrove forest and therefore to local islands, which serve as protected tiger habitats

and as home to local communities. According to Colette et al. (2007), the global rise of sea level together with other stressors can cause destruction of three quarters of mangroves in Sundarbans by the end of the century. To protect local tigers, it is necessary to use appropriate GIS – based spatial analyses and identify and protect new islands, which can serve as potential tiger habitats, despite the fact that currently they might seem unimportant or undeveloped (Hansen et al. 2010).

## Case of Snow Leopard

Apart from examples presented in this paper, taking place by the coastline, climate-smart conservation has a great potential to be used in the mountain areas as well. More specifically, in the Himalayan region of Nepal, in conservation of snow leopard. Local population of snow leopard is scattered across the entire country and in many cases the area of their movement also crosses the national boundaries. Climate-smart conservation can be used to protect their possibility of movement by protecting areas of future corridors, which are likely to shift due to the climate change, and therefore contribute to snow leopard's better resilience. However, to determine future corridors we need to estimate potential suitable habitats for snow leopards.

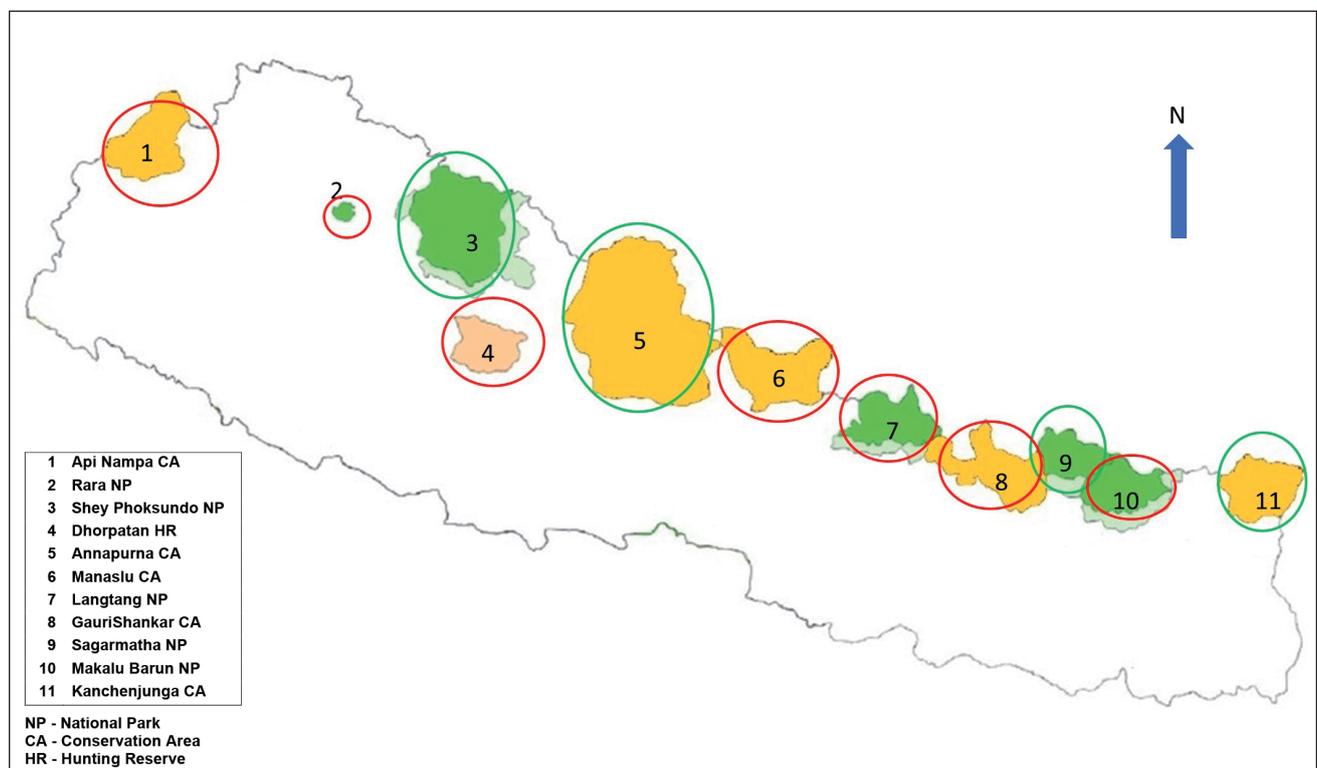
Snow leopards in Nepal currently find refuge within some designated conservation areas and national parks

(Fig. 1). Nevertheless, in the future this might simply not be enough, as they will be most likely forced to search for prey and habitats elsewhere.

Another region considering implementing climate-smart conservation into their conservation plans to protect local snow leopard population is Central Asia (Kyrgyzstan and Tajikistan). In this area, there is an ongoing programme of Vanishing Treasures, an UNEP (United Nations Environment Programme) project to protect iconic species (Vanishing Treasures 2021). Also, in this case, one of the conservation points, where they are using this adaptation strategy, is “Integrating climate-smart measures into conservation planning, including ecological connectivity measures that account for shifting and changing habitats and other changes as a result of climate change” (Vanishing Treasures 2021).

## Conclusions

Relatively new adaptation strategy, climate-smart conservation, offers an approach, which can help the world's biodiversity and us to prepare for changes inevitably coming with the climate change. Despite still being described in various ways, with every author the core stays the same, and in every way, it is meant to be implemented in a wide range of projects to help navigate the conservation into the future. The future might not seem too bright for the biodiversity, but the entire life is about



**Fig. 1** Protected areas of Nepal in mountain range and possible snow leopard range: yellow colour – Conservation Area, dark green colour – National Park, pink colour – Hunting Reserve; green circle – area with snow leopard abundance/density data using genetic analysis or camera trap survey, red circle – area with no sufficient/rigorous data.

adaptation, and we are right now standing at a tipping point, where we must decide how we want our future to look like and how we want our future generations to remember us. Climate change is happening and if species are able to adapt to a certain point, we are obliged to help them to make a step even further for them to be able to survive.

## Acknowledgements

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# THE ROLE OF NATURA 2000 NETWORK IN PROTECTING THE ORCHID FLORA OF EAST MACEDONIA (NE GREECE)

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## ABSTRACT

East Macedonia (northeast Greece) is a relatively small part of Greece, where a considerable number of orchid taxa occurs. Some of these orchids can only be found there and this fact makes the specific area of Greece unique. In this study, an up-to date database of orchid records was used to assess the effectiveness of the existing Natura 2000 network. Specifically, the effectiveness of the Natura 2000 network was evaluated by identifying the number of orchids whose distribution is overlapping to a lesser or greater extent with the network, which chorological categories are included/excluded from it, and whether the rare and threatened orchid taxa are adequately distributed within that. Out of the 73 orchid taxa recorded in East Macedonia so far, 14 taxa are exclusively distributed outside the Natura 2000 network. Specifically, the Natura 2000 network is not overlapped with a number of Balkan and Mediterranean orchid taxa, which are only sparsely found in East Macedonia. Moreover, most of the orchid taxa that have been classified in the threat categories of the IUCN are distributed within the Natura 2000 network of East Macedonia, and specifically, some of the most threatened ones are almost exclusively distributed within that network. Consequently, although the Natura 2000 network is not congruent with the distribution of a number of species of southern origin, which are widely distributed elsewhere in Greece, it can conserve important floristic elements of Greece, which are orchid taxa of northern or central European origin.

**Keywords:** conservation; East Mediterranean; Orchidaceae; threatened species

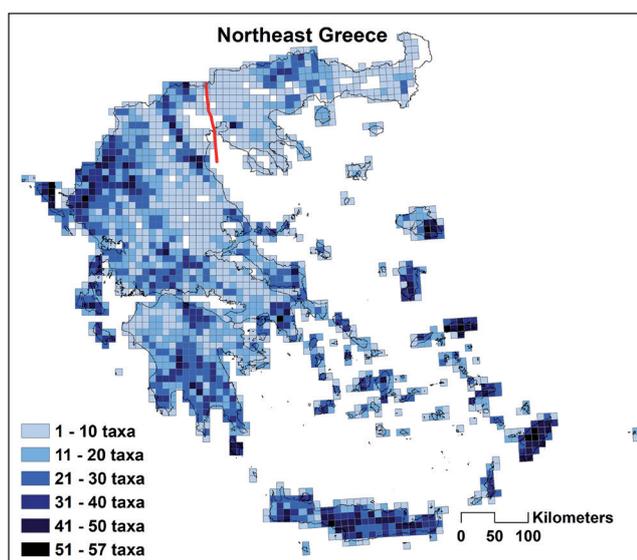
## Introduction

The human impact and activities over the past few decades have caused serious declines in organisms all around the world, and as a result, governments have signed environmental agreements to reverse these declines (Rogalla von Bieberstein et al. 2019). Globally, the Convention on Biological Diversity to which 196 countries are contracting parties, is one of the most important agreements, whose Aichi Biodiversity Targets referred to its Strategic Plan for Biodiversity 2011–2020 (Decision X/2) include the protection of 17% of the earth and 10% of the oceans (strategic goal C: target 11). Thus, all around the world, protected areas, such as national parks and nature reserves, constitute a key strategy for conserving biodiversity (Geldmann et al. 2019).

In Europe, the 27 member states of the European Union have established a network of protected areas called “Natura 2000 network” by applying two Directives; the Birds (Directive 79/409/EEC, which was amended by the Directive 2009/147/EC) and the Habitats (Directive 92/43/EEC) Directive (European Commission 2020). The Natura 2000 network in Europe covers more than 18% of the EU’s land area and more than 8% of its marine territory, and it is considered the largest coordinated network of protected areas in the world. Conservation of plant species and/or habitats is subject to the Habitats Directive (Directive 92/43/EEC) through the establishment of a network of Special Areas of Conservation (SACs). In Greece, this network is composed of 241 sites (SACs), covering 21.27% of the terrestrial area of the country, which is among the highest among the European countries.

Although networks of protected areas are considered to be the most important measures that governments take to conserve biodiversity, the effectiveness of these networks is still uncertain in several cases (Watson et al. 2014; Joppa et al. 2016). Looking at the geographical location of the Special Areas of Conservation of the Natura 2000 network in Greece, one can identify that most of these have been established in high-altitude areas. This could be attributed to two different reasons: (a) the high species diversity of the mountainous areas in Greece, and (b) the rather low human activities in these areas compared to areas of lower altitudes, where natural habitats are more degraded. Based on this, it is unclear, whether SACs in Greece can adequately conserve populations of specific subsets or groups of plant species. For example, the Natura 2000 network in the Peloponnese was only partly congruent with a theoretical network of areas for the protection of the endemic flora of the Peloponnese (Trigas et al. 2012). However, this study is not the only one, where Natura 2000 network did not fully overlap with the distribution of all the target plant species. Similar results presented by Dimitrakopoulos et al. (2004), who worked with the plant species of Crete, and by Tsiftsis et al. (2009; 2011), who explored the effectiveness of the Natura 2000 network using the orchids of a subarea of East Macedonia and Crete, respectively.

The orchid family is characterized by a complex biology and an especially high speciation rate, but many orchid taxa are at the verge of extinction (Swarts and Dixon 2009). These characteristics make orchids an important group in biological conservation and because of the threats and danger that many orchids face they are protected in several countries (in Greece, as well) by



**Fig. 1** Distribution of the orchid taxa of Greece (red line represents the limit among the northeast and northcentral floristic regions of Greece).

legislation. Under general legislation, many orchid taxa are protected by the Directive 92/43/EEC (21-5-1992), whereas the whole family of Orchidaceae is included in the three Appendices of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora).

Greece is especially rich in orchids (193 orchid taxa have been recorded so far) and one of the most orchid rich countries in Europe (Delforge 2006; Tsiftsis and Antonopoulos 2017). However, as in all plant families, orchids are not evenly distributed throughout Greece (Tsiftsis et al. 2019). As it can be seen in Fig. 1, northeast Greece is relatively poor in number of orchid taxa compared to the other areas of Greece. East Macedonia constitutes an exception to this general trend, as the

specific area hosts quite a large number of orchid taxa. Based on Tsiftsis et al. (2007), 62 orchid taxa were found in East Macedonia in 2007 and additional orchid taxa were recorded later (e.g. *Gymnadenia odoratissima*: Lafranchis and Sfikas 2009; *Pseudorchis albida*: Tsiftsis and Antonopoulos 2011). East Macedonia unique in Greece, because specific orchid taxa, some of which cannot be found elsewhere in the country, have been recorded in the high-altitude mountains. Under this perspective, the reassessment of the effectiveness of the Natura 2000 network in the area is desirable.

It is well-known that the effectiveness of a network of protected areas in conserving a set of target species is influenced (a) by the degree of representation of the target species within these areas, and (b) the management actions that will be focused on these species (González-Maya et al. 2015; Geldmann et al. 2019; Neugarten et al. 2020). Compared to the database used by Tsiftsis et al. (2009), the database of orchid records of East Macedonia was enriched by additional species distribution data obtained after 2009. Thus, the queries I tried to answer were:

- How effectively is the Natura 2000 network conserving the orchid flora of East Macedonia in the light of the new data?
- Which chorological categories of orchid taxa does the Natura 2000 network conserve?
- What is the significance of the Natura 2000 network of East Macedonia in conserving rare and threatened orchid taxa of Greece?

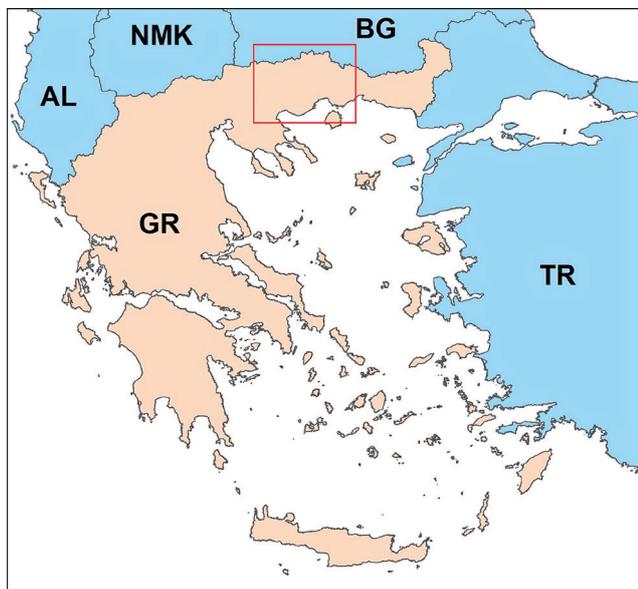
## Material and Methods

The study area comprises the whole of East Macedonia (longitude 23°17' to 24°54' E, latitude 40°38' to 41°34' N)

**Table 1** Special Areas of Conservation in East Macedonia (NE Greece).

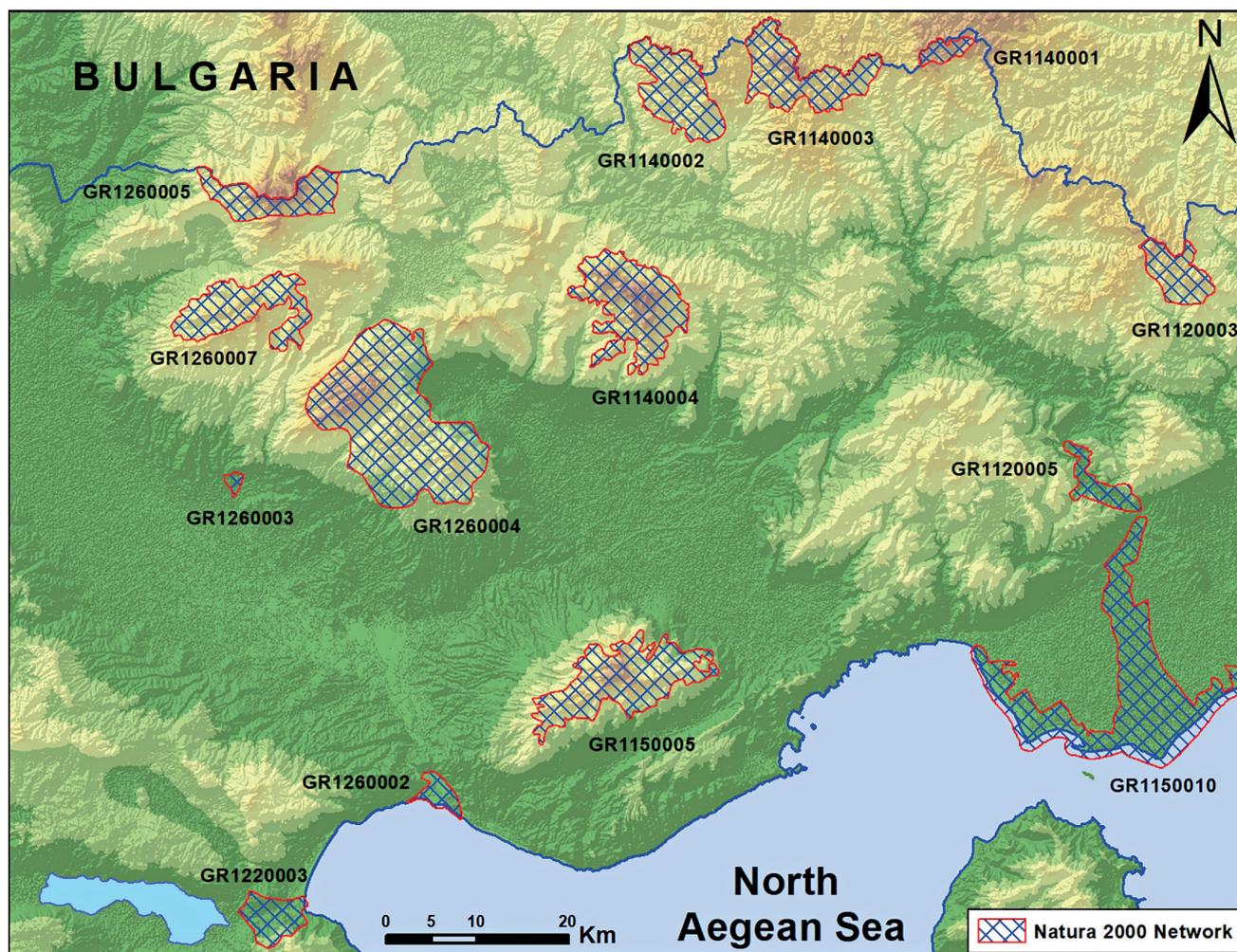
	Special Areas of Conservation	Area (ha)	Official name
1	GR1260002	1,297.10	Ekvoles Potamou Strymona
2	GR1120003	3,491.99	Oros Chaintou – Koula and Gyro Koryfes
3	GR1120005	2,335.87	Aisthitiko Dasos Nestou
4	GR1140001	1,090.05	Dasos Fraktou
5	GR1140002	6,715.45	Rodopi (Simyda)
6	GR1140003	7,447.10	Periochi Elatia, Pyramis Koutra
7	GR1140004	9,845.62	Koryfes Orous Falakro
8	GR1150005	10,345.47	Koryfes Orous Pangaio
9	GR1150010	22,484.64	Delta Nestou kai Limnothalasses Keramotis – Evryteri Periochi kai Paraktia Zoni
10	GR1220003	2,905.16	Stena Rentinas – Evryteri Periochi Spilaio Drakotrypa – Spilaio Lakkia kai Rema Neromana
11	GR1260003	327.29	Ai Giannis – Eptamyloi
12	GR1260004	23,288.69	Koryfes Orous Menoikion - Oros Kouskouras – Ypsoma
13	GR1260005	4,871.04	Koryfes Orous Orvilos
14	GR1260007	6,799.47	Ori Vrontous – Lailias – Epimikes – Spilaia Zesta Nera kai Katarrakton

(Fig. 2), including all the high mountains of North-East Greece (Mts Menikion, Orvilos, Falakron, Pangeon, Vrontous, Lekani, Simvolo, Kerdilion and Rodopi). The area comprises of fourteen Special Areas of Conservation (SAC) of the European Ecological Network Natu-



ra 2000 (Table 1). The network of these areas covers the summits and the high altitudinal zones of Mts Falakron, Pangeon, Menikion, Vrontous and Orvilos, four areas of Rodopi mountain range (Simyda, Elatia, Frakto, Koula), and a part of Nestos river (two SACs) (Fig. 2). Moreover, three other small-sized SACs include riparian areas in the lowlands (GR1260002, GR1260003 and GR1220003). The total area of these SACs is approximately 103,244.92 Ha (Dafis et al. 1996).

East Macedonia, as most parts of Greece, presents a high variability of vegetation types (from maquis-pseudomaquis to *Picea abies* forests and subalpine grasslands) and the geological substrates (e.g. limestones, granites, schists). The combinations of these factors, together with the human impact observed during the last few decades, creates a mosaic of different habitats, where many orchids can occur (Tsiftsis et al. 2007; Tsiftsis and Antonopoulos 2017). Another factor that has a positive effect on the number of orchid taxa is the geographical position of the study area. As a part of northern Greece, which shares some common mountainous ranges with Bulgaria (e.g. Rodopi mountain range, Mt. Orvilos), the area hosts orchids of northern origin, some of which are endemic here (e.g. *Neottia cordata*; Tsiftsis et al. 2019).



**Fig. 2** Map of East Macedonia (NE Greece) (GR: Greece; AL: Albania, NMK: North Macedonia; BG: Bulgaria; TR: Turkey). The official names of the Special Areas of Conservation are presented in Table 1.

The distribution data used for the analyses were based on the database that was built for the purposes of the Orchid Flora of Greece project (Tsiftsis and Antonopoulos 2017), which is still being updated with new orchid records based on recent literature cites and unpublished data (records made up to now). The nomenclature of the orchid taxa follows Dimopoulos et al. (2013), Antonopoulos and Tsiftsis (2017) and Tsiftsis and Antonopoulos (2017). In total, distribution data (8,788 records) of 73 orchid taxa occurring or being reported for the study area were used. *Ophrys insectifera*, recorded by Zaganariaris (1940), was excluded from the total number of orchids in East Macedonia, because this very old record has not been confirmed recently and could not be georeferenced with sufficient accuracy. To assess the effectiveness of the Natura 2000 network in conserving all the orchid taxa of the study, a  $1 \times 1$  km resolution Universal Transverse Mercator (UTM) grid was used in the analyses. Although the size of almost all grid cells was  $1 \text{ km}^2$ , the size of a number of cells was different. Specifically, the grid cells that were adjacent to the Greek-Bulgarian borders, those close to the sea shoreline and close to the borders between different UTM coordinate zones (34T and 35T zones in the UTM projection) were of slightly different size.

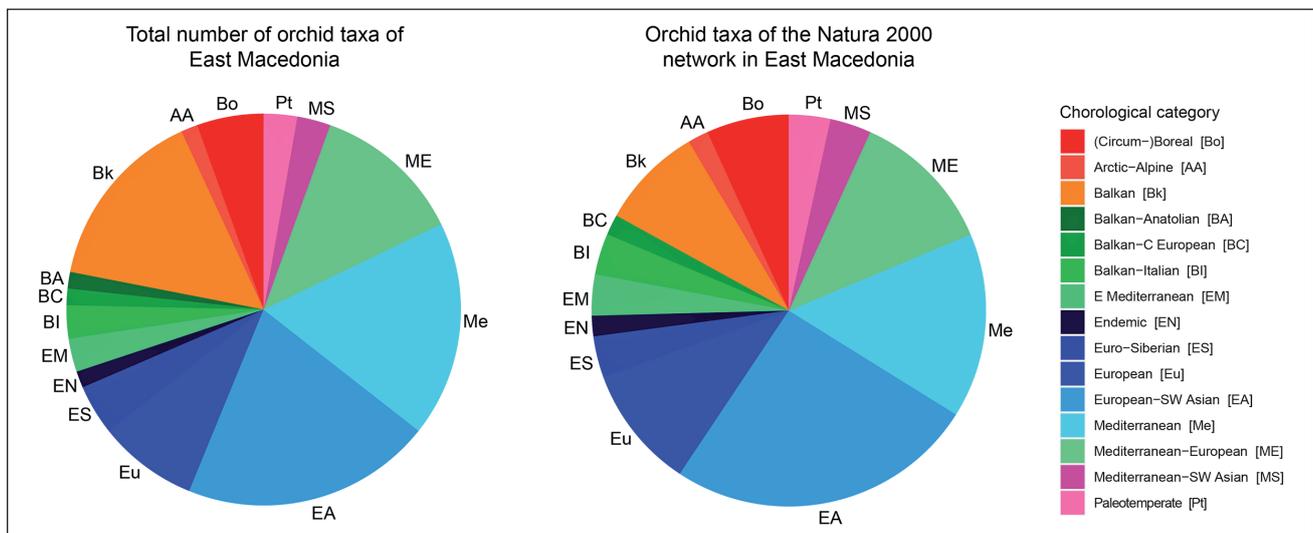
The geographical coordinates of all orchid records were transformed into the UTM projection and then a matrix “species  $\times$  grid cells” was generated. Afterwards, the grid cells that were totally within the Natura 2000 network, or of which more than half was included into the network, were characterized as grid cells of the Natura 2000 network. Thus, the grid cells with orchid occurrences in East Macedonia were divided into two categories: the Natura 2000 grid cells and the non-Natura 2000 grid cells. Based on this, the effectiveness of Natura 2000 in conserving the orchid flora in East Macedonia was assessed. To compare the number of orchids of the  $1 \times 1$  km grid cells inside and outside the Natura 2000 network, the Mann-Whitney U test was used.

To answer the second query, the chorological categories of the orchids of East Macedonia were adopted, as referred by Dimopoulos et al. (2013). For a few orchid taxa, not referred to by Dimopoulos et al. (2013), the chorological category was determined by taking into consideration their general distribution. The third query requires information about the threat categories of the Greek orchids, which were obtained from Tsiftsis and Tsiropidis (2016). One orchid taxon (*Epipactis helleborine* subsp. *distans*), not referred by Tsiftsis and Tsiropidis (2016), was evaluated at a regional scale using the IUCN Red List Criteria (IUCN 2012a) and the guidelines for application of IUCN Red List Criteria at a National Level (IUCN 2012b).

## Results

Out of the 73 orchid taxa recorded in East Macedonia, 14 taxa are exclusively distributed outside of the Natura 2000 network. Orchid taxa absent within Natura 2000 network are mainly found in the southern and central part of Greece. Such orchids (e.g. *Neotinea lactea*, *N. maculata*, several *Ophrys* taxa, *Serapias cordigera* subsp. *cordigera*, *S. parviflora*) have been recorded in a large number of  $1 \times 1$  km grid cells in Greece, but their distribution in East Macedonia is very restricted (Table 2). Apart from these, two species – *Epipactis pontica* and *Epipogium aphyllum* – although found in high altitude areas of Mt. Rodopi, were found in areas outside the Natura 2000 network. The Mann-Whitney U test has shown that the  $1 \times 1$  km grid cells of the Natura 2000 network host more orchid taxa compared to the grid cells outside the Natura 2000 network ( $p < 0.05$ ).

In total, the orchid taxa of East Macedonia are classified into 15 chorological categories (Fig. 3; Table 2). Most of them belong to the European-SW Asian (20.55%), Mediterranean (17.81%) and Balkan (15.07%) categories, followed by the Mediterranean-European taxa (12.33%).



**Fig. 3** Chorological spectrum of all orchid taxa recorded in East Macedonia (left graph) and of those recorded within the Natura 2000 network (right graph).

**Table 2** Orchid taxa recorded in East Macedonia, their chorological category and the number of 1 × 1 km grid cells in which they have been recorded.

Taxon	Chorological category	IUCN category	Total number of 1 × 1 km grid cells	1 × 1 km grid cells within Natura 2000 network	Total number of 1 × 1 km grid cells in Greece
<i>Anacamptis coriophora</i> subsp. <i>coriophora</i>	EA		18	2	137
<i>Anacamptis coriophora</i> subsp. <i>fragrans</i>	Me		69	18	2,212
<i>Anacamptis laxiflora</i> subsp. <i>laxiflora</i>	Me		32	11	1,703
<i>Anacamptis morio</i> subsp. <i>caucasica</i>	MS		378	46	2,389
<i>Anacamptis palustris</i> subsp. <i>elegans</i>	BA	NT*	3	0	70
<i>Anacamptis papilionacea</i> subsp. <i>papilionacea</i>	MS		53	13	446
<i>Anacamptis pyramidalis</i>	Eu		253	41	4,121
<i>Cephalanthera damasonium</i>	ME		216	70	914
<i>Cephalanthera longifolia</i>	EA		202	47	1,033
<i>Cephalanthera rubra</i>	EA		214	66	1,130
<i>Coeloglossum viride</i>	Bo		43	35	80
<i>Corallorhiza trifida</i>	Bo		116	54	257
<i>Dactylorhiza cordigera</i> subsp. <i>cordigera</i>	Bk		44	22	122
<i>Dactylorhiza incarnata</i>	EA	EN*	8	4	13
<i>Dactylorhiza macedonica</i>	EN	VU*	17	8	40
<i>Dactylorhiza romana</i>	Me		21	4	448
<i>Dactylorhiza saccifera</i>	Me		41	21	862
<i>Dactylorhiza sambucina</i>	Eu		244	125	696
<i>Epipactis atrorubens</i>	EA		46	36	199
<i>Epipactis helleborine</i> subsp. <i>distans</i>	Eu	EN**	3	3	5
<i>Epipactis helleborine</i> subsp. <i>helleborine</i>	Pt		371	145	1,317
<i>Epipactis leptochila</i> subsp. <i>naousaensis</i>	BI	EN*	7	6	40
<i>Epipactis leptochila</i> subsp. <i>neglecta</i>	Eu	VU*	6	3	7
<i>Epipactis microphylla</i>	EA		54	21	506
<i>Epipactis palustris</i>	EA		15	7	179
<i>Epipactis persica</i> subsp. <i>exilis</i>	BI		109	19	297
<i>Epipactis pontica</i>	ME	NT*	8	0	12
<i>Epipogium aphyllum</i>	ES		5	0	48
<i>Goodyera repens</i>	Bo		61	38	65
<i>Gymnadenia conopsea</i>	EA		146	82	410
<i>Gymnadenia frivaldii</i>	Bk	NT*	5	2	22
<i>Gymnadenia odoratissima</i>	Eu	CR*	1	1	1
<i>Gymnadenia rhellicani</i>	AA	CR*	4	4	7
<i>Himantoglossum jankae</i>	BC		269	23	755
<i>Limodorum abortivum</i>	Me		146	18	1,708
<i>Neotinea lactea</i>	Me		1	0	931
<i>Neotinea maculata</i>	Me		2	0	1,467
<i>Neotinea tridentata</i>	Me		251	83	1,194
<i>Neotinea ustulata</i>	Eu		77	36	209
<i>Neottia cordata</i>	Bo	VU*	18	11	18
<i>Neottia nidus-avis</i>	EA		413	138	1,051
<i>Neottia ovata</i>	EA		90	13	487
<i>Ophrys apifera</i>	ME		27	3	798

Taxon	Chorological category	IUCN category	Total number of 1 × 1 km grid cells	1 × 1 km grid cells within Natura 2000 network	Total number of 1 × 1 km grid cells in Greece
<i>Ophrys attica</i>	Bk		1	0	556
<i>Ophrys epirotica</i>	Bk		12	0	442
<i>Ophrys grammica</i>	Bk		46	15	547
<i>Ophrys hansreinhardii</i>	Bk		2	1	43
<i>Ophrys hebes</i>	Bk		13	2	168
<i>Ophrys helenae</i>	Bk		1	0	518
<i>Ophrys leucophthalma</i>	Bk		1	0	71
<i>Ophrys mammosa</i>	ME		268	39	2,600
<i>Ophrys oestrifera</i>	ME		171	26	2,108
<i>Ophrys reinhardiorum</i>	Bk		1	0	34
<i>Ophrys reinholdii</i>	EM		8	4	475
<i>Ophrys sicula</i>	ME		1	0	5,502
<i>Ophrys zeusii</i>	Bk		11	0	125
<i>Orchis italica</i>	Me		70	9	2,770
<i>Orchis mascula</i> subsp. <i>mascula</i>	EA		155	72	976
<i>Orchis militaris</i> subsp. <i>militaris</i>	EA	VU*	10	9	13
<i>Orchis pallens</i>	ME		25	17	233
<i>Orchis pauciflora</i>	Me		11	4	660
<i>Orchis provincialis</i>	ME		7	4	836
<i>Orchis purpurea</i> subsp. <i>purpurea</i>	EA		122	20	474
<i>Orchis quadripunctata</i>	Me		59	29	2,002
<i>Orchis simia</i> subsp. <i>simia</i>	EA		53	13	616
<i>Platanthera bifolia</i>	Pt		24	6	145
<i>Platanthera chlorantha</i> subsp. <i>chlorantha</i>	ES		326	80	1,100
<i>Pseudorchis albida</i>	ES	CR*	1	1	1
<i>Serapias bergonii</i>	EM		13	1	3,515
<i>Serapias cordigera</i> subsp. <i>cordigera</i>	Me		2	0	212
<i>Serapias parviflora</i>	Me		1	0	1,056
<i>Serapias vomeracea</i>	ME		69	4	1,419
<i>Spiranthes spiralis</i>	EA		134	6	1,260

CR: critically endangered; EN: endangered; VU: vulnerable; NT: near threatened

\* Evaluation according to Tsiftsis and Tsiropidis (2016); \*\* Evaluation based on recent distribution data and not by Tsiftsis and Tsiropidis (2016)

The categories with the smallest number of orchid taxa where the Balkan-Anatolian, Balkan-Central European, Arctic-Alpine and the Endemics, with one orchid taxon each. Similarly, in the total orchid flora of East Macedonia, the richest categories within the Natura 2000 network were the European-SW Asian taxa (25.42%), the Mediterranean taxa (15.25%) and the Mediterranean-European taxa (11.86%). On the contrary, 6 Balkan and 4 Mediterranean orchid taxa have not been recorded within the Natura 2000 network.

Out of the 37 Greek orchid taxa that have been classified in the threat categories (Critically Endangered, Endangered and Vulnerable) of the International Union for the Conservation of Nature (IUCN), nine occur in East Macedonia (Table 2), whereas 3 out of 11 orchid

taxa were classified as Near Threatened. One more taxon (*Epipactis helleborine* subsp. *distans*), is classified as Endangered (criterion D).

The three critically endangered taxa (*Gymnadenia odoratissima*, *G. rhellicani*, *Pseudorchis albida*) are either exclusively distributed in the Natura 2000 network, or have their highest populations there (Table 2). Among the endangered species, *Dactylorhiza incarnata* and *Epipactis helleborine* subsp. *distans* are mainly found in East Macedonia, whereas *Epipactis leptochila* subsp. *naousaensis* has some viable populations in the Natura 2000 network. From the vulnerable orchid taxa recorded in East Macedonia, two (*Epipactis leptochila* subsp. *neglecta* and *Dactylorhiza macedonica*) can be found elsewhere in Greece. Out of the areas where they have been recorded in East

Macedonia, about one-half is within the Natura 2000 network. The other two orchids (*Neottia cordata* and *Orchis militaris* subsp. *militaris*) are almost exclusively distributed in East Macedonia with their larger distribution being within the Natura 2000 network of the area.

Another species category, whose species could not be classified in any of the three threat categories of the IUCN, is the category of the Near Threatened species. East Macedonia hosts three orchid taxa of this category (*Anacamptis palustris* subsp. *elegans*, *Epipactis pontica* and *Gymnadenia frivaldii*), from which only *G. frivaldii* is found within the Natura 2000 network (Table 2).

## Discussion

As also stated in the introduction, the Natura 2000 network in Europe has been designed to ensure the long-term persistence of a large number of species (valuable and threatened) and habitats of European importance (European Commission 2020). Fourteen Special Areas of Conservation (SACs) of the Natura 2000 network have been established in East Macedonia (Table 1), aiming at the protection of the local flora and habitats (Dafis et al. 1996). Here I show that the Natura 2000 network fails to protect the total orchid flora in the area, because the distribution of 14 orchid taxa is not overlapping with any of the Special Areas of Conservation established in East Macedonia, similarly to Tsiftsis et al. (2009), whose results were based on fewer data, corresponding to a more restricted area. However, East Macedonia is not the only exception in this respect. Dimitrakopoulos et al. (2004), Tsiftsis et al. (2011) and Trigas et al. (2012) show that the spatial overlap of the Natura 2000 network with the important areas for the endemic species of the Peloponnese, the orchids of Crete and the plant biodiversity of Crete, respectively, was low in all cases.

Most orchid taxa that are not found within the Natura 2000 network in East Macedonia belong to the chorological category of the Balkan species, followed by the Mediterranean species. These Balkan species belong to the genus *Ophrys* and are mainly distributed in central and northwestern Greece (Antonopoulos and Tsiftsis 2017; Tsiftsis and Antonopoulos 2017), whereas Mediterranean species belong to the genera *Serapias* and *Neotinea*, with a wider distribution both in Greece and Europe (Delforge 2006; Kretzschmar et al. 2007). The most important part of the Natura 2000 network in East Macedonia exists in the high mountainous areas, characterized by cold climatic conditions and analogous to such climate vegetation. Orchid taxa preferring milder climatic conditions, such as several Balkan and Mediterranean species, cannot therefore be found in the high-altitude areas of East Macedonia. Species of these genera are disturbance-tolerant, widely distributed in open habitats at low or medium altitudes, where human activities are rather intense (Dafni 1987; Tsiftsis et al. 2019). Although

such areas are not included in the Natura 2000 network, strict protection of the habitats where these orchid taxa occur might cause a reduction to their populations as the result of the natural vegetation succession.

Contrary to the orchid taxa mentioned above, *Epipactis pontica* is a Mediterranean-European taxon, whose most populations, and among them the largest ones in size, are distributed in East Macedonia. However, the sites where it has been recorded so far are not overlapping with the Natura 2000 network. This species is categorized as Near Threatened according to the classification of the IUCN, with the possibility to become Vulnerable or even Threatened under improper management (Tsiftsis and Tsiripidis 2016).

*Anacamptis palustris* subsp. *elegans* is another taxon, whose distribution in East Macedonia is not overlapping with the Natura 2000 network. It was recorded in several sites all around Greece (70 grid cells; Table 2), mostly in low or medium altitudes. A number of these sites has been severely degraded and its total populations greatly reduced during the last two decades.

A great advantage of the Natura 2000 network in East Macedonia is that the Special Areas of Conservation established here strongly overlap with the distribution of the IUCN red listed orchid taxa. Such orchids usually reach their southernmost distribution limits in northern Greece (e.g. *Gymnadenia odoratissima*, *G. rhellicani*, *Neottia cordata*, *Pseudorchis albida*) and their distribution is mainly driven by climate. Thus, these orchid taxa are sensitive to increasing temperatures and might be influenced by climate change (Kolanowska and Jakubska-Busse 2020).

Except of the high overlap between the distribution of the IUCN red listed orchids and the Natura 2000 network in East Macedonia, the higher number of orchid taxa per grid cell in the network compared to the grid cells outside it, is another advantage. The natural conditions of the Natura 2000 network create suitable circumstances for the existence of most orchid taxa. This confirms the design and the establishment of the Special Areas of Conservation in East Macedonia as this network offer, under suitable management actions, multiple possibilities for the future survival of such an important group of plant species.

## Conclusions

Natura 2000 network established in East Macedonia hosts a significant number of orchids (59 out of the 73 orchid taxa). Among them, the distribution of almost all orchids of central and northern European origin (e.g. *Coeloglossum viride*, *Dactylorhiza incarnata*, *Goodyera repens*, *Gymnadenia rhellicani*, *Neottia cordata*, *Orchis militaris* subsp. *militaris*) and the distribution of those that have been classified in the threat categories of the IUCN is highly overlapped with the Special

Areas of Conservation in East Macedonia. Although this demonstrates the significance of the Natura 2000 network in conserving the orchid taxa in East Macedonia, I have used only a small area of Greece. A similar study should be conducted for the whole of Greece.

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# CAUSES OF THE DIEBACK OF LITTORAL STANDS IN AN OVERPOPULATED WATER BIRD RESERVE: ROLE OF EUTROPHICATION, FISH AND GEESE

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## ABSTRACT

European fishponds can serve as refuges for water birds if the fish stocks are limited, but the effects of other ecological factors on their ecological stability are rarely considered. The aim of this study is to determine the causes of marked dieback of littoral stands dominated by *Typha angustifolia* L. in a hypertrophic fishpond that is also a valuable water bird reserve. A field study and two experiments were conducted in order to separate the effects of mineral nutrient availability, redox conditions, fish and water birds. The physico-chemical characteristics of the water and sediments confirmed hypertrophic conditions in the fishpond, but a mesocosm experiment did not indicate it had a negative effect on plant growth. On the other hand, a field enclosure experiment showed that in sparse stands, unfenced parts had a significantly smaller shoot density than fenced parts. This was attributed to grazing by greylag geese (*Anser anser* L.). In addition, damage to belowground parts of plants were ascribed to large individuals of albeit a few large common carp (*Cyprinus carpio* L.). This study highlights a conservation dilemma as large numbers of geese destroy littoral stands in fishpond nature reserves, which then become unsuitable nesting sites for other species of water birds.

**Keywords:** carp; fishpond; grazing damage; nature reserve; sediment; *Typha angustifolia*

## Introduction

European fishponds are important refuges of aquatic and wetland organisms in intensively used agricultural landscapes (IUCN 1997). They are man-made shallow water bodies of various sizes, with an area ranging from several hundred square meters to more than 1 km<sup>2</sup>. Many fishponds have well developed littoral plants, which provide breeding sites for water birds, including rare species such as the great crested grebe (*Podiceps cristatus* L.), grey heron (*Ardea cinerea* L.), greater white-fronted goose (*Anser albifrons* Scopoli) and great bittern *Botaurus stellaris* L. (e.g. Švařas and Stanevičius 1998; Janda and Ševčík 2002; Polak 2007; Gergely et al. 2009; Nieoczym 2010; Flis and Gwiazda 2018). Large fishponds, which have an area greater than 1 km<sup>2</sup>, also serve water birds as staging areas during migration and wintering grounds (Miklín and Macháček 2016). European fishponds are important for maintaining the biodiversity of water birds and are nature reserves, which are protected areas according to the EU Directive No. 2009/147/EC (Birds Directive) or Wetlands of International Importance according to the Ramsar Convention (Bird Life International 2001).

Although European fishponds resemble natural shallow lakes in many aspects, their ecology is largely determined by the rearing of fish, mainly cyprinids (Kestemont 1995; Pechar 2000; Schlumberger and Girard 2013). Fish production is promoted by manuring, liming and feeding the fish, which in turn increase nutrient availability in the

water column and promote the accumulation of nutrient rich organic sediments at the bottom (Baxa et al. 2019). Large stocks of fish control the food chains in the water and at the bottom by feeding on large zooplankton and benthos. This results in steep vertical gradients in oxygen content, pH, chlorophyll content and light penetration in summer (Bíró 1995; Potužák et al. 2007; Weber and Brown 2009).

In addition to open water, dense littoral stands of plants, such as the common reed (*Phragmites australis* [Trin. Exd Steud.]) and cattails (*Typha* spp.), provide habitats for many species of water birds, especially ducks. They use them as shelter, source of material for building nests and also feed on these plants and/or the large populations of invertebrates and fish fry that inhabit the littoral stands. On the other hand, some species can destroy these stands if they are abundant. This is documented both for birds (Bakker et al. 2018) and fish (Crivelli 1983). In addition, the size and stability of the littoral stands can be reduced by many other factors such as floods, high water levels (Ostendorp 1989), eutrophication (van der Putten 1997; Čížková et al. 1999), toxic substances in the sediment (Armstrong et al. 1996; Armstrong and Armstrong 2001) and, finally, mechanical damage caused by human activities.

All of the injurious effects listed above can occur in fishponds and many of them are closely related to fishpond management (Hejný et al. 2002; Francová et al. 2019a). In spite of the importance of both open water

and littoral stands of plants for water birds, most ecosystem studies focus on only one of these two components. There are several studies assessing the relationship between fishpond management and biodiversity (Broyer and Calenge 2010; Broyer and Curtet 2012; Francová et al. 2019b), but they do not specifically deal with the stability of the littoral stands of plants. In addition, studies on the factors affecting the dynamics of littoral stands of plants are almost exclusively on *Phragmites australis* (e.g. Ostendorp 1989; van der Putten 1997; Armstrong and Armstrong 2001), while knowledge on that of other common species, including *Typha* spp., is scarce.

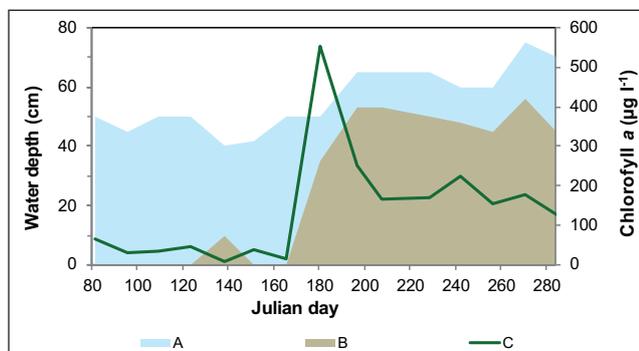
In order to resolve this, an ecosystem study was undertaken of a hypertrophic fishpond and valuable water bird reserve, in which the littoral stands of mainly *Typha angustifolia* L. markedly declined both in area and shoot density between 2004 and 2013. This study started in 2013 and ended in 2016, and had the following specific aims:

1. to document the extent of the decline in the area of the littoral stand of plants that occurred between 2004 and 2013 and determine the condition of the remaining stands in terms of their horizontal pattern and plant morphology;
2. to assess the physico-chemical characteristic of the water and sediments as possible determinants of the condition of the different *T. angustifolia* stands: dense (compact), sparse (declining) and absent;
3. to assess the potential toxicity of the fishpond sediment on plant growth and root morphology in a mesocosm;
4. to determine whether particular animals are destroying the littoral stands of plants using enclosures.

## Materials and Methods

### Site description

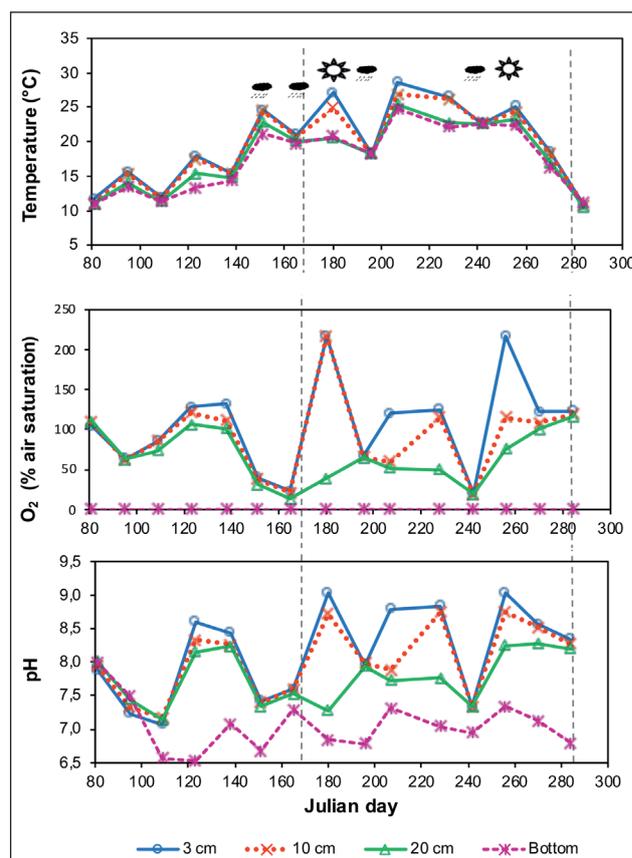
The ecosystem studied was the Bažina fishpond (49.0092322 N, 14.4393331 E), which is part of a valuable water bird reserve, Vrbenské rybníky (Vrbenské fishponds), near the city of České Budějovice in the Czech



**Fig. 1** Seasonal changes in water transparency and chlorophyll *a* content at different depths. The dashed vertical lines separate spring from summer (Julian day 173) and summer from autumn (Julian dan 267), respectively. A – photoc zone; B – dark zone; C – chlorophyll *a* content.

Republic. The Vrbenské rybníky is a system of shallow water bodies (mean depth 1–2 m) used for rearing fish, mainly the common carp (*Cyprinus carpio* L.), which makes up about 90% of the fish community. Because of its importance as a bird habitat, the area has been a nature reserve since 1990, a special area of conservation according to Directive 92/43/EEC since 2005 and a part of an Important area for Birds, Českobudějovické rybníky, according to EU Directive 2009/147/ES since 2009. The Bažina fishpond (6.13 ha) is of special conservation value because various species of duck, e.g. great crested grebe (*Podiceps cristatus* L.) and red-necked grebe (*Podiceps grisegena* Boddaert), nest in its littoral plant stands (Albrecht 2003). The fishpond is also an important gathering site for greylag geese (*Anser anser* L.) prior to their winter migration.

Bažina fishpond is hypertrophic and the water is turbid and there are no submerged macrophytes. From 2013 to 2016 the water in spring was clear and the photic zone extended down to the bottom and oxygen content was near 100% saturation in the whole water column. When the mean temperature of the water column increased above 20 °C in late spring (usually at the end of May), a marked vertical stratification developed (Fig. 1).



**Fig. 2** Seasonal changes in the physico-chemical features of the water column. Vertical dashed lines separate spring from summer (Julian day 173) and summer from autumn (Julian day 267). The legend indicates the depths at which measurements were recorded. Values for 30 cm are very similar to those for 20 cm so only the latter is shown. Symbols of a cloud and a sun represent rainy and sunny weather, respectively, on the days sampled.

The photic zone decreased to less than 0.2 m (Fig. 2). This was associated with a strong peak in chlorophyll *a* content, reaching a seasonal maximum of over 500  $\mu\text{g l}^{-1}$ , an extremely high value even for hypertrophic fishponds in the same geographic region (cf. Šimek et al. 2019). The vertical stratification was most pronounced in sunny weather, when the oxygen content in the water column was more than 200% air saturation near the surface and hypoxic below a depth of 20 cm (Fig. 2).

The extent of the hypoxic zone corresponded with the depth of loose sediment. On rainy days, the water column was hypoxic up to the water surface due to the mixing action of rain (Fig. 3). The bottom was always anoxic. The pattern in pH values was similar to that of oxygen content. Near the water surface, it fluctuated between 9.0 on sunny days and 7.3 on rainy days, whereas at the bottom it ranged from 7.3 to 6.5. In autumn the oxygen content and pH levels were similar to those recorded in spring, but water transparency remained low (Fig. 1).

The fishpond was used mainly for rearing carp fry or one-year-old fingerlings. In the years preceding this study (i.e. from 2004 to 2011) and also in 2015 and 2016, it was stocked every spring with a total biomass of up to 50  $\text{kg ha}^{-1}$ , which is ten times less than the usual stock in production fishponds. The fish was harvested every year during a draw-down in October or November. The draw-down lasted for about one week. In 2012–2014, the fishpond was not stocked. This was because in autumn

2011, some adult carp (about 2 kg) arrived in Bažina from an upstream fishpond. Their total weight in spring 2012 was estimated to be less than 50  $\text{kg ha}^{-1}$ , which corresponds to the biomass of fish stocked in other years. These carp were successively harvested in the autumns of 2012–2014. No supplementary food was added for the fish and the fishpond was not limed at least from 2002, (i.e. before the die-back of littoral stands was first noticed). The mean seasonal height of the water column was about 0.5 m with the summer minimum of 0.4 m occurring usually in July.

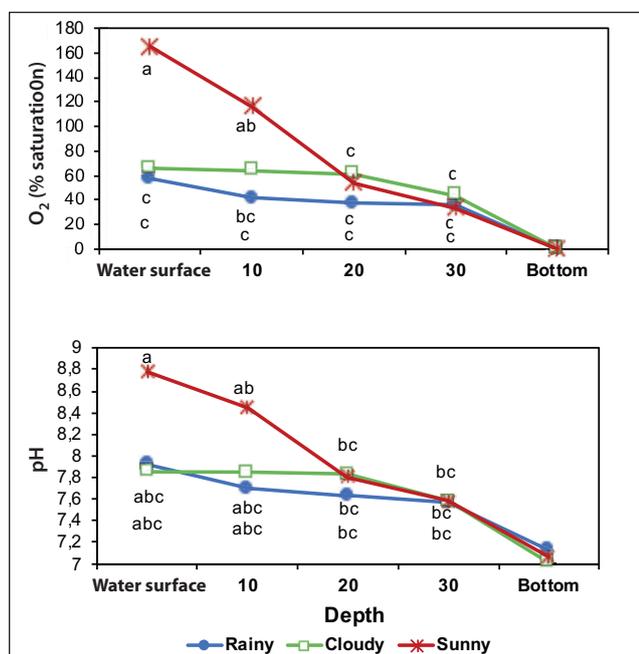
### Assessment of littoral vegetation

A vegetation map was made in 2013 in order to document the past and current area of the littoral stands. Background information included a local topographic map and orthophotographs of the area from 2004 (i.e. before the die-back of the littoral stands was first observed in 2013). In August 2013 borders of vegetation units were recorded in the field using an Ashtech GPS Magellan mobile mapper 10. Five vegetation units were defined: (1) *Phragmites australis* dominated stands, (2) sparse (cover  $\leq 35\%$ ) and (3) dense ( $>35\%$ ) stands dominated by *Typha angustifolia*, and (4) sparse (cover  $\leq 35\%$ ) and (5) dense ( $>35\%$ ) dominated by *T. latifolia*. After the fishpond was drained in October 2013 (in order to harvest the fish), remnants of the dead stands were identified on the fishpond bottom and also recorded. The data were further processed using ArcGIS ESRI 10 in order to create the map and assess the area of particular vegetation units.

A preliminary investigation of plant condition was carried out in August 2013. It focused on signs of damage to plants (uprooting of clumps, the intensity of grazing damage and the proportion of living and dead shoots or leaves in clumps). Three 0.5 m diameter clumps that were only loosely connected to the bottom were dug up in order to determine the incidence of dead roots and rhizomes. In addition, the presence of horizontal rhizomes, ensuring vegetative expansion, was recorded in a 0.1 m thick surface layer of the sediment in close vicinity of ten shoots growing in sparse stands. Subsequently, a photographic documentation of the current condition of the littoral stands was taken at two-week intervals throughout the study.

### Survey of water and sediment characteristics

Basic physico-chemical parameters were investigated in 2013 in areas formerly or currently overgrown with *T. angustifolia* L.; we distinguished three types of biotope (1) dense (compact) stands of *T. angustifolia*, (2) sparse stands of *T. angustifolia* and (3) open water in the area where *T. angustifolia* was formerly present (dead stands). In all three biotopes, the vertical profile consisted of three layers: (1) aquatic photic zone consisting of clear water determined using a Secchi disk, (2) dark zone of sludge consisting of water and fine loose sediment and (3) solid bottom sediment. In order to keep the vertical stratifica-



**Fig. 3** Changes in oxygen content and pH with depth in Bažina fishpond when the mean water temperatures were  $\geq 20^\circ\text{C}$ . Different lines and symbols indicate rainy, cloudy and sunny weather, respectively. Different small letters denote significant differences for a particular type of weather, based on repeated measures ANOVA followed by post-hoc Tukey tests. Bottom values were not included in the analysis because of extremely low variance. For original data see Fig. 2.

tion intact, all *in situ* measurements and sampling were performed from a boat.

Two sampling sites were established in each type of biotope and investigated at the time of the seasonal maximum aboveground biomass (13 August 2013). Prior to the sampling, the depths of the photic and dark zones were measured. Water conductivity and pH were recorded in the middle of the photic zone using a portable meter WTW Multi 3430 set D. Water samples were taken from the photic zone using a 0.4 m long plexiglass tube of 0.05 m internal diameter. Two replicate samples of water were collected at each site. Loose sediment was sampled within the dark zone in the same manner. The volume of these samples was approximately 1 litre. The solid bottom sediment was sampled using a PVC tube with an 0.1 m internal diameter. The samples were stored in a refrigerator overnight and analysed as described below. There were three replicates of each sediment sample.

Alkalinity of the water was determined by sample titration with hydrochloric acid to pH 4.5. The content of chlorophyll *a* was estimated immediately after sampling with a fluorometer Aquafluor 8000-001 (Turner Designs, Canada) using a standard procedure. The concentrations of total N, total P, K, Ca and Mg were determined for samples of fishpond water and water in the loose sediment, which was obtained by centrifugation of the loose sediment followed by filtration of the supernatant. Total nitrogen concentration was determined using a modified Kjeldahl method (ISO 11261) by mineralization to ammonium ion. Total phosphorus concentration was estimated according to Kopáček and Hejzlar (1995) as phosphate ion after mineralization in perchloric acid. Ammonium and phosphate ions were estimated calorimetrically in a flow-through Flow Injection Analyzer Lachat QC 8500 (Lachat Instruments, USA) using standard protocols. The concentrations of cations (Ca, Mg, K) were determined by ion chromatography using 881 Compact IC Pro-Cation (Metrohm AG, Switzerland).

Air-dried samples of the fine loose sediment and the bottom sediment, respectively, were used for analysis of main mineral nutrients. The concentrations of cations (Ca, Mg, and K) were determined in soil solution obtained using the Mehlich II extraction method (Zbiral 1995; Zbiral and Němec 2002) and atomic absorption spectroscopy (spectrometer contraAA, Analytik Jena AG, DE). Total phosphorus and nitrogen were estimated after mineralization of ground samples using the same method as in the case of water samples. The content of organic matter in the sediments was based on the loss on ignition at 550 °C in a muffle furnace for 4 hours. The results were expressed per unit dry mass obtained by drying partial samples of sediment at 105 °C.

Redox potential (Eh) was investigated *in situ* in 2013 at the same sites where water and sediments were sampled for chemical analyses. We used two approaches: a preliminary survey and a quantitative estimate. The prelim-

inary survey was based on the colour change from reddish to dark grey associated with the reduction of ferric to ferrous iron ( $\text{Fe}^{\text{III}} \rightarrow \text{Fe}^{\text{II}}$ ) (Mitsch and Gosselink 2015). In August 2013, iron sticks with an oxidized reddish layer on their surface were placed at the same sites used for sampling sediment. Altogether, 18 sticks of 0.5 m in length were inserted to a 10 cm depth in the solid bottom sediment, their upper parts extended through the layer of the fine loose sediment into clear water. In November 2013, the sticks were checked for the presence of the grey colour. The quantitative measurements of the redox potential were taken at the same sites on 13 August 2013. Eh was estimated as the electric potential between a Pt electrode installed at the surface of the solid sediment and an Ag/AgCl (3M KCl) reference electrode immersed in the water. The reference electrode had a potential of 210 mV against the standard hydrogen electrode (Friis et al. 1998).

### Mesocosm

In 2014, a mesocosm was set up in order to determine whether anaerobic fishpond sediment affects the growth of the roots of *Typha angustifolia* and/or causes injury to them. There were two treatments, which differed in the type of the rooting substrate: (1) anaerobic sediment taken from the bottom of Bažina fishpond and (2) washed river sand. The latter was used as a control substrate in which there was negligible microbial activity. The sand was enriched with a slow-dissolving fertilizer Osmocote in order to compensate for the lack of mineral nutrients necessary for plant growth.

The plants were propagated from seeds collected at the Bažina fishpond. Twenty-four plants of similar size were selected for the experiment. They had one shoot with 3–4 leaves with a total length of  $1.94 \pm 0.40$  m (mean  $\pm$  standard deviation,  $n = 24$ ). The initial biomass was determined for five additional plants, which were separated into shoots, rhizomes and roots and their dry weight recorded after drying at 85 °C to constant weight. The resulting dry mass of shoots, rhizomes and roots were  $15.1 \pm 8.4$ ,  $9.1 \pm 5.1$  g and  $2.8 \pm 1.9$  g, respectively (mean  $\pm$  standard deviation,  $n = 5$ ).

Each of the 24 plants selected was planted in a 32-liter basket-type container. Twelve baskets were filled with the fishpond sediment, which contained 1584 mg total N, 238 mg total P, 67 mg K and 139 mg Mg per 1 litre. The other twelve baskets were filled with the washed river sand supplemented with 6 g Osmocote per litre, which contained 900 mg total N, 600 mg total P, 720 mg K and 1200 mg Mg. An iron stick was inserted in each basket as an indication of the redox potential (in the same manner as in the survey of the fishpond sediment characteristics, see above). Baskets with the same type of substrate were randomly distributed between two fibre-glass tubs and all filled with substrate to the same height. In summary, there were two types of substrate, each placed in two tubs and a total of four tubs. Each tub contained six plants

with a total of 24 plants in the four tubes. The tubs were kept outside and water was added to the tubs to keep the water level at about 0.1 m above the surface of the substrate. Algal growth was prevented by a 0.05 m thick layer of perlite on the water surface.

The experiment lasted from 30 July to 20 October 2014. At the end of the experiment, the plants were harvested and the following characteristics were determined: number of shoots, number and length of live and dead leaves, respectively, and also the biomass of shoots, rhizomes and roots after drying to constant weight at 85 °C.

### Enclosures

In 2015, enclosures were established in stands of littoral plants in order to assess the effect of mechanical damage by waterfowl and fish on shoot density. Four pairs of transects were established in the littoral area perpendicular to the shoreline. They were 1.2 m wide and 13–22 m long, depending on the width of the littoral zone. There were two transects in both the sparse and dense stands of *T. angustifolia*, of which one was fenced and the other was left open. The fence was made of 1 m wide, green LLDPE-coated diamond-mesh netting with a 0.02 m mesh size, which was attached to wooden poles hammered to a depth of 0.5 m in the solid bottom sediment. It extended from the bottom sediment to a height of 0.5–0.6 m above the water level. The experiment lasted from March 1 (i.e., before the start of the growing season) to July 31, 2015 (after the aboveground biomass achieved its seasonal maximum). At the end of the experiment, the transects were divided into 1-m long segments, in which the number of green shoots were counted.

### Density and activity of geese

The counts of the greylag geese on the fishpond in the years 2002–2016 were obtained from the Nature Conservation Agency of the Czech Republic. The bird censuses were made from the ground using a standard approach every month. For the purpose of this study, the data were separated according to the annual cycle in the activity of the geese, i.e. spring migration (February–March), breeding (April–July) and autumn migration (August–November). Monthly mean counts and monthly maximum counts were calculated for each season within each year.

As the geese were often observed in the sparse stand of *T. angustifolia*, an infrared camera was installed there from August to November 2013 in order to record whether they were feeding on the plants. We used a SG-007 (ScoutGuard, USA) set to take 9 photographs in 30 s following activation of the motion sensor.

### Statistical analyses

In order to determine the differences between the biotopes in terms of the chemical characteristics of the water and sediments, the following statistical hypothesis was set:

(H0) There are no differences between the biotopes in terms of the chemical characteristics of the water and sediments.

The data were assessed using one-way ANOVA followed with a Tukey post-hoc test. The data were tested for normality using a normal probability plot and for homogeneity of variance using the Levene test.

With regard to the potential harmful effect of the sediment on the growth characteristics of *T. angustifolia* in the mesocosm, the hypothesis tested was:

(H0) The sediment does not have a harmful effect on the plant growth characteristics. The effect was tested using hierarchical ANOVA, where the factor “substrate” was set superior to the “tub” factor.

In order to determine whether fencing protects *T. angustifolia* plants from damage in the enclosure experiment, the hypothesis tested was:

(H0) The shoot density is the same in the fenced transects as in the control (unfenced) transects, within each type of stand (dense or sparse).

The data were then evaluated using Chi-square tests.

The inter-annual trend in geese counts was tested using linear regression, applied to monthly means in a particular period of the geese annual cycle (i.e. spring migration, breeding and autumnal migration). the hypothesis tested was:

(H0) There was no inter-annual trend in the monthly mean counts. The same procedure was applied to monthly maxima.

All the statistical analyses were done using Statistica 12 (Statsoft, Inc., USA).

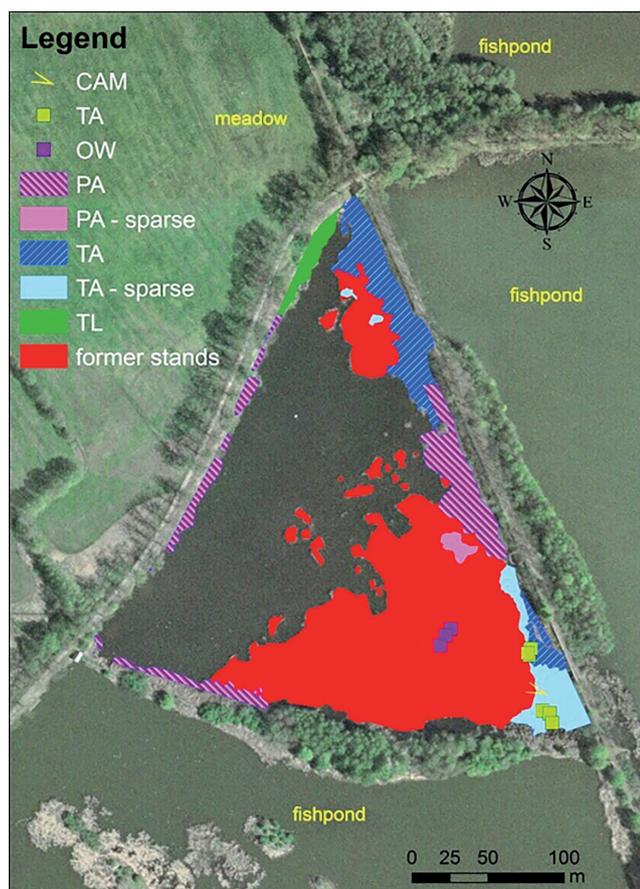
## Results

### Extent and condition of the littoral vegetation

The dominant plants in the littoral vegetation were *Typha angustifolia* (growing in depths of water of 0.3–0.4 m, the minimum water level in summer), *T. latifolia* (in depths 0.1–0.2 m) and *Phragmites australis* (forming a narrow belt along the dams) (Fig. 4). Other species of plants were scarce and made up less than 5% of the stands. The area covered by helophytes suitable for birds to nest in was 28,721 m<sup>2</sup> and 9,457 m<sup>2</sup> in 2004 and 2013, respectively, which corresponds to 48 and 16% of the total area of the fishpond and indicate that the area covered by helophytes decreased by 2/3rds in ten years. This decline was most pronounced in the south-eastern part of fishpond, where dense stands of *T. angustifolia* originally covered much of the area.

This marked reduction was associated with the apparent die-back of the current stands of *T. angustifolia*. The shoot densities frequently decreased towards the waterward edge of the stands. At some sites, the whole stands were sparse, formed by isolated islets several meters apart. The smallest islets consisted of individual clumps with about 10–20 shoots, of which several were flowering. Shoots at the perimeters of clumps were often damaged by grazing (Fig. 5a) and there were no horizontal rhizomes growing from these clumps within a 10 cm upper layer of the solid sediment or apical rhizome buds protruding from the bottom of the fishpond near the clumps. Some clumps were uprooted (Fig. 5b). They occurred along the water edge of the littoral belt and throughout the stands in the south-eastern part of the fishpond. Although the clumps had green shoots, their belowground parts were severely damaged (Fig. 5c). In the south-eastern part of the fishpond, the belowground systems of the isolated clumps formed compact blocks about 0.5 m in diameter and 0.6 to 0.8 m in height. They had very weak or no connection with the bottom. Much of their surface was exposed to water and loose sediment.

No living rhizomes extended from them. Roots extending from the blocks were scarce, short and thick and often had necrotic tips. The blocks smelt of organic acids.



**Fig. 4** Vegetation map of littoral stands of plants in 2013 plotted on the orthophoto map from 2004. CAM – camera, TA – stands of *Typha angustifolia*, TL – stands of *T. latifolia*, OW – open water. Squares indicate positions where water and sediments were sampled. Former stands disappeared between 2004 and 2013.

### Physico-chemical conditions in the littoral biotopes

The nutrient contents in the photic zone (clear water column) were similar in the three littoral biotopes (dense, sparse and dead littoral stands of *T. angustifolia*, respectively) (Table 1).

**Table 1** Nutrients in the photic zone, water and dry matter in the loose sediment and dry matter in the solid sediment in August 2013. TN – total nitrogen, TP – total phosphorus, LOI – organic matter content based on loss on ignition. The numbers are means for the water in the photic zone ( $n = 2$ ) and mean  $\pm$  standard error for the sediments ( $n = 3$ ). Significant differences within a row are given in bold and indicated by different small letters.

Parameter	Biotope		
	Dense stand	Sparse stand	Open water
Water in photic zone			
TN ( $\text{mg l}^{-1}$ )	2.29	2.29	2.18
TP ( $\mu\text{g l}^{-1}$ )	278	293	323
K ( $\text{mg l}^{-1}$ )	7.4	7.5	7.4
Ca ( $\text{mg l}^{-1}$ )	33.7	35.2	34.9
Mg ( $\text{mg l}^{-1}$ )	6.3	6.6	6.5
Water in loose sediment			
TN ( $\text{mg l}^{-1}$ )	<b>5.38 <math>\pm</math> 0.50<sup>a</sup></b>	<b>9.25 <math>\pm</math> 1.46<sup>b</sup></b>	<b>3.41 <math>\pm</math> 0.21<sup>a</sup></b>
TP ( $\mu\text{g l}^{-1}$ )	<b>274 <math>\pm</math> 98<sup>a</sup></b>	<b>900 <math>\pm</math> 275<sup>b</sup></b>	<b>143 <math>\pm</math> 29<sup>a</sup></b>
K ( $\text{mg l}^{-1}$ )	7.6 $\pm$ 0.1	9.4 $\pm$ 0.8	7.7 $\pm$ 0.4
Ca ( $\text{mg l}^{-1}$ )	35.5 $\pm$ 2.0	42.3 $\pm$ 2.5	37.8 $\pm$ 1.4
Mg ( $\text{mg l}^{-1}$ )	7.0 $\pm$ 0.4	8.5 $\pm$ 0.5	7.1 $\pm$ 0.4
Fe ( $\text{mg l}^{-1}$ )	<b>0.44 <math>\pm</math> 0.06<sup>a</sup></b>	<b>0.19 <math>\pm</math> 0.01<sup>b</sup></b>	<b>0.26 <math>\pm</math> 0.04<sup>b</sup></b>
Dry matter in loose sediment			
TN ( $\text{mg kg}^{-1}$ )	13,691 $\pm$ 2,800	18,400 $\pm$ 1,713	17,851 $\pm$ 953
TP ( $\text{mg kg}^{-1}$ )	<b>858 <math>\pm</math> 67<sup>a</sup></b>	<b>1,381 <math>\pm</math> 152<sup>b</sup></b>	<b>1,228 <math>\pm</math> 114<sup>ab</sup></b>
K ( $\text{mg kg}^{-1}$ )	351 $\pm$ 47	420 $\pm$ 36	456 $\pm$ 70
Ca ( $\text{mg kg}^{-1}$ )	<b>4,559 <math>\pm</math> 594<sup>a</sup></b>	<b>6,902 <math>\pm</math> 707<sup>ab</sup></b>	<b>7,503 <math>\pm</math> 301<sup>b</sup></b>
Mg ( $\text{mg kg}^{-1}$ )	509 $\pm$ 95	746 $\pm$ 82	904 $\pm$ 113
LOI (%)	<b>40.4 <math>\pm</math> 2.8<sup>ab</sup></b>	<b>30.3 <math>\pm</math> 2.1<sup>a</sup></b>	<b>48.5 <math>\pm</math> 3.6<sup>b</sup></b>
Dry matter in solid sediment			
TN ( $\text{mg kg}^{-1}$ )	1,705 $\pm$ 3	3,055 $\pm$ 1,228	2,470 $\pm$ 762
TP ( $\text{mg kg}^{-1}$ )	257 $\pm$ 20	412 $\pm$ 167	187 $\pm$ 12
K ( $\text{mg kg}^{-1}$ )	96 $\pm$ 6	175 $\pm$ 61	87 $\pm$ 23
Ca ( $\text{mg kg}^{-1}$ )	1,609 $\pm$ 202	3,025 $\pm$ 1,494	3,212 $\pm$ 918
Mg ( $\text{mg kg}^{-1}$ )	217 $\pm$ 13	330 $\pm$ 95	259 $\pm$ 56
LOI (%)	12.2 $\pm$ 2.0	17.7 $\pm$ 6.0	6.0 $\pm$ 1.3

Some differences between biotopes in nutrient content were recorded in the loose sediment. Its thickness was 0.30–0.40 m in both the dense and sparse stands (Table 2). It filled the space between the clumps, thus surrounds the plant root and rhizomes. The water in the loose sediment had higher contents of both total nitrogen and total phosphorus in sparse than in dense and dead stands (Table 1). In addition, the loose sediment in dense stands had a significantly lower dry matter content, and lower contents of total P and Ca than the sparse and dead stands. It had also lower contents of other nutrients (total nitrogen, K, Mg), but these differences were not

significant (Table 1). We found no significant differences between biotopes in the nutrient contents in the dry matter of the solid sediment.

**Table 2** Sediment characteristics of *T. angustifolia* biotopes. The redox potential was measured at the interface between the loose and the solid sediments. The numbers denote medians (minimum; maximum).

Biotope	Depth of loose sediment (cm)	Redox potential (mV)
Dense stand	35 (34; 38)	30 (-87; 332)
Sparse stand	36 (30; 37)	-13 (-140; 10)
Dead stand	21 (14; 26)	-155 (-195; -52)

The numeric values of redox potential of the solid sediment varied within each biotope and mostly overlapped. Most values ranged from -100 to +100 mV (Table 2). The long-term assessment of redox potential using the iron sticks revealed predominantly reducing conditions in both the loose and solid sediments in all littoral biotopes. This was indicated by the colour of the iron sticks, which was dark grey (indicating prevalence of reduced forms of iron) on the parts inserted into both the solid and the loose sediments, and was reddish (indicating the prevalence of oxidised iron forms) only on the part in the photic zone (Fig. 5e).



**Fig. 5** Photographs of the condition of *T. angustifolia* biotopes; a – shoots damaged by grazing in a sparse stand; b – uprooted clumps in a sparse stand; c – broken rhizome apex of an uprooted plant; d – greylag geese grazing in a sparse stand (night photograph taken by camera); e – a green fibre-glass stick labelling a site and an iron stick indicating the redox conditions in water and sediments. The arrows indicate the interface between the oxidised and reduced zones on the sticks.

**Table 3** Growth characteristics of *T. angustifolia* plants grown in sand and fishpond sediment in a mesocosm. DW – biomass dry weight. Belowground biomass comprises the biomass of rhizomes and roots. Means  $\pm$  standard deviations, n = 12. Statistically significant differences between the substrates are given in bold.

Growth characteristic	Substrate		F	p
	Sand	Sediment		
Total length of leaves (cm)	<b>1374 <math>\pm</math> 551</b>	<b>2297 <math>\pm</math> 613</b>	<b>19.63</b>	<b>&lt;0.001</b>
Final shoot DW (g)	<b>34.82 <math>\pm</math> 7.40</b>	<b>52.62 <math>\pm</math> 15.08</b>	<b>20.68</b>	<b>&lt;0.001</b>
Final rhizome DW (g)	43.82 $\pm$ 14.91	49.82 $\pm$ 17.50	0.79	0.384
Final root DW (g)	15.09 $\pm$ 3.57	13.55 $\pm$ 3.76	0.97	0.336
Belowground / shoot ratio	<b>1.74 <math>\pm</math> 0.50</b>	<b>1.29 <math>\pm</math> 0.59</b>	<b>4.75</b>	<b>0.041</b>
Rhizome / shoot ratio	3.58 $\pm$ 1.31	3.13 $\pm$ 1.14	2.70	0.115
Root / shoot ratio	0.39 $\pm$ 0.13	0.31 $\pm$ 0.10	<b>29.92</b>	<b>&lt;0.001</b>

### Mesocosm

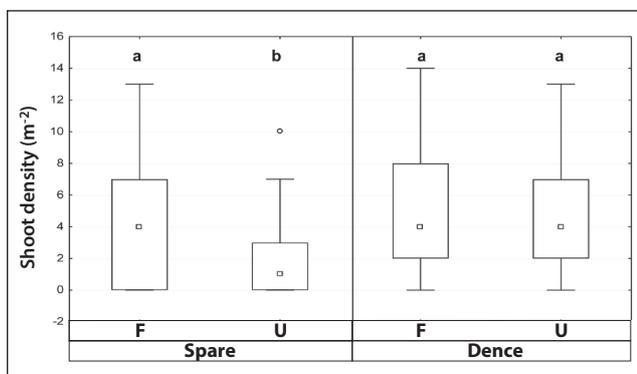
The fishpond sediment differed from the sand in re-dox conditions as indicated by the colour of the iron sticks, which was reddish in the sand and dark grey in the sediment.

The type of substrate (fishpond sediment or sand enriched with mineral nutrients) had pronounced effects on plant growth. The aboveground parts of the plants grew generally better in the fishpond sediment. The plants had a significantly greater length of newly produced live leaves, and significantly greater shoot biomass at the end of the experiment than the plants cultivated in sand (Table 3).

No significant effects were recorded for either root or rhizome biomass or the ratio of rhizome: shoot biomass. Nevertheless, the ratios of belowground/shoot biomass and root/shoot biomass were significantly smaller in the fishpond sediment (Table 3), indicating a shift in biomass allocation from roots to shoots. No dead root tips or other symptoms of root injury were observed in either substrate.

### Enclosures

In the fenced enclosures the shoot densities were significantly greater than in unfenced (control) areas in



**Fig. 6** Total number of shoots in fenced (F) and unfenced (U) areas in sparse and dense stands of *T. angustifolia*. The box plot shows medians (squares), quartiles (boxes), maximum and minimum values (whiskers) and an outlier (circle).

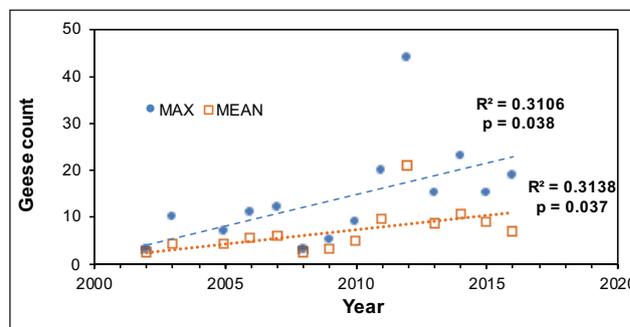
sparse stands ( $\chi^2 = 109$ , d.f. = 1,  $p < 0.001$ , Fig. 6). In dense stands, the shoot densities were similar in fenced and unfenced stands. They were also similar to those in fenced areas in sparse stands. In addition, grazing damage on shoots was recorded in the unfenced areas in sparse stands, but not dense stands.

### Geese and fish activity

In 2013 damage to plants was frequently recorded, especially in sparse stands of *T. angustifolia*. As documented by photographs, this damage was done by geese (Fig. 5d).

Apart from one exception, the geese were recorded at the fishpond only when they were breeding. Their numbers increased considerably over the period of this study. While the seasonal maximum counts were 2–12 in 2002–2010, 44 were recorded in 2012, which corresponds to more than seven individuals per hectare. The trends in the numbers of geese from 2001 to 2016 were highly significant in terms of both the monthly means and maximum counts during the breeding season (Fig. 7).

In addition to geese grazing on shoots, adult carp (about 2 kg) were repeatedly observed shaking small isolated clumps of plants (up to 0.25 m<sup>2</sup>) in the sparse stand of *T. angustifolia* in 2012–2014.



**Fig. 7** Trends in numbers of greylag geese (*Anser anser*) on Bažina fishpond during the breeding period (March to July) from 2002 to 2016. MAX – monthly maximum numbers, MEAN – monthly mean numbers. Dotted lines indicate linear regressions. The data were provided by the Nature Conservation Agency of the Czech Republic.

## Discussion

### Role of abiotic factors

The physico-chemical characteristics of water quality confirmed the extremely high trophic status of the fishpond (cf. Pechar 2000; Šimek et al. 2019). The same was true for both the water and dry matter of the loose sediment (cf. Čížková et al. 2001). The low redox potentials, recorded in the solid bottom sediment are also reported in other similar hypertrophic or eutrophic fishponds in the region (Čížková et al. 2001).

On the other hand, the dark layer containing the loose sediment, which formed in late spring as the temperature increased above 20 °C (Fig. 1), is to the best of our knowledge not previously reported occurring in fishponds in central Europe. Although there is some oxygen in this layer, possibly a result of water mixing, the conditions are predominantly anaerobic based on iron stick measurements (Fig. 5e).

Of the environmental components investigated (i.e. fishpond water, water and dry matter in loose sediment and dry matter in the solid sediment) water and dry matter in the loose sediments differed in the biotypes. Higher contents of total nitrogen and total phosphorus were recorded in sparse, rather than dense stand (Table 1), which may indicate a high degree of eutrophication. Nevertheless, it is questionable whether these differences affect the plants because nutrient values were extremely high in all the biotopes. In addition, it is unlikely that high nutrient levels would have a deleterious effect *per se*. As has been documented for *Phragmites australis*, the decline of its stands at eutrophic sites is not due to nutrient levels but the secondary effect of eutrophication, i.e. toxic substances originating from the anaerobic decomposition of the organic matter present (Armstrong et al. 1996; van der Putten 1997; Čížková et al. 1999; Armstrong and Armstrong 2001). It seems reasonable to assume that the same mechanism would also hold for *T. angustifolia*.

Reducing conditions promote the formation of substances toxic to plants (e.g. Reddy and De Laune 2008; Lamers et al. 2012). One of them, iron in the reduced state (Fe<sup>II</sup>), might occur in both the loose and the solid sediments, as is indicated by the grey colour of the surface of iron sticks (Fig. 5e) and redox potential values below +100 mV (Table 2). Toxic effects or the reduced forms of iron are reported for other wild wetland plants (Lucassen et al. 2000; Li et al. 2017) and rice (Becker and Asch 2005). On the other hand, very low Fe contents (below 1 mg l<sup>-1</sup>) were recorded in the water in loose sediment in all three biotopes and, therefore, it is unlikely it would have had a toxic effect.

Although the fishpond sediment was reduced and therefore potentially toxic, it did not adversely affect the growth of the plants in the mesocosm (Table 3). On the contrary, the plants prospered even better in fishpond sediment than in the control substrate (sand enriched with the mineral nutrients). This might have been due to

a more balanced nutrient supply in the organic fishpond sediment, which could not be fully simulated by adding inorganic fertilizer to the sand.

These results indicate that *T. angustifolia* is well adapted to anaerobic conditions. This is in accordance with the finding of Čížková et al. (1996) that it can grow in strongly reducing black mud in fishpond littoral zones. This is probably due to this plant having extraordinarily high numbers of spaces containing gas in both its shoots and roots (Seago et al. 2005; Schweingruber et al. 2020).

### Role of biotic factors

The results of the enclosure experiment (Fig. 6) indicate that fencing can have a marked effect on shoot density in sparse stands. In contrast, the results indicate there was no grazing damage in dense stands because there were similar densities of plants in fenced and unfenced areas.

Generally, fish (Hroudová et al. 2010; Francová et al. 2019a) and/or water birds (Chaichana et al. 2011; Bakker et al. 2018) can destroy the littoral stands of plants when they are abundant. The design of the enclosures does not allow the separation the effects of fish and water birds. However, the effect of fish can be excluded because there was only fry in the fishpond in 2015. The *T. angustifolia* clumps were not damaged by fry because they are small (10–20 g, 6–10 cm) and feed on small zooplankton. It can, therefore, be concluded that the destruction of the littoral plants by geese was the sole cause of the decline in stands of littoral plants in 2015 and probably also in the other years when only fry was present in the fishpond. Increasing pressure on the littoral stands of plants by geese is supported by the four-fold increase in geese counts over the period of this study (Fig. 7). Moreover, photographs taken at night (Fig. 5d) revealed that geese graze *T. angustifolia* plants in late summer and autumn (August to October), when they aggregate prior to migration. At that time, their presence was not detected by the daytime censuses because they are then feeding in surrounding fields and are only present at the fishpond at night.

In addition to geese, some damage could have been caused by adult carp present in the fishpond in 2012–2014. Because of lack of large zooplankton in summer, carp feed on zoo benthos, which is present on root surfaces in greater densities than in the surrounding anaerobic mud as oxygen leaks from the roots (Hargeby et al. 1994; Ságová-Marečková and Květ 2002). While moving around the isolated clumps, the carp probably broke buds extending from the rhizomes (Fig. 5c) and thus prevented vegetative spreading. Compared with the solid mineral bottom, the loose organic sediment may facilitate the searching for food by fish on root surfaces in already damaged stands of plants. In addition, Boarman and Fuller (1981) and Weisner (1991) propose that littoral plants may be anchored less well in soft sediments than in solid ones, which makes them

more vulnerable to disturbance. Insufficient anchorage of the plants would ultimately lead to uprooting, such as observed in this study (Fig. 5b).

It is interesting to note that fencing only protected sparse stands (cf. Fig. 6). This is probably due to the fact that geese can easily move around in the gaps between the clumps in sparse stands. On the other hand, they only damaged dense stands on their waterward side. This was observed repeatedly, but the experiments were not designed to detect it.

### Possible management measures

Overstocking with cyprinid fish has commonly been seen as a constraint on fishponds in terms of supporting populations of water birds (Broyer and Calenge 2010; Broyer and Curtet 2012). This study draws attention to an additional conservation dilemma, i.e. how to preserve littoral stands plants that are being damaged by increasing densities of greylag geese (Fig. 7). Herbivorous birds such as geese do not compete with fish for food because they feed in surrounding crops and grasslands. They are also legally protected in nature reserves. As geese numbers increase, they increasingly destroy littoral stands of plants, where species of rare water birds nest. This may increase in importance in the near future owing to changes in migratory behaviour in response to ongoing climate change (Podhrázský et al. 2017).

As Bakker et al. (2018) point out, the conservation of both the littoral stands of plants and high geese numbers appears to be impossible. There are two possible solutions: (1) negative effects of geese can be limited by the regulation of their population size. Lethal scaring (i.e. shooting some geese to scare other members of the flocks) has been reported as a successful measure to prevent geese damage in agricultural crops (Månsson 2017). This measure may be worth considering for preventing the destruction of littoral stands of plants in late summer (i.e., from the beginning of August), after the fledging of the young of the protected species of birds and the geese gather in nature reserves in order to escape from hunting. Nevertheless, this may be hard to achieve in a nature reserve because local inhabitants and tourists are likely to protest. Using lasers to scare geese has been used, but so far with limited success (Clausen et al. 2019).

An alternative to scaring geese may be the regeneration of the littoral stands of plants by a partial lowering of the water level once in several years, which would expose the littoral zone to air, which promotes both the vegetative and generative reproduction of the plants (Rea 1996; Hroudová et al. 2010). It also prevents geese from feeding on the young plants provided the water table remains low till autumn, when the shoots are tall enough and no longer an attractive food for geese. During the draw-down, another fishpond in the vicinity should be filled with water in order to provide nesting habitats for the protected species of water birds.

## Conclusions

In a hypertrophic fishpond, which is also a nature reserve for rare species of water birds, the area suitable for nesting decreased by 2/3rds over a period of 10 years. Although chemical analyses of water and sediments confirmed hypertrophic conditions, the results of a mesocosm study indicated that sediment quality did not negatively affect the quality of the plants. In contrast, field enclosures revealed a significant biotic effect, which was attributed to shoot grazing by large numbers of geese. Damage to the belowground parts of plants, recorded in isolated clumps in disintegrated stands, was ascribed to large carp.

This study revealed that, although fish production was strictly limited in the fishpond because the conservation of water birds was a management priority, damage caused by fish could not be fully prevented. More importantly, it reveals a conservation dilemma in that the large numbers of geese destroyed littoral stands of plants, which resulted in the loss of nesting sites for other species of water birds.

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# SALT NO LONGER TRAVELS THROUGH THE BOHEMIAN FOREST ALONG THE GOLDEN TRAIL, BUT HALOPHYTIC NEOPHYTES DO

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## ABSTRACT

The Golden Trail, used for the transport of salt from alpine mines to the Czech Kingdom for centuries, was a crucial ancient trade route in the Central European region. The contemporary road I/4, copying the Golden Trail, plays the same role today. The I/4 is used year round with deicing salt, a common standard, applied for winter maintenance. Deicing salt is often used, even in sections where the I/4 passes through the Bohemian Forest and its protected areas. The effects of applying deicing salts on ecosystems in the region is well documented. In addition to many other effects, high concentrations of salt along the roads cause significant changes in plant communities. Plant species sensitive to salinity disappear and the abundance of halophytes increases. Roads are also trajectories for the migration of neophytes. Seeds or other propagules are transported with cargo or in car tires.

In this paper, we present our findings on the pilgrimage of *Plantago coronopus*, a true halophyte, into the Bohemian Forest. We also describe the species' colonisation strategy and human measures supporting its successful migration in a region of high conservation value.

**Keywords:** Bohemian forest; deicing salt; management of protected areas; National Park; neophytes; *Plantago coronopus*

## Introduction

Trade trails have passed throughout Europe since ancient times, ensuring the exchange of many regional commodities. These routes have traversed densely populated landscapes and crossed mountain ridges or other hard to reach areas. The Golden Trail, used for bringing salt from alpine mines to the Czech Kingdom for centuries, was one of the most important historical trade trails in this region (Kubů and Zavřel 2007, 2015). Caravans of mules and horses crossing vast forested areas of the Bohemian Forest sought the most feasible routes and several branches of the Golden Trail gradually emerged. Today, modern roads copy some branches of the Golden Trail and the international road I/4 Prague–Passau (hereinafter I/4 throughout), marked B12 in Bavaria, is one of the most important in the area. Of course, transported commodities have changed, as well as modes of transport. Fast trucks and cars replaced slowly traveling horses and mules. 21st century populations require a different quality of trails (i.e. roads) and hence their requisite maintenance, too. Roads must have year round accessibility and we understand for their winter maintenance deicing salt is a common standard.

We should not forget that the effects of applying deicing salts on ecosystems are well known and documented in both urban and natural areas in many countries (Findlay and Kelly 2011). In cold regions, deicing salts applied to roads are a major contributor to local and regional contamination of terrestrial ecosystems, while salts from water softeners and septic systems are less important. In

addition to the many other effects on ecosystems, high concentrations of salt also cause significant changes in plant communities (i.e. Angold 1997; Wrobel et al. 2006). More sensitive species can disappear and result in gaps in vegetation cover. The abundance of halophytes and facultative halophytes (i.e. subhalophytes), which produce many seeds and invade neighbouring plant communities, may also increase. Facultative halophytes can tolerate salty soils, but they are not more advantageous for them. Obligate halophytes, however, not only tolerate salinity, but their growth can be stimulated by NaCl (Flowers et al. 1977) and they are good indicators of salinity.

Because of the well-known negative effects of applying deicing salt in many countries, their application to roads in protected areas is prohibited by law in the Czech Republic. There are special exceptions for several of the central arteries in the Šumava National Park (Šumava NP) and Šumava Protected Landscape Area (Šumava PLA) though, and deicing salts are applied when particular conditions prevail. The first monitoring of the effects of deicing salt on ecosystems in the Šumava NP was done in the 1990's and routine monitoring began in 2003. Recently, phytosociological monitoring is conducted and soil samples for chemical analysis are collected annually (Zýval et al. 2015).

Dataloggers recording water level, temperature and conductivity are used for monitoring the impact of deicing salt on water ecosystems in the Šumava NP since 2016 (Zýval et al. 2018). There was little detailed information on the effects of these salts on ecosystems in the Bavarian Forest NP, since the first preliminary study was

only carried out in 2011 and 2012 (Křenová et al. 2012). This study, as well as repeated monitoring conducted in 2015 and 2018 showed that ecosystems along the main road running through the old part of the Bavarian Forest NP, the “Nationalparkstrasse” were significantly affected by deicing salts (Křenová et al. 2018). Vegetation analyses revealed significant differences in the occurrence of halophytes and sub-halophytes among studied sites with increasing numbers of halophytes and sub-halophytes recorded at sites along this main road, particularly between Altschönau and Spiegelau. We have recorded more than thirty halophytic and subhalophytic species that were previously known from the Czech side of the Bohemian Forest (Zýval et al. 2015). As early as 2012, we also found *Plantago coronopus*, a new species that had not been mentioned before in this area. We began looking for this species in the Bohemian Forest and a central question was whether this species also occurred in adjoining Czech regions.

In this paper, we report the pilgrimage of the Buck's-horn plantain in the Bohemian Forest, from Bavaria to Czechia. We also elucidate the species' colonisation strategy and human measures supporting its successful migration.

## Methods

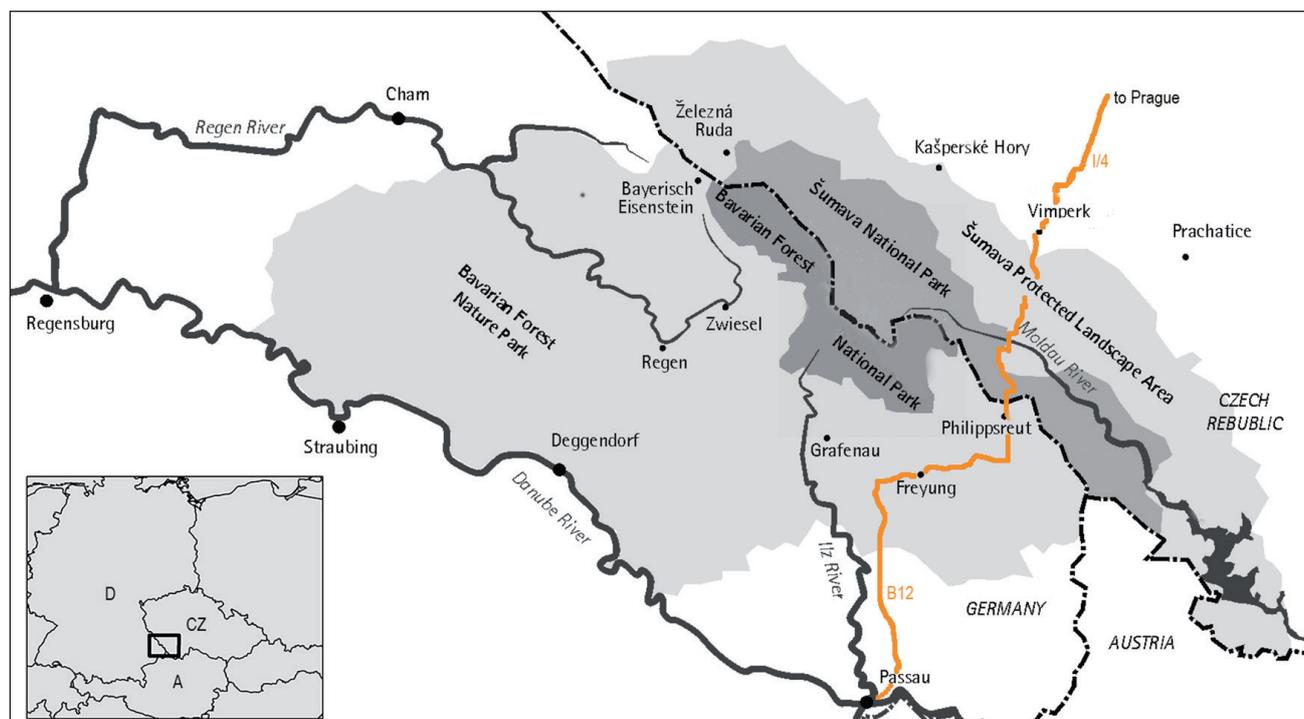
### Study area

The Bohemian Forest is one of the largest forested landscape in Central Europe and covers the highest ridges of the low mountain range that forms the watershed

between the Danube and Elbe catchment areas. Two national parks, the Bavarian Forest National Park (Bavarian Forest NP, Germany) and the Šumava National Park (Šumava NP, Czech Republic) were established in the Bohemian Forest in 1970 and 1991, respectively, and protect the area with the highest conservation value (Křenová and Kindlmann 2015, 2018). Two national parks together with the Bavarian Forest Nature Park (300,800 ha, established 1967) and the Šumava Protected Landscape Area (99,624 ha, established 1963) create the great Bohemian Forest ecosystem. The latter two can be understood as buffer zones of the two national parks (Fig. 1).

The greater area is a unique mosaic of natural and secondary habitats of exceptional natural value with European-wide significance. Each type of habitat hosts numerous rare and protected plant and animal species. Forests cover over 80% of the Šumava NP, the largest national park in the Czech Republic (68,064 ha), and peat bogs and natural wetlands are the second most important habitat in this area. The Bavarian Forest NP (24,235 ha) is approximately 98% covered by forest (Elling et al. 1987) and peat bogs are again its second most important habitat in this area. The region is characterized by montane and high montane areas within a vertical range of roughly 800 m (from 650 to 1,420 m a.s.l.). The soils are nutrition poor with cambisols, cryptopodzols, podzols, and organic soils being the most common (Kočárek 2003). At all altitudes, but especially in the valleys, there is characteristically a persistent or intermittent water-table in both mineral and organic soils (Bässler et al. 2009).

At a larger scale, the Bohemian Forest is in the temperate zone and subject to both Atlantic and continental



**Fig. 1** Great Bohemian Forest ecosystem. Four large protected areas and the main international road Prague – Passau (marked I/4 in CZ and B12 in Germany) are shown.

influences. The cold mountain climate of the region is characterised by an average annual temperature of 4.2 °C and average rainfall of 1,090 mm (Czech Hydrometeorological Institute – Churáňov station, 1,118 m a.s.l.; <http://www.chmi.cz/>). The total annual precipitation on the Bavarian site is between 1,200 and 1,800 mm depending on altitude. Annual mean air temperature ranges from 5.1 °C in the valleys, 5.8 °C on hillsides and 3.8 °C in the high montane zones (Noack 1979; Bässler 2004). Snow cover differs from year to year. In Bavaria, continuous snow cover over the last ten years has averaged about twenty weeks with an average snow depth of 45.7 cm. Several days per year with more than 100 cm of snow cover are also common in this area (data from the Bavarian Forest NP Authority). The richest in snow are the positions at the highest altitudes around the state border. Continuous snow cover occurs on average during 90 to 100 days a year in the lowest positions and in more than 200 days in the highest positions of the Bohemian Forest. The first day with snow cover in the lowest positions is at the end of October, in the highest positions at the beginning of the month (exceptionally also in September). The last day with snow cover in the lowest altitudes is at

the end of April, with the highest positions in mid-May, however there is still a discontinuous snow cover until the end of May and exceptionally until mid-June. Snow cover acquires the greatest mass in lower positions in February, in the peaks of Šumava in March. The average maximum height of snow cover ranges from 40 cm in the lowest positions to 150 cm and more in the highest (data from the Šumava NP Authority).

### Study species

*Plantago coronopus* (L.), Buck's-horn plantain, is a wind-pollinated, self-compatible annual or short-lived perennial herb that mainly occurs in southern Europe and North Africa. It also occurs in north-western Europe along the Atlantic and North Sea coast and all the way to south-east Asia (Fig. 2; Hultén and Fries 1986; Villellas et al. 2013). Records of this species from inland sites refer to secondary habitats, usually of anthropic origin.

*P. coronopus* is a true halophyte (Flowers et al. 1977). The species remains viable and completes its life cycle at seawater salinity; however, its germination decreases with increasing concentration of NaCl, i.e. a facultative halophyte (Bueno et al. 2017). Ellenberg (1991)

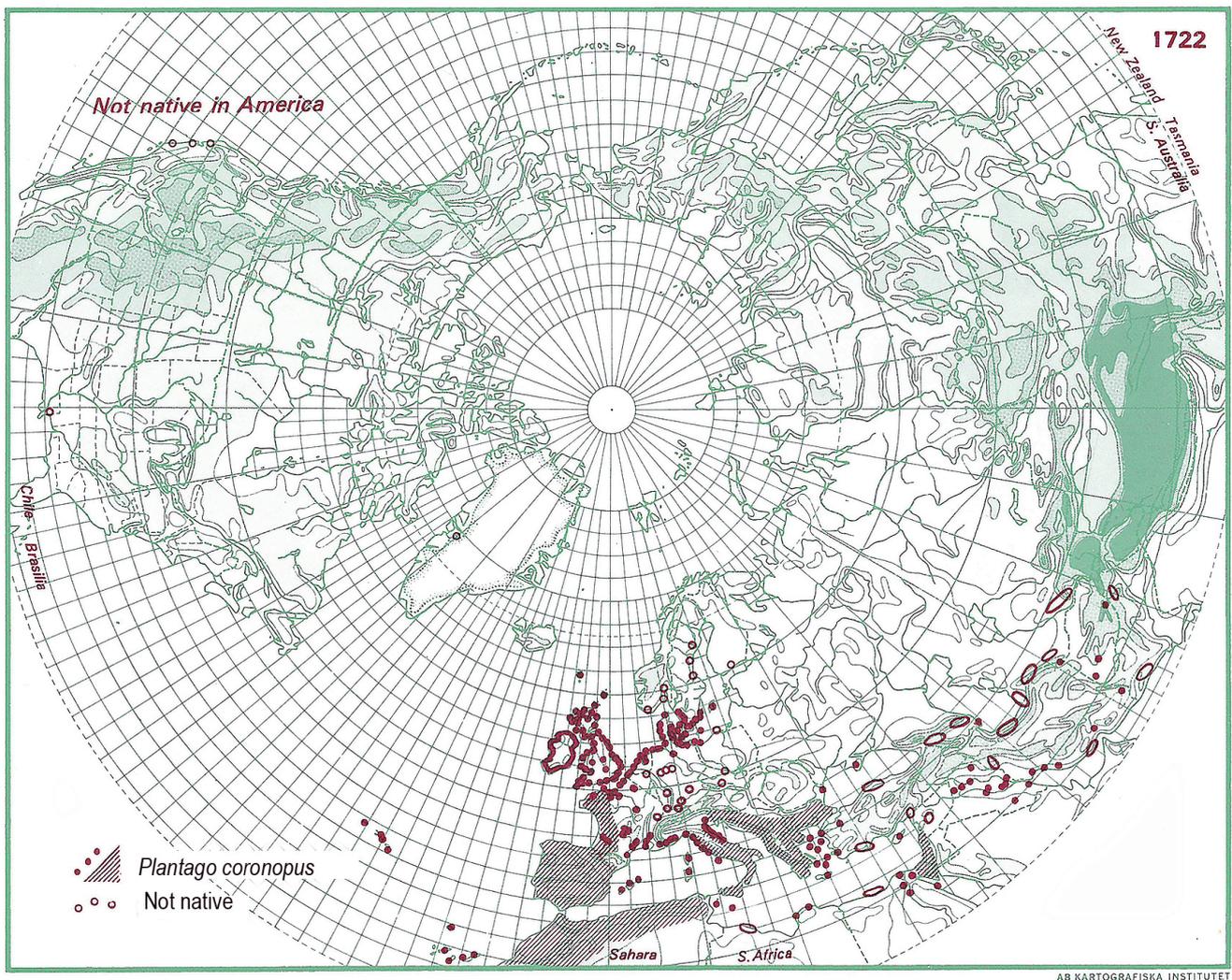


Fig. 2 Natural range of *Plantago coronopus* (adapted from Hultén and Fries 1986).

classifies *P. coronopus* as a mesohalophytic species usually colonizing soils with a low to a moderate chloride content (0.5–0.7% Cl<sup>-</sup>). It is a drought-tolerant and drought-adapted plant species (<http://www.sussex.ac.uk/affiliates/halophytes/>). It grows in sandy or gravelly soils and cracks in rocks, in sunny places in dry soils usually near the sea (Clapham et al. 1962). It is one of the first colonizers of bare sand flats in the Netherlands (Koelewijn 2004). It is edible and occasionally grown and used in herb salad and as a garnish. This plant is commonly harvested and eaten in the Maritimes and it is currently a popular frost-hardy winter crop for farmers in northern climates where it is usually grown in unheated tunnels (Baldwin et al. 2012).

Individuals contain one or several basal rosettes from which flowering stalks can emerge with an inflorescence. Each leaf axil has an axillary meristem that can produce either a spike or a lateral rosette, or remain dormant.

It is a gynodioecious and predominantly outcrossing species. *P. coronopus* hermaphroditic flowers are protogynous, the stigma emerges first, and after approximately 3–4 days the anthers appear (Koelewijn and van Damme 1995). Flowering and subsequent maturation occur from the base of the ear upwards (Koelewijn and van Damme 2005). This species flowers from the beginning of May through to September and can overwinter as a rosette.

The species physiology (Koyro 2006), reproduction (Dinnézt and Jerling 1997), germination (Waite and Hutchings 1978), demography (Waite 1984) and ecological characteristics (Smekens and Tienderen 2001; Koelewijn 2004) have been previously studied. However, all these studies were done with samples from native populations growing in the coast or inland salt marshlands. Ecology of this species growing in secondary habitats, i.e. along roads treated by deicing salt, have not been studied previously.

### Search methods of the species occurrence

We began with a literature survey and analysis of floristic databases in Germany, Austria and Czech Republic to learn more about the historical distribution of the Buck's-horn plantain (*Plantago coronopus*) in Central Europe.

We have searched for *P. coronopus* in the Bohemian Forest region since 2012. We started with permanent study sites, which were established in the Bavarian Forest NP in 2011 (Křenová et al. 2012). In addition to those sites, sixty-five transects (200 meters long and 60 cm wide) were established along the central segment of the Nationalparkstrasse (730–830 m a.s.l.) in order to quantify a proportion of the area along the road affected by deicing salt. All vascular and bryophytes as well as their abundances (Braun-Blanquet 1951, 5 degrees scale) were recorded along all these transects. Monitoring of these 65 transects first took place in 2012 and repeated in 2015 and 2018. Data about abundances of *P. coronopus* were used for this study.

After the first record of the Buck's-horn plantain (*P. coronopus*), we also searched for the species in other parts of the Bohemian Forest region. Since 2012, we have walked all the roads treated by deicing salt in the region and searched for the species. GPS coordinates of newly found individuals or small colonies were recorded. We primarily focused on the roads in protected areas, i.e. in Bavarian Forest NP and in Šumava NP and Šumava LPA, where applying of deicing salt is regulated. We also searched along the central roads outside protected areas. The I/4, international road Prague – Passau, the most trafficked in the region, was the centre of our interest.

Newly, 16 transects of the same design (200 meters long and 60 cm wide) were set in 2015 with the aim of monitoring the Buck's plantain occurrence and its abundance along the I/4 from the border to inland, where the species was first recorded on the Czech side of the Bohemian Forest in 2014. The monitoring along the I/4 was repeated in 2018 and again in 2020 and 2021, after the completion of large road reconstructions in 2019.

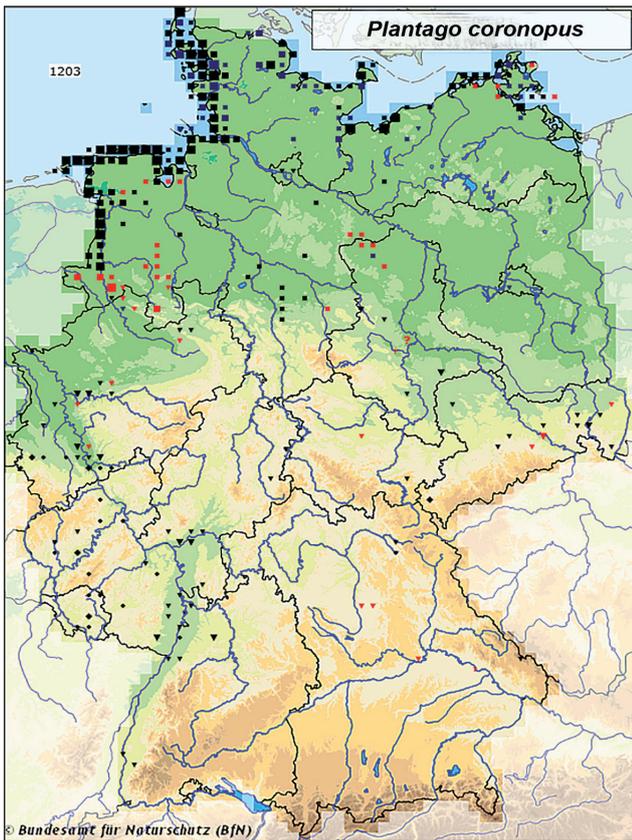
## Results

### Distribution and historical records in Germany and the Czech Republic

Reviewing the literature, we found that in Germany (Fig. 3) there are records of *Plantago coronopus* subsp. *coronopus* (Rothmaler 2005) growing along the coast, in Lower Saxony and Schleswig-Holstein. This species naturally occurs in salt meadows from Mecklenburg-Vorpommern to Nordrhein-Wistfalen and Saxony-Anhalt. According to Oberdorfer (2001) this species occurs along the North Sea and Baltic coast, in Harzrandmulde – Magneburger Börde – Saxony, Lower Saxony, Fränkisches Keuper and Liasland, Haßberge and Frankenalbvorland and Oberrheinebene. *Plantago coronopus* is not included in the Austrian Flora (Adler et al. 1994).

Before 2016, there were few records of *Plantago coronopus* in the Czech Flora (Slavík 2000) and the Czech main botanical database ([www.florabase.cz](http://www.florabase.cz), Chytrý and Rafajová 2003). In the database, the first record dates from 1959, when this species was found at the industry complex Vítkovické železářny KG in Ostrava, North Moravia, 420 m a.s.l. (Kilián and Krkavec 1961). The second record is from 30 November, 1987, when Dr. Pyšek found this species on an industrial dump at the Kovosvit factory, Sezimovo Ústí, South Bohemia (Štech et al. 1988); this location was confirmed again in 2005. Both the Czech Flora (Slavík 2000) and Kubát et al. (2002) state that this species was first recorded on slag heaps at Třinec, North Moravia.

The new database of the Czech flora and vegetation (<https://pladias.cz/>, Chytrý et al. 2021) has more records (Fig. 4), most of them from last five years. There are several records from our region. All of them are from 2016 or younger.



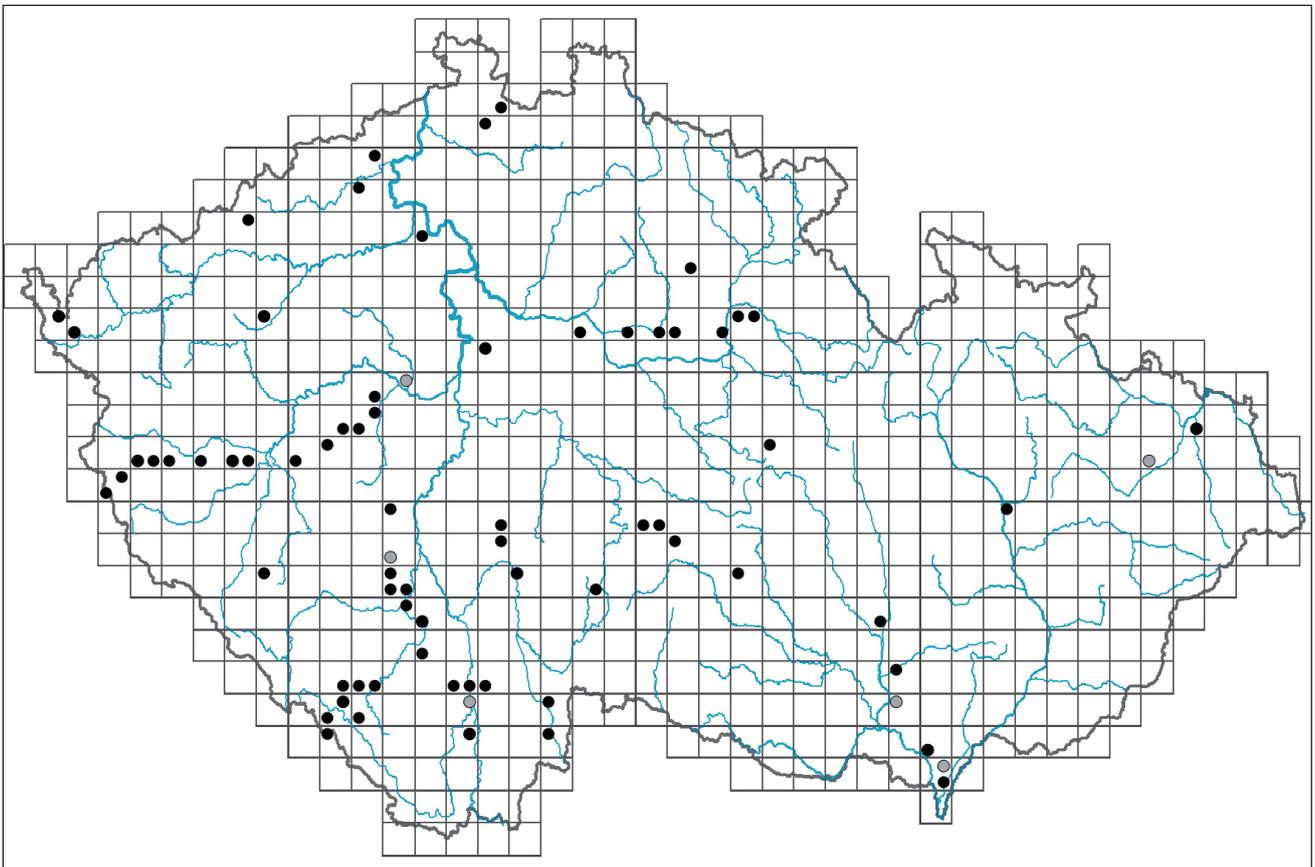
**Fig. 3** Occurrence of *Plantago coronopus* in Germany (adapted from [www.floraweb.de](http://www.floraweb.de)). Red points show data recorded before 1950, blue marks are data from 1950–1990 and black points are data since 1991 and later.

The database of the Czech Nature Conservation Agency (AOPK ČR 2021) has 18 records of *P. coronopus* from 2013–2021 (Fig. 5). Only one record is from our region. The species was recorded along the I/4 near Kortusova Huť in 2018.

### New species recordings in the Bohemian Forest

In 2011, during the first year of the monitoring project, we recorded *Plantago coronopus* at three sites from nine we monitored along the Nationalparkstrasse in the Bavarian Forest NP (Křenová et al. 2012). Two locations were close to the Grosse Ohe River (location S4: N48° 55.416' E13°24.862', 765 m a.s.l.; location S5: N48°55.406' E13°24.817', 765 m a.s.l.) and the last was near Altschönau (S9: N48°54.664' E13°27.977', 748 m a.s.l.). The species always occurred only at the edge of the asphalt road and it was the dominant species there. No older records of this species were found from this area or other parts of the Bohemian Forest.

During the first year of our survey, we were not sure if *P. coronopus*, a species natural in moderate maritime climate, could survive at such cold locations. The temperature was less than  $-20^{\circ}\text{C}$  on several days in February 2012 in parts of the Bavarian Forest NP; nevertheless, the occurrence of *P. coronopus* was confirmed in 2012 and again in 2015 and 2018. Since summer 2012 we have also recorded this species at two other permanent study sites – Grosse Filz (S7: N48°55.338' E13°25.210', 777 m a.s.l.) and Guglöd (S8: N48°55.388' E13°25.106', 769 m a.s.l.).



**Fig. 4** Occurrence of *Plantago coronopus* in the Czech Republic ([www.pladias.cz](http://www.pladias.cz)).

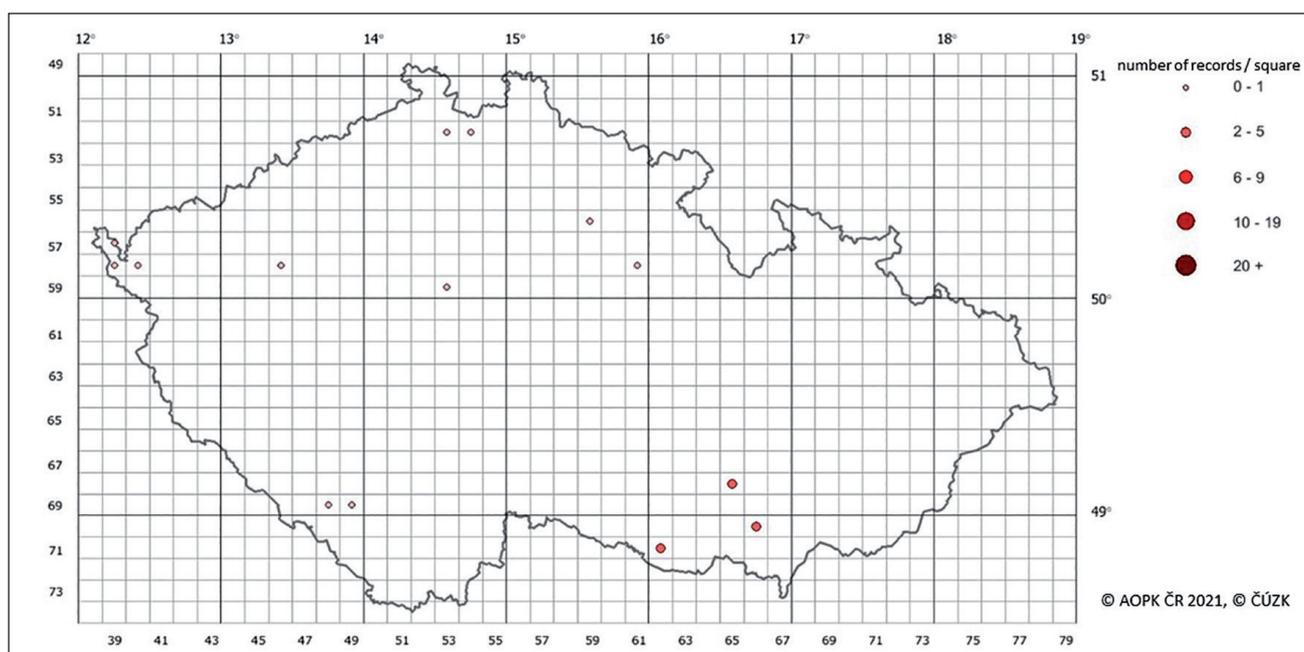


Fig. 5 Occurrence of *Plantago coronopus* in the Czech Republic (www.portal.nature.cz).

Chemical analysis of soil samples from these locations confirmed an increasing conductivity and concentration of  $\text{Na}^+$  and  $\text{Cl}^-$  ions (Křenová et al. 2018).

With the first monitoring of 200 meter long transects in Bavaria in 2012, we recorded *Plantago coronopus* in 33 transects (51% of all transects) established along the main part of the Nationalparkstrasse. *P. coronopus* was recorded in 42 transects in 2015 and in 47 transects in 2018, it was found in 65% and 72% of transects respectively. In all cases, *P. coronopus* plants occurred only at the edge of the asphalt road maintained by deicing salt (max 15 cm from the edge of the road). Plants grew in different densities, from several individuals to hundreds of plants in some places. It was a dominant species in some transects. In the control transects, established along the parallel road not maintained by deicing road, there were



Fig. 6 Individuals of *Plantago coronopus* growing along the Nationalparkstrasse road are small and grow very close to the asphalt road edge.

no individuals of *P. coronopus*. The morphology of these plants was the same as in 2011 and later. All plants were small, up to 10 cm tall with a rosette of maximum 10 cm in diameter. Leaves in rosettes were ascending and small. Individuals had usually only five or six stems with cobs per plant (Fig. 6).

In May 2013, we found several small plants of *P. coronopus* along the I/4. We checked the occurrence of this species more carefully in summer 2013. There were dozens of plants at an altitude of 950 m a.s.l., the highest point along the I/4, where it crosses the main ridge of the Bohemian Forest and watershed between the Danube and Vltava Rivers (N 48°52.411', E 13°41.357'). Closer to the Czech border this species was less abundant and the last few individuals or small groups of *Plantago coronopus* were recorded 1.5 km from the border, on eastern edges of the road (N 48°52.411', E 13°41.357'). We carefully searched for the species along the same road in 2013 in Czechia but no individuals of the Buck's-horn plantain were found.

In summer 2014, the first small individuals of this species were recorded along the I/4, 0.5 km inside the Czech Republic (N 48°53.122', E 13°42.316'). A more or less continuous 10 cm wide and more than 1 km long strip of hundreds of plants was found on the right side of the road in the direction from the Czech-German border to Strážný (N 48°54.106', E 13°43.221').

In 2015, we established fifteen 200 meter transects on the south edge of this road from the border to the Czech interior and we recorded *P. coronopus* in 11 transects. Usually we found only several individuals and small groups of *P. coronopus* plants in each transect. Several individuals of *P. coronopus* were also recorded along the north edge of the I/4, near the petrol station in Strážný (N 48°54.101', E 13°43.188'). A habitus of these

*P. coronopus* plants was similar to plants found in Germany. They were small, max 10 cm in diameter and had only few flowering stems. They grew in a narrow strip (max 20 cm wide) along the asphalt road edge. In summer 2018, we have repeated monitoring of 11 transects on the south edge of the I/4 from the border to the Czech interior and we recorded *P. coronopus* in 14 transects. Abundances of plants in several transects significantly increased. More or less, there were no differences in conditions between the locations with and without *Plantago coronopus* occurrences. Plantains grow along the edges of the roads, often in places where there were low abundances of other plant species and that were regularly mechanically disturbed by snow plowing, mowing or sweeping. We could summarise that there are many similar locations but only some are colonized by this species. It seemed likely that occurrences of *P. coronopus* are a result of seed distribution and many new locations will be colonized in the future.

### Road reconstruction and surface disturbances support the spread of the species

In summer 2018, we found four new locations of *P. coronopus* along the I/4 and we learned that this species profits from disturbances along road edges.

The first new location was a colony with many small plants on the Czech-German border (N 48°52.848', E 13°42.271'). We have already had this species occurrence records to the north and south of this site but not precisely from here. Plantains newly colonised a 1.5 × 10 m plot where the grass was removed and bare soil mixed with recycled gravel in autumn 2016. *P. coronopus* and *Puccinellia distans*, another halophytic species, were the only two species creating a sparse vegetation of this new substrate. *P. coronopus* individuals were small (max 5 cm in diameter) with only one or two flowering stems (Fig. 7). Recycled gravel with a high proportion of old asphalt limited the growth of the plants. At the second, dozens of plantains were found in Řasnice (N 48°54.653', E 13°45.124'), where *P. coronopus* plants

used the widened edge of the road and its torn turf. This place was modified to improve the entrance to agricultural land. We found also a new population of *P. coronopus* north of Horní Vltavice (N 48°57.7613', E 13°45.2641'). Finally, the species was also recorded near Kortusova Huť (N 49°0.49048', E 13°45.98530'), where widening of the roadsides created a new suitable habitat for *P. coronopus*. Plantains growing in Řasnice, Horní Vltavice and Kortusova Huť were max 10 cm in diameter and usually had 6–8 flowering stems. These three locations were 3.5, 9.5 and 16 km from the nearest location where the species was recorded in 2015. We carefully searched along the I/4 among all known locations, however no further *P. coronopus* individuals were found in 2018.

A complete reconstruction of the I/4 between the Czech-German border and a petrol station near Strážný was conducted in summer 2019. The old road surface was milled and a new asphalt carpet created. The edges of the roadway were changed too. Old road curbs were torn down and the road edges (about 60 cm wide) were covered with a new mixture of sand and gravel. Fifteen 200 meter transects established in 2015 disappeared with this reconstruction.

Nevertheless, we observed *P. coronopus* individuals colonizing a new substrate at the road edges in summer 2020. We restored our transects along the south edge of the I/4 and we added another 15 transects (again 200 m long and 60 cm wide) along the north edge of the road. In summer 2020, we recorded *P. coronopus* in seven of 15 transects on the south edge and in eight transects of 15 from the north edge of the road. We recorded only one or two plantains in several 200 meter transects. These plants were robust (more than 20 cm in diameter), with many leaves and often 15–25 flowering stems (Fig. 8). We did not find the remnants of the original population near these new colonists. The seeds were transported from a greater distance. In two transects, we found dozens of *P. coronopus* individuals growing in a new substrate. They likely originated from remnants of



**Fig. 7** Tiny plants of *Plantago coronopus* and *Puccinellia distans* created a sparse vegetation in the plot covered with recycled gravel and old crushed asphalt.



**Fig. 8** *Plantago coronopus* individuals colonising a new substrate at the road edges were more than 20 cm in diameter and had many flowering stems. This species was one of the first colonists of the new road edges.



**Fig. 9** Remnants of old *P. coronopus* population, which survived outside of the reconstructed road edges, produced many seeds and their offsprings successfully colonised a new substrate. Several dozens of new colonists appeared only a year after the road edge reconstruction was completed.

old populations surviving outside of the reconstructed road edge. New colonists were again more robust than *P. coronopus* individuals growing in a neighboring remnant population (Fig. 9).

In summer 2021, we repeated our monitoring and recorded *P. coronopus* in eight of 15 transects on the south edge of the I/4 and in nine of 15 transects on the north edge. An abundance of the species increased in several transects. In 2021, we again carefully searched for *P. coronopus* along the I/4 and found that migration of the species slowly continues. New individuals or smaller groups were recorded between a petrol station in Strážný (N 48°54.106', E 13°43.221') and Hlinišť, an intersection of the I/4 and II/39 (N 48°56.0952', E 13°45.996'). No plants were found between this intersection and a location south of Kortusova Huť, from where the species was already reported in 2018. Hundreds of plantains were found on both sites of the I/4 south of Kortusova Huť, between the village and the forest. Some plants of *P. coronopus* were also recorded north of Kortusova Huť (N 49°1.246', E 13°46.073'). In all new locations visited in 2021, *Plantago coronopus* occurred on the edges of the I/4 in open landscapes. We never found the species inside villages and in sections where the I/4 passes through forest. *P. coronopus* successfully colonized wider curbs with open

vegetation, mixtures of sand and gravel, spots with open bare soil and drying substrates. The species avoids moister forest sections and old (i.e. un-reconstructed) areas of the I/4 with narrow curbs covered in dense vegetation.

## Discussion and Conclusions

Buck's-horn plantain (*Plantago coronopus*), the new species of a local flora, has appeared in the Bohemian Forest during the last decade. The I/4, international road, Prague – Passau is the primary migration corridor of the species. In this paper, we described the gradual spread of this species along the I/4 from the Czech-German border to within the Czech Republic. The initial 25 km of the road passes through a region of high conservation value (Šumava NP and Šumava PLA). We can state that the Golden Trail is no longer a corridor for the delivery of salt to the Czech Republic, but rather, a suitable migration corridor for halophytic species. *P. coronopus* is one of these halophytic species and its occurrence confirms the synanthropisation of the region.

Intensification of traffic and increase in the application of deicing salt to roads after the collapse of the Iron Curtain resulted in colonization by several other halophytic species. The best documented is a pilgrim of the weeping alkali grass (*Puccinellia distans*), a species which was unknown in the Bohemian Forest region until 1990. The first occurrences of this species were recorded in the locality of Hlinišť – the I/4 in 1995 (Hadinec and Hadincová in FLDOK – Flora Database of the Czech Republic). In 1997, a local botanist František Procházka reported this same species already “from several locations along main roads in the region” (Procházka in FLDOK). Thirty years later, *Puccinellia distans* is a common and often dominant species along all regional roads maintained by deicing salts. In addition, *Spergularia salina*, another halophytic species, was not recorded in this region before 2000. This species' first records are from 2006 and 2007 where it was found in permanent transects along several roads in the region regularly treated with deicing salt (Zýval and Chochołušková 2006). Currently, it is a common and widespread species.

There are no differences in habitus or morphology of the previously introduced species, *Puccinellia distans* and *Spergularia salina*, growing at natural or synantropic locations. The habitus of *Plantago coronopus* occurring in the Bohemian Forest, however, is somewhat different. Many individuals occurring in the region are smaller and of lower vitality than plants in natural habitats along the sea coast (personal observation in several sites). However, the *P. coronopus* individuals colonising new substrates, for examples mixtures of sand and gravel used for reconstruction of the road edges, can be robust, vigorous and produce many flowering stems and seeds. The seeds ripen, overwinter and germinate successfully. *P. coronopus*, a species natural in moderate maritime climates, can suc-

successfully reproduce in the cold climate of the Bohemian Forest region.

It is likely that all these halophytic species have similar strategies. Migration vectors for their seeds are cars, more precisely the tread of their tyres, which effectively scatter the seeds along the edges of roads where abundance of other species is limited due to road maintenance (snow plowing, sand sweeping, reconstruction etc.). *Puccinellia distans* and *Spergularia salina* are very successful at producing seeds but they can as well colonize certain places by vegetative reproduction. *P. coronopus*, strictly dependent on seed reproduction, successfully colonises open bare sites along the roads maintained by deicing salt, from which salt-intolerant species have left and vegetation cover is often mechanically disturbed. The species prefers dry sites and avoids more moist forest sections of the roads with dense vegetation along road edges.

We conclude that intensification of traffic and more active maintenance of the roads including applying deicing salt during the last 30 years have affected the flora and vegetation in the Bohemian Forest. Some changes in the vegetation are still only visible near roads that are regularly treated with deicing salts (Zýval et al. 2015), but successful colonization by several halophytic species has already occurred. It is unquestionable that this increased biodiversity is not welcome in this region of high nature conservation value. We believe that regulation of the use of deicing salt in the national parks and responsible road management together with careful monitoring of ecosystems along the roads could help protect sensitive habitats and minimize the synanthropization of the Bohemian Forest flora.

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# EFFECT OF AQUEOUS EXTRACTS OF THREE DIFFERENT PLANTS ON MYZUS PERSICAE SULZER (HEMIPTERA: APHIDIDAE) INFESTING PEPPER PLANTS UNDER LABORATORY CONDITIONS

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## ABSTRACT

This study aimed to determine the percentage infestation and population parameters of the green peach aphid *Myzus persicae* under laboratory conditions and evaluate the effect of aqueous extracts of three different plants (pot marigold: *Calendula officinalis*, mint: *Mentha viridis* and rosemary: *Salvia rosmarinus*) on the mortality of this aphid. Extracts of these plants were used at three concentrations (C1 = 15%, C2 = 30% and C3 = 45%). Results indicate a percentage infestation of 76.9 ± 9.4%, a mean relative growth rate of 0.062 ± 0.007 and generation time of 11.12 ± 1.42 days. All treatments reduced the numbers of aphids and statistically significantly reduced the number ( $\alpha < 0.01$ ) recorded after treatment with C1, C2 and C3 of each extract. *C. officinalis* extract was more effective than those of *M. viridis* and *S. rosmarinus*. The highest mortality (69.82 ± 5.23%) and efficacy (61.71 ± 4.46%) were recorded for the C3 of aqueous extract of *C. officinalis*, whereas the lowest mortality (38.24 ± 2.42%) and efficacy (32.41 ± 1.23%) were recorded for the C1 of extract of *M. viridis*. The data provided indicate that aqueous extracts of *C. officinalis*, *M. viridis* and *S. rosmarinus* have an insecticidal effect on *M. persicae* and can be integrated into a pest management strategy to reduce *M. persicae* abundance on pepper plants.

**Keywords:** botanical extracts; efficacy; mortality; *Myzus persicae*; population parameters

## Introduction

Pepper (*Capsicum* spp.) is an annual herbaceous plant belonging to the Solanaceae family, which includes commercial vegetables such as tomato, eggplant, tobacco and potato (Tripodi and Komar 2019). It is one of the most popular vegetable crops throughout the world and a source of vitamins C and E, provitamin A, carotenoids and phenolic compounds that offer health benefits for consumers (Agwu et al. 2018). Despite the importance and popularity of pepper, several pests, such as insects, fungi, bacteria, viruses and nematodes adversely affect its production worldwide.

Among the insects, the green peach aphid, *Myzus persicae* Sulzer (Hemiptera, Aphididae) is one of its most serious pests (Blackman and Eastop 2006; van Emden and Harrington 2007; Mdellel et al. 2019). It can occur on the underside of the leaves and on young plants at high densities, which causes deformation of leaves, reduces plant growth and infects the plants with virus, which reduce yield and the quality in terms of its nutritional content (Blackman and Eastop 2006; Bass et al. 2014). The green peach aphid is controlled mainly by using chemical insecticides.

However, the use of chemical insecticides has serious disadvantages, such as human and animal poisoning, water contamination, development of pest resistance, death of pollinating agents and natural enemies (Ofuya and Okuku 1994; Bass et al. 2014). Therefore, there is a need to investigate alternative means of control that can reduce

chemical pesticide-related problems (Nahusenay and Abate 2018). Among these, botanical insecticides are an important group of naturally occurring pesticides, which are usually safer for humans, animals, environment and natural enemies than chemical insecticides (Devi et al. 2016).

Currently, the use of plant extracts and essential oils to control insect pests is widely used. Indeed, some plants are chemically protected against insects or contain various biologically active compounds that can be toxic to insect pests, anti-feedants, inhibit growth, inhibit oviposition and even sterilize insects (Sayed et al. 2020). In addition, plant extracts are less toxic to humans, easily degrade in the environment and less persistent than chemical pesticides (Mwine et al. 2011; Silva et al. 2016).

Among the biological active compounds, pyrethrin, which is extracted from *Chrysanthemum* spp. kills insects at low concentrations and has many others advantages such as rapid degradation and is less toxic to beneficial insects (Shawket et al. 2011). Many other plants like neem (*Azadirachta indica*), tinjot (*Otostegia integrifolia*), crinum (*Crinum ornatum*) and common sage (*Salvia officinalis*) have been used to control insects (Nahusenay and Abate 2018; Sayed et al. 2020). Unfortunately, insecticidal effects of aqueous extracts of many others plants, such as pot marigold (*Calendula officinalis*), mint (*Mentha viridis*) and rosemary (*Salvia rosmarinus*) on aphids and the most effective concentrations are unknown.

In this study, the biological parameters of *M. persicae* under laboratory conditions and pesticidal effects of

aqueous extracts of *C. officinalis*, *M. viridis* and *S. rosmarinus* applied as a foliar spray at three different concentrations were investigated.

## Materials and Methods

### Insects and plants

*M. persicae* collected from pepper plants cultivated in greenhouses at the National Organic Agriculture Centre, Unaiza, Kingdom Saudi Arabia, in January 2020. This aphid was reared on pepper plants in Plexiglas cages (50 × 50 × 50 cm) at 25 ± 2 °C, relative humidity of 60 ± 10% and a photoperiod of 14 L: 10 D h for several generations (60 days) before use in the experiments. The cultivar of pepper used was Shakira. Pepper plants (Cultivar: Shakira) were reared in the laboratory at day/night temperature ranging from 18 to 25 °C, 60–80% RH, and under ambient light conditions. Plants at eight leaf stage of development, were transferred to 500 mL pots containing a substrate consisting of 1/3 sand and 2/3 peat in a greenhouse at 25 ± 2 °C, relative humidity of 60 ± 10% and a photoperiod of 14 L: 10 D h and were watered on alternate days and no pesticides were used during the experiments.

### Infestation rate and population parameters of *M. persicae* on pepper

At the twelve-leaf stage, wingless adult aphids were collected from the colony and transferred to pepper plants in a greenhouse. Fifteen plants were each infested with four adult aphids. The experiment was replicated 3 times and total number of plants used was 45 plants. Data collection started after 2 days after infestation and total number of leaves, infested leaves, and aphid number/cm<sup>2</sup> of leaf were counted every seven days over a period of two months. The mean relative growth rate (MRGR) and generation time (T) of *M. persicae* were determined according to Leather and Dixon (1984), and the F1 and F2 formulas of Ramade (2003):

$$\text{MRGR} = (\ln N(t_n) - \ln N(t_{n-1})) / (t_n - t_{n-1}) \quad (\text{F1})$$

$$T = \log_2 / \text{MRGR} \quad (\text{F2})$$

where  $N(t_n)$  is aphid number/cm<sup>2</sup> of leaf at time  $t_n$ ,  $N(t_{n-1})$  is aphid number/cm<sup>2</sup> of leaf at time  $t_{n-1}$ .

### Plant samples and extracts

Fresh leaves of marigold, Mint and rosemary were collected in March 2020 from plants growing in natural habitats at Al-Qassim as indicated in Table 1. Collected leaves were washed with water and dried, well ventilated in the shade for two weeks (Sarwar 2015). The dried leaves were cut and ground to a fine powder using an electrical grinder. Thirty percent stock solution was prepared for each plant separately. The mixtures were stirred thoroughly with a repeated agitation at 3 h intervals for

24 h. Three concentrations (15, 30, and 45%) were prepared from the final extracts.

**Table 1** Description of the three plants tested in the present study.

No.	Common name	Scientific Name	Family name	Part used
1	Marigold	<i>Calendula officinalis</i>	Asteraceae	Leaves
2	Mint	<i>Mentha viridis</i>	Lamiaceae	Leaves
3	Rosemary	<i>Salvia rosmarinus</i>	Lamiaceae	Leaves

### Bioassay

To determine the insecticidal effect of aqueous extracts of selected plants, three leaves of each plants were marked and number of *M. persicae* on each marked leaf was counted 1 hour before bioassays. Using a hand-held sprayer, aphids on pepper plants were sprayed with different concentrations (C1 = 15%, C2 = 30%, and C3 = 45%) and the control (untreated) with water. Three infested plants were used for each concentration. Three, six, nine and 12 days after spraying the number of living aphids on each of the marked leaves was counted. Mortality of *M. persicae* population after 12 days were determined and the corrected efficacy percentage was calculated using Henderson and Tilton (1955) formula:

Corrected efficacy (%) = 1 – (n in Co before treatment × n in T after treatment / n in Co after treatment × n in T before treatment) × 100. Where: n = number of *M. persicae*/selected leaf, T = Treated and Co = Control.

### Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA) using SPSS (2015) software program, version 23. Separation of means separation was done using DMRT (Duncan's multiple range tests) test (P < 0.01).

## Results

### Percentage infestation and population parameters of *M. persicae* on pepper

The percentage infestation and mean relative growth rate of *M. persicae* on pepper plant cultivar Shakira under laboratory conditions are shown in Table 2. During this experiment, total number of leaves/pepper plant was 52.6 ± 13.8 and total number infested 39.8 ± 7.24. Per-

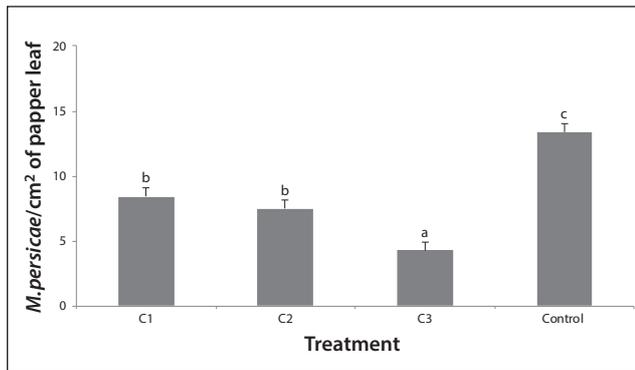
**Table 2** Biological parameters of *Myzus persicae* on pepper plants in green house.

Parameters	Average ± SD
Total leaves number	52.6 ± 13.8
Infested leaves number	39.8 ± 7.24
Infestation rate (%)	76.9 ± 9.4%
Aphid number/leaf cm <sup>2</sup>	11.24 ± 2.3
Mean relative Growth Rate (MRGR)	0.062 ± 0.007
Generation time (T)	11.12 ± 1.42 days

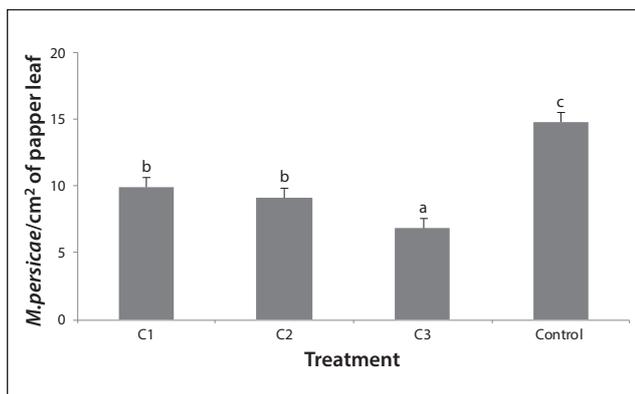
centage infestation was  $76.9 \pm 9.4\%$ . The aphid population survey resulted in an average of  $11.24 \pm 2.3$  aphids/cm<sup>2</sup> of leaf, mean relative growth rate of  $0.062 \pm 0.007$  and generation time of  $11.12 \pm 1.42$  days.

### Effect of aqueous extracts on *Myzus persicae* population

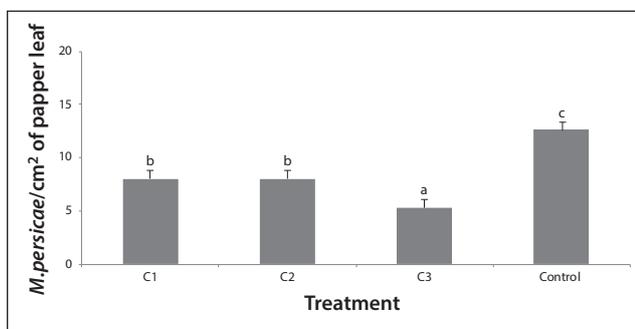
Aphid numbers/cm<sup>2</sup> of leaf on treated and none treated pepper plants were recorded 3, 6, 9 and 12 days after



**Fig. 1** Effect of an aqueous extract of marigold aqueous on the numbers of *Myzus persicae*/cm<sup>2</sup> of leaf of pepper plants. (Means followed by the same letter are not significantly different ( $\alpha < 0.01$ , Duncan's multiple range test).



**Fig. 2** Effect of an aqueous extract of mint on the numbers of *Myzus persicae*/cm<sup>2</sup> of leaf of pepper plants. (Means followed by the same letter are not significantly different ( $\alpha < 0.01$ , Duncan's multiple range test).



**Fig. 3** Effect of an aqueous extract of rosemary on the numbers of *Myzus persicae*/cm<sup>2</sup> of leaf of pepper plants. (Means followed by the same letter are not significantly different ( $\alpha < 0.01$ , Duncan's multiple range test).

spraying. Results indicate that all treatments decreased the numbers/cm<sup>2</sup> compared to untreated plants. Aphid numbers/cm<sup>2</sup> of leaf after treatment with C1, C2 and C3 of the *C. officinalis* extract were  $8.37 \pm 1.33$ ,  $7.43 \pm 1.46$  and  $4.33 \pm 1.24$ , respectively, and  $13.37 \pm 2.81$  for untreated plants. That is, all concentrations in this treatment resulted in significant reductions ( $F = 48.57$ ;  $\alpha < 0.01$ ) in the numbers/cm<sup>2</sup> (Fig. 1). For *M. viridis* extract, the numbers were  $9.95 \pm 1.12$ ,  $9.11 \pm 1.86$ ,  $6.83 \pm 1.66$  and  $14.81 \pm 1.21$ , respectively, and this treatment resulted in a significant reduction in the number of aphids ( $F = 43$ ;  $\alpha < 0.01$ ) (Fig. 2). A reduced number/cm<sup>2</sup> was also recorded after treatment with the extracts of *S. rosmarinus*:  $8.05 \pm 2.31$ ,  $7.79 \pm 0.98$  and  $5.27 \pm 1.32$ , respectively, which are significantly different from the  $12.61 \pm 1.93$  recorded on untreated plants (Fig. 3) ( $F = 39.76$ ;  $\alpha < 0.01$ ).

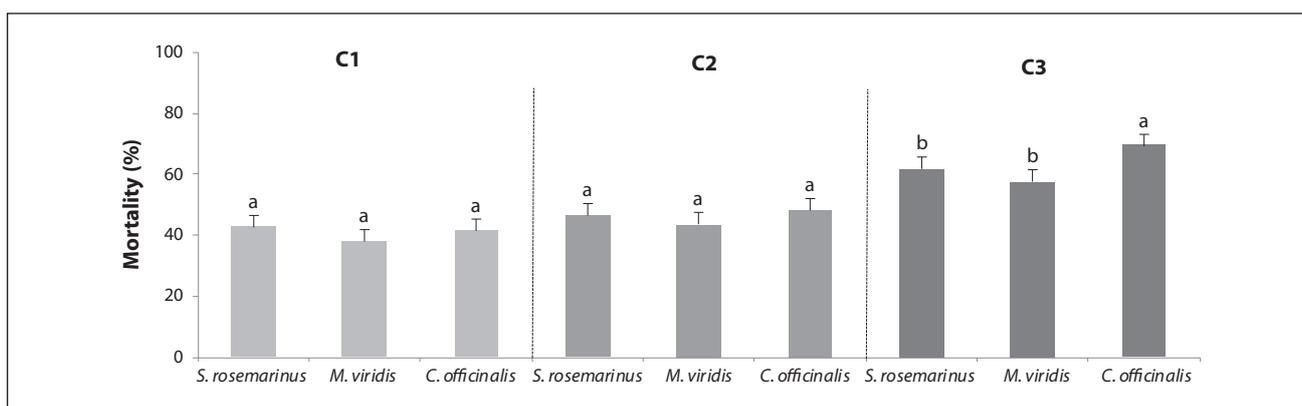
### *Myzus persicae* mortality and efficacy of plant extracts

The efficacy of the different concentrations of aqueous extracts of *C. officinalis*, *M. viridis* and *S. rosmarinus* plants in killing *M. persicae* was determined. Results in Table 3 indicate that the treatments resulted in from 38.24 to 69.82% mortality. The percentage efficacy ranged between  $32.41 \pm 1.23$  and  $61.71 \pm 4.46$ . The highest mortality ( $69.82 \pm 5.23\%$ ) and efficacy ( $61.71 \pm 4.46\%$ ) were recorded for treatment with C3 of the aqueous extract *C. officinalis* (Table 3). Whereas, the lowest mortality ( $38.24 \pm 2.42\%$ ) and efficacy ( $32.41 \pm 1.23\%$ ) were recorded for the treatment with C1 extract of *M. viridis*. Treatments with the three different concentrations of the extracts of *C. officinalis*, *M. viridis* and *S. rosmarinus*, resulted in a significant difference ( $\alpha < 0.01$ ) in mortality and efficacy (Table 3). No significant differences ( $\alpha > 0.01$ ) in mortality and efficacy were recorded for the treatments with C1 and C2 of *C. officinalis*, *M. viridis* and *S. rosmarinus* (Fig. 4, 5), but a significant higher ( $\alpha < 0.01$ ) mortality and efficacy was recorded for the C3 extract of *C. officinalis* treatment compared to that of *M. viridis* and *S. rosmarinus*.

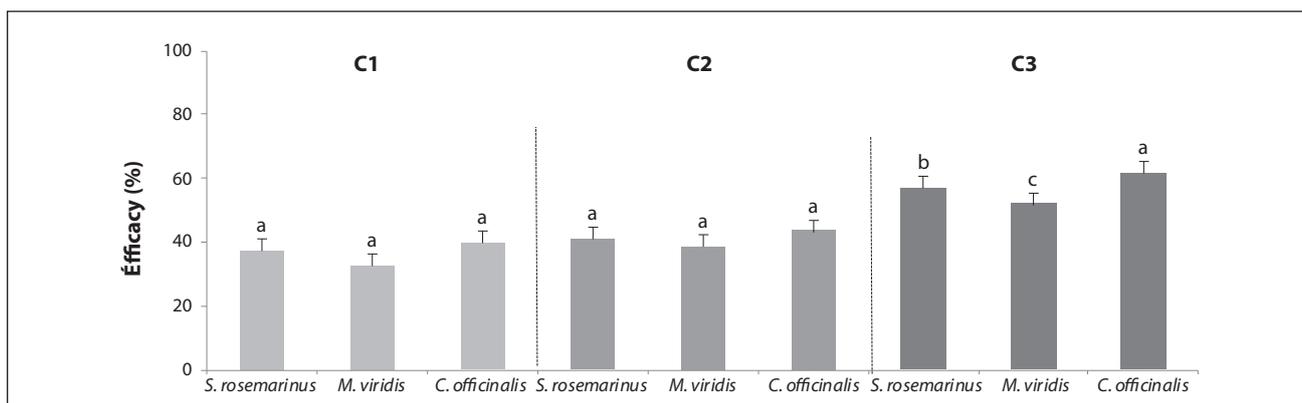
**Table 3** *Myzus persicae* mortality and efficacy obtained across treatments with concentrations of extracts of selected plants.

Selected plant	Concentration	Mortality (%)	Efficacy (%)
<i>C. officinalis</i>	C1	41.76 ± 2.15c	39.53 ± 2.42c
	C2	48.20 ± 3.45b	43.64 ± 2.57b
	C3	69.82 ± 5.23a	61.71 ± 4.46a
<i>M. viridis</i>	C1	38.24 ± 2.42c	32.41 ± 1.23c
	C2	43.34 ± 3.74b	38.74 ± 0.98b
	C3	56.66 ± 4.37a	51.86 ± 1.74a
<i>S. rosmarinus</i>	C1	42.71 ± 3.35c	37.23 ± 0.79c
	C2	46.33 ± 2.53b	40.85 ± 1.37b
	C3	61.76 ± 4.46a	56.81 ± 1.67a

Means followed by the same letter are not significantly different ( $\alpha < 0.01$ , Duncan's multiple range test). C1, C2 and C3: Concentrations.



**Fig. 4** Percentage mortality of *Myzus persicae* recorded treatments using three different concentrations of aqueous extracts of three species of plants. (Means followed by the same letter are not significantly different ( $\alpha < 0.01$ , Duncan's multiple range test).



**Fig. 5** Efficacy of three concentrations of aqueous extracts of three different species of plants in reducing the abundance *Myzus persicae* on pepper plants. (Means followed by the same letter are not significantly different ( $\alpha < 0.01$ , Duncan's multiple range test).

## Discussion and Conclusions

Many species of plants contain bioactive compounds that are used as botanical insecticides for controlling pests. These botanical insecticides are naturally safe and harmless for consumers, but for insects are toxic, repellent, antifeedants or growth regulators (Sertkaya et al. 2010; Erdogan and Yildirim 2016). Of the insects, *M. persicae*, is classified as a serious pest of pepper plants worldwide due to its high potential growth rate.

In this study, percentage infestation and fitness of *M. persicae* were determined. Results indicate that *M. persicae* have a high reproductive potential on pepper, with a mean relative growth rate and a generation time of 0.062 and 11.12 days, respectively. Results also indicate a high percentage infestation ( $76.9 \pm 9.6\%$ ). Fast development of *M. persicae* on pepper is reported with the generation time ranging between 10.23 and 13.51 days at temperatures between 20 and 25 °C (Satar et al. 2008).

Similarly, Ali et al. (2021) report that *M. persicae* develops faster on pepper (9.96 days) than on cabbage (14.2 days) or crown daisy (10.9 days). In addition, Mdellel et al. (2019) reports that the MRGR of *M. persicae* on pepper at 25 °C ranges between 0.046 and 0.068 depending on soil fertility. Several factors can influence the population growth of *M. persicae*, such the nitrogen level in the soil,

which has a direct effect on host plant quality, which affect the growth rate of *M. persicae* (Mdellel and Ben Halima 2014). Similarly, Dixon (1987) shows that nitrogen is an important factor affecting the fitness of aphids.

The bioassays of the effectiveness of extracts of three species of plants (*C. officinalis*, *M. viridis* and *S. rosmarinus*) in reducing the abundance of *M. persicae* on pepper plants revealed they were effective. Extracts at the highest concentration, C3, in all treatments was the most effective. Of the plant extracts, that of *C. officinalis* was more effective in reducing the abundance of *M. persicae* than that of *M. viridis* and *S. rosmarinus*. This might be attributed to differences in the insecticidal effects on aphids of the chemicals in these plants.

There is a report that flowers of *C. officinalis* contain flavonol glycosides, triterpene oligoglycosides, oleanane-type triterpene glycosides, saponins and a sesquiterpene glucoside (Ukiya et al. 2006). These compounds are highly toxic to plant sucking insects such as aphids and whiteflies and can inhibit feeding and growth of insect pests (Jankowska and Wilk 2011; Murrovhi et al. 2020). The other plant extracts (*M. viridis* and *S. rosmarinus*) were also effective in reducing the abundance of *M. persicae*. Samarasekera et al. (2008) report that the essential oil of *Menthus* spp. contains menthol which can adversely affect insects. Similarly, Ebadollah et al. (2020)

report that essential oils isolated from *Menthus* plants can adversely affect insects and could act as a repellent and antifeedant. In addition, same authors indicate that essential oils of *Menthus* spp. and other Lamiaceae contain a monoterpenoid component, which damages pests.

Studies on *Rosemarinus* reveal that it contains volatile compounds, which can be extracted and used as an effective fumigant against various insects such as coleopteran (*Tribolium* spp.; *Callosobruchus chinensis* (L.)) and lepidopteran pests like *Cadra cautella* (Walker) (Lee et al. 2002; Isikber et al. 2006). Terpenes and monoterpenes of *R. officinalis* affect the central nervous system of insects by inhibiting acetylcholinesterase enzymes (Krzyzowski et al. 2020).

Our study indicates that extracts of three plants can markedly reduce the abundance of *M. persicae* on pepper plants, especially the highest concentration extract of *C. officinalis*, and consequently reduce the damage caused by this pest. Thus, plant extracts could potentially be used to control aphid pests on different crops and as an effective biological control agent for use in an integrated management strategy for controlling aphids.

Further studies on the chemical components of the extracts of the plants tested are needed in order to determine how they affect insects and their effect on the yield and quality of crops. In conclusion, the present study confirms that the potential growth of *M. persicae* on pepper plants is very high and likely to result in serious damage. Extracts of three different species of plants namely: *C. officinalis*, *M. viridis* and *S. rosmarinus*, at three different concentrations, increased the mortality of this aphid. All the plant extracts tested were effective in reducing aphid populations. The extract of *C. officinalis* was more effective than that of *M. viridis* and *S. rosmarinus*. The highest concentration of all extracts, especially that of *C. officinalis*, were effective in reducing aphid numbers and thus in decreasing the damage done by this pest. Therefore, it is recommended that these plant extracts are used to manage aphid abundance instead of chemical insecticides. The use of these plant extracts can reduce the cost and minimize the negative effects of chemical pesticides on consumers and the environment.

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# A NOVEL APPROACH OF USING SHED SKINS OF THE GREEN TREE PYTHON, *MORELIA VIRIDIS*, FOR FORENSIC PURPOSES

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## ABSTRACT

Green tree python (*Morelia viridis*, Schlegel 1872) is a highly sought-after Indonesian/Papuan NG/Australian species in terms of the international trade in reptile pets. As the trade in wild animals is mostly prohibited nowadays, captive breeding supplies the international pet trade. There is evidence that captive breeding might be used as a cover for specimen's illegally sourced from the wild, as there are very few possibilities of distinguishing wild from captive-bred animals. These rely on invasive sampling (cutting off the end of the tail in order to obtain a sample of blood/muscle/bone tissues) or presence of ecto- and/or endoparasites (method overcome by breeders housing animals in semi wild conditions). Therefore, we examined the possibility of using stable isotope analysis for determining: either the place of origin or diet as a means of defining whether they are captive bred or illegally sourced from the wild. We also review the use of non-invasive samples of shed (moulted) skins. We conclude that shed skins that are currently not used for identifying the source of green tree python could be used as forensic evidence, subject to the development of a viable method.

**Keywords:** captive vs. wild; forensics; *Morelia viridis*; shed snakeskin

## Introduction

Live reptiles are among the most favourite animals in the world. They are widely traded as pets (Auliya 2003; Nijman and Shepherd 2011; Bush et al. 2014; Auliya et al. 2016; CITES trade database 2021). Therefore, they are subject to the Convention on International Trade in Endangered Species of Flora and Fauna, CITES. Czech Republic, despite its relatively low population (approx. 10 million people), plays an important role in the international trade in species protected by CITES (CITES trade database 2021).

One of the most popular internationally traded pets are live reptiles (Bush et al. 2014), with more than 6 million live CITES listed snakes exported over the period of 44 years (1975–2018) of CITES existence (Hierink et al. 2020), among them the green tree python, *Morelia viridis* (Wilson et al. 2006b). It is in the top 10 species traded internationally to and from the Czech Republic, with thousands of live specimens traded over the period 1998–2020 (CITES trade database 2021; Kufnerová, in prep.).

As the trade in wild animals is mostly prohibited nowadays, captive breeding supplies the international pet trade. There is evidence that captive breeding might be used as a cover for specimen's illegally sourced from the wild (Lyons and Natusch 2011), as there are very few possibilities of distinguishing wild from captive-bred animals. These rely on invasive sampling (cutting off the end of the tail in order to obtain a sample of blood/muscle/bone tissues) or presence of ecto- and/or endoparasites, which is unreliable because breeders often keep animals in semi-wild conditions and thus wild living and captive kept animals can host the same parasite species (Witter et al. 2016). In this paper, we therefore examined the pos-

sibility of using stable isotope analysis for determining: either the place of origin or diet as a means of defining whether they are captive bred or illegally sourced from the wild. We also review the use of non-invasive samples of shed (moulted) skins. We conclude that shed skins that are currently not used for identifying the source of green tree python could be used as forensic evidence, subject to the development of a viable method.

## The Green Tree Python

Although it is included in the *Morelia viridis* complex (still recognised by CITES (UNEP-WCMC and CITES-Secretariat 2013), based on genetic variability and slight morphological differences, it should be actually split into two distinct species: the more southern *Morelia viridis* and northern *Morelia azurea* (discussed in Maxwell 2005; Hynkova et al. 2008; Natusch et al. 2020). The border line between the distributions of these two species is the central mountain massive in New Guinea (Natusch et al. 2020). There are possibly three subspecies of *M. azurea*: *M. azurea azurea*, *M. azurea utaraensis* and *M. azurea pulcher* (Rawlings and Donnellan 2003; Hynkova et al. 2008; Barker et al. 2015; Natusch et al. 2020).

*M. viridis* is very popular among breeders (Wilson et al. 2006b). Its rarity and unusual colour are reflected in its high price (Auliya 2003; Maxwell 2005; Lyons and Natusch 2011). Its popularity resulted in many papers and books on its natural history, ecology, captive breeding and genetics of colour morphs (e.g., Maxwell 2005; Wilson 2011; Julander and Phillip 2017; Kyle 2020 etc.).

Green tree python is a tropical, nonvenomous, (mostly) arboreal, nocturnal snake of average size (up to 2 m

total length, 1.8 kg body weight), which is a native of New Guinea, a few surrounding islands (Normanby, Schouten Islands, Aru, Salawati, Mosool, Biak etc.) and northern tip of Australia (Cape York Peninsula). It prefers forest (both primary and secondary), which provides it with enough light and sufficient prey (Maxwell 2005). However, its distribution is highly patchy, possibly due to patchiness in prey abundance and/or illegal hunting, as on the Cape York Peninsula (Natusch and Natusch 2011).

Green tree pythons generally live up to 15 years in captivity and exceptionally even longer. Field studies indicate that only 50% of the adults breed each year and they are not sexually dimorphic, but females are slightly bigger than males (Wilson et al. 2006b; Natusch and Lyons 2014). The red or yellow young animals become vivid green on reaching adulthood at the age of 6 months to one year and are 53–59 cm in length. This change can be very fast and does not correspond with the shedding of skin (Wilson et al. 2006a; Wilson and Heinsohn 2007). The bright and conspicuous colour of juveniles seems to be adaptive (Natusch and Lyons 2021), as it possibly enables them to blend into the background of rain forest edges making them less visible to avian predators (Wilson et al. 2007).

Neither juveniles, nor adult males are territorial, but adult females live in territories about  $6.21 \pm 1.85$  ha in size. Females are more mobile than males, despite the fact that males search for females; during the wet season the pythons move more than during the dry season, which might correspond to mate searching; juveniles move similar distances to adult snakes despite their smaller size (Wilson et al. 2006a).

Green tree python is oviparous (18–24 eggs per clutch), eggs are brooded by the female for about 48–50 days (Maxwell 2005), but facultative thermogenesis recorded in other boid species to enhance embryonic development is not reported (Brashears and Denardo 2015). Hatchlings are either yellow (in *M. viridis*) or yellow or red (in *M. azurea*) (Natusch et al. 2020). The adult snakes are usually vivid green, with some geographical variation (Natusch et al. 2020) that takes the form of blue or white spots, white line along back or different coloured tail or labia, which makes this snake more “collectable” for the pet trade. These differences in morphology, colour, patterning and/or scalation are associated with particular geographical locations: “Aru”, “Biak”, “Merauke”, “Sorong”, “Irian Jaya”, “Jayapura”, “Kofiau”, “Cyclops”, “Manokwari”, “Wamena”, “Lereh” etc., and are either kept as separate pure bloodlines or crossbred to provide new interesting colour morphs that are traded for a higher price (Anonymous, personal communication 2021). However due to cultural differences, the “locality” name might not always be correct (Maxwell 2005).

Adult green tree pythons feed mainly on rodents, but they are reported feeding on birds and lizards (Natusch and Lyons 2014). Juveniles feed mostly on smaller prey such as beetles and moths (Maxwell 2005), but Natusch

and Lyons (2014) report them feeding on small lizards. The change in the diet corresponds with the change from the arboreal hunting of youngsters to ground hunting of adults, which is accompanied by changes in the anatomic structure of the snake’s head and ontogenetic changes in colour (Natusch and Lyons 2012).

The green tree python is not considered to be globally threatened according to the International Union for Conservation of Nature’s Red List of Threatened Species (Talowin et al. 2018). The threat to this species are the vast numbers caught for the international trade in pets, which could result in the extinction of small island populations (Natusch and Natusch 2011).

*M. viridis* is listed in Annex II to the CITES convention, as a member of the family Pythonidae (CITES Convention Appendices 2021), meaning that international trade is regulated by at least export permits issued by the relevant CITES management authority in the country of origin. Czech Republic became a CITES signatory in 1998, there was a separate national act No. 16/1998 Coll, on CITES, setting the national rules among others for export and import of CITES listed specimen. After the Czech Republic had joined the European Union in July 2004, Czech national legislation was replaced by EU legislation, represented by the main “Council Regulation (EC) No. 338/97 of 9 December 1996 on the protection of species of wild fauna and flora by regulating trade therein”, amended regularly by appendices containing species lists (called A, B, C, D, which correspond to CITES appendices I, II and III, and Annex D is for species not listed in CITES) and by implementing regulations. However, the legal status of *M. viridis* in the Czech Republic remained the same, this species is still listed in the second annex, both EU 338/97 “Annex B” and CITES “Annex II” listed. This listing allows international trade, providing the exporting country issues CITES export permits and importing country issues import permits (this rule is a bit stricter under EU 338/97 than the rule in the CITES convention, where only an export permit for CITES II species is required).

## Distinguishing Legal and Illegal Specimens

Distinguishing the legality of a specimen is one of the key factors in international trade regulated by CITES. Very often trade in wild-caught specimen is forbidden, whereas that in captive-bred specimens is legal. Indonesia banned the export of wild specimen in 1979 (Maxwell 2005), but allows trade in captive-bred specimens and other states do not allow the export of captive-bred or wild *M. viridis* for commercial purposes. Australia protects its native species by banning export for commercial purposes and allowing export for the purpose of research and for ZOOs under the Environment Protection and Biodiversity Conservation Act 1999, that became valid in 2000 (Australian Government 2021). However, misdec-

laration of wild-caught animals as captive-bred animals poses problems (Maxwell 2005; Lyons and Natusch 2011) wherever wild animals might and do occur (Natusch et al. 2020) and threatens the legal trade in captive-bred animals (Nijman and Shepherd 2009; Lyons and Natusch 2011). Therefore, we need good tools for distinguishing legal and illegal specimens.

There are only a few studies on distinguishing legal and illegal specimens, but research on this topic is growing. It includes use of endoparasites, genetic analysis, X-ray fluorescence elemental analysis and stable isotopes.

One of the methods proposed for *M. viridis* is the presence of intracellular parasites, *Sarcocystis* sp. that are more likely to be present in wild-caught animals or those reared under conditions in which they have access to free-living rodents (*Microtus*). Free-living mice serve as an intermediate host of *Sarcocystis*, but not house mice. Mice breeders declare that their mice are free from parasites and safe to feed to pet snakes. Snakes that are fed with in-house produced mice that are not an intermediate host for *Sarcocystis* sp. are unlikely to be infected (Moré et al. 2014).

Pernetta (2012) proposes monitoring breeding facilities and/or controlling the animals offered for sale in order to exclude wild-caught animals. He suggests microsatellite genotyping and parentage assignment techniques as suitable tools, because there is a library of polymorphic markers and the accuracy of parentage assignment techniques is well understood for this species, which makes the genetic approach readily available. This idea seems to be accurate and well scientifically supported. However, genetic methods are costly for the consumer and can be overcome by harvesting eggs from the animals in the wild, if only eggs are collected, or if gravid females are caught and released after laying eggs (personal observation).

The third method (elemental analysis) might be using high resolution X-ray fluorescence (XRF), which is already used for identifying echidnas (Brandis et al. 2018). It could also be used to distinguish legal and illegal specimens, or might also be using the inductively coupled plasma mass spectrometry (ICP-MS), when differences in elemental composition were found in skin of specimen of naturally dead (= wild living) snakes compared with for snakeskin trade slaughtered (= captive bred) snakes of 2 species (reticulated python *Malayopython reticulatus* and burmese python *Python bivittatus*) bred or found dead in Vietnam and Indonesia (Natusch et al. 2017). They also proposed to use this difference as one of the tools for distinguishing captive bred from wild living snakes of the two species studied.

Stable isotopes analysis (SIA) is used in ecology (Canarini et al. 2020; Liancourt et al. 2020; Kubásek et al. 2021), animal husbandry, forensic science and for consumer protection. It helps trace the routes of migratory species (Hobson and Wassenaar 2019), identify where they forage, their dietary habits and niches (Fry 2006;

Durso and Mullin 2017; Martín et al. 2017; Lobos et al. 2020), identify the source of meat (Bong et al. 2010; Kabalika et al. 2020). In human forensic science (Kramer et al. 2020; Chesson and Berg 2021) it also helps in identifying dead people. Natusch et al. (2017) described differences in isotopic ratios ( $^{13}\text{O}$ ,  $^{15}\text{N}$ ,  $^2\text{H}$ ) in raw snakeskins of captive bred and wild *P. reticulatus* and *P. bivittatus*. In their experiment, 5 different diets were fed to the captive bred animals resulting in statistically different isotopic ratios found 13 months later when the snakes were slaughtered for skin trade and the samples from their skins were analysed. Natusch et al. (2017) therefore propose the use SIA to differentiate captive bred from wild caught reticulated and burmese pythons; however, they propose to study in detail other python species and do agree that sampling the skin (in their analysis the skin was descaled) is an invasive method.

Because of all the above-mentioned findings, we foresee that for identifying legal and illegal specimens of *M. viridis* stable isotope analysis would appear to be a potential method.

#### Potential of shed skin as a forensic tool

SIA analysis in wildlife research is very often based on analysis of epidermal derivatives, such as feathers or hairs (Hobson and Wassenaar 2019). Snakeskin that serves many different life-protecting barrier functions for which it is well adapted (Tu et al. 2002; Klein and Gorb 2012, 2014, 2016; Torri et al. 2014; Kovalev et al. 2016) contains keratines. Keratines, like many other proteins, are composed of hydrogen, carbon, nitrogen, oxygen and sulphur, all of which have traceable stable isotopes ( $^1\text{H}$ ,  $^2\text{H}$ ;  $^{12}\text{C}$ ,  $^{13}\text{C}$ ;  $^{14}\text{N}$ ,  $^{15}\text{N}$ ;  $^{16}\text{O}$ ,  $^{18}\text{O}$ ;  $^{32}\text{S}$ ,  $^{33}\text{S}$ ,  $^{34}\text{S}$ ,  $^{36}\text{S}$ ) that are already used in SIA. Shed snakeskins also contain keratin and, therefore, potentially could be used for forensic purposes.

Shed skin is composed of several layers, rich in either  $\alpha$ -keratin,  $\beta$ -keratin, proteins or lipids. The sloughing of the skin is a complex process (Maderson et al. 1998; Alibardi 2005; Dalla Valle et al. 2007a, b), involving several enzymes (acid phosphatase, esterase) resulting in hydrolysis and death of cells in the lower layer (Singh and Mittal 1989). It is important to stress that all the above-mentioned structures and processes occur in both captive bred and wild-captured snakes, and involve biogenic elements (H, C, N, O, S) that can be used in SIA.

Green tree pythons shed their first skin about 10 days after hatching, then every 6-8 weeks during the first year of life. Later it is shed several times a year, depending on size, but usually every 2 months. Gravid females shed skin 20 days before they lay eggs. The whole shedding process takes about 10 days (Maxwell 2005) and is a predictable and detectable process. The shed skins, in addition to being a source of stable isotopes, could also be used for genetic analysis (Pernetta 2012) or elemental analysis (Brandis et al. 2018). However, we are not aware

of shed skins of *M. viridis* being used for forensic purposes.

Shed skins are easily collected without disturbing the snake. However, enforcement officers would have to wait for a sample and “waiting” for a snake to shed its skin might be seen as a disadvantage, but in the meantime, faeces could be searched for parasites providing the snakes were not treated medically to rid them of gut parasites before shipping. The behaviour of the animal can be also observed, as “domesticated” snakes are calmer than wild-caught animals, which are nervous and more likely to bite (Maxwell 2005). Therefore, in spite of the problem posed by having to wait for snakes to shed skins they are nevertheless an ideal source of stable isotopes for forensic evidence.

### Stable isotopes in wildlife research and as a forensic tool

Stable isotope analysis is used to identify counterfeit products, such as adulterated oils, juices etc. (Angerosa et al. 1997; Figuiera et al. 2011) and its use as forensic evidence is widespread (Gunn 2019). Isotopic ratios ( $^{13}\text{C}$  and  $^{15}\text{N}$ ) depend on what animals feed on, with a higher proportion of heavier isotopes in the tissues of predators than their prey (Perkins et al. 2014), and is already used in wildlife research. Stable isotopes in bird feathers can be used as an indicator of whether a bird is captive-bred or imported from elsewhere (Gunn 2019). The isotopic ratio in sea snakes changes with age and depends on the species of prey it has fed on (Brischoux et al. 2011).

Van Schingen et al. (2016) report that differences in food result in pronounced differences in the isotopic signatures of the scales taken from the ends of tails of captive-bred and wild living Vietnamese crocodile lizards, *Shinisaurus crocodilurus*. The stable isotope values of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  for the scales differed between the two groups (captive vs wild), with those for semi-captive animals intermediate, but closer to the captive group. The ranges in the values of both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  were lower in captive-bred animals than wild-caught ones, which feed on a much broader range of prey than captive-bred specimen that are usually fed a few species like crickets and mealworms. Van Schingen et al. (2016) conclude that stable isotopes can be used for distinguishing captive-bred from wild-caught lizards and in other species. Similar results were found by Natush et al. (2017) in skins of dead/killed *M. reticulatus* and *P. bivittatus*.

The main advantage of using shed skins is that most other methods are invasive and involve cutting the tip of the tail. However, even a “little cut” of 0.5 cm in length is often considered to be “non-invasive”, because the tip of the tail regenerates rather fast (van Schingen et al. 2016), but if it damages blood vessels it might result in infection and handling is stressful for the animal. Stable isotope analysis of shed skins, however, is non-invasive and potentially suitable for determining whether an animal was born in captivity or caught in the wild.

## Conclusions

There are still doubts about the origin of specimens of *M. viridis* and *M. azurea* supposedly reared in captivity, therefore tools to distinguish captive-bred from wild-caught specimens are needed.

Using stable isotopes as forensic evidence is based on the values of isotopes of oxygen, hydrogen and sulphur differing geographically and those of carbon and nitrogen depending on the food of the animal. This is well understood and already in place. Shed skins of snakes contain, among other things, keratins (rich in sulphur, nitrogen, carbon and oxygen) and histidine (rich in nitrogen, carbon and oxygen). This chemical composition is ideal for stable isotope analysis, aimed at determining the origin of specimens. Using shed skin, which is non-invasive, is an added advantage. Its use for forensic purposes needs further testing and determining how to overcome the problem of “waiting for the sample” that is needed for the test. Further research on *M. viridis* is needed because it is extensively internationally traded as a pet and there are doubts about the true origin of specimens supposedly “bred in captivity”, because many wild-caught animals are still being channelled through breeding farms.

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# TECHNICAL NOTE: DEVELOPMENT OF DNA QUANTITATION AND STR TYPING SYSTEMS FOR *PANTHERA TIGRIS* SPECIES DETERMINATION AND INDIVIDUAL IDENTIFICATION IN FORENSIC CASEWORK

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## ABSTRACT

The aim of this technical note is to provide an overview of methodical approaches used to develop molecular systems for species determination/DNA quantification called *Ptig Qplex* and individual identification called *Ptig STRplex* of *Panthera tigris* samples. Both systems will help to combat the illegal trade of endangered species and create a worldwide shared database of DNA profiles.

**Keywords:** CITES organism; DNA quantitation; forensic genetics; STR multiplex

## Introduction

Human DNA identification for forensic purposes is a valuable tool for identifying suspects and proving guilt in criminal cases. Forensic genetics evolved significantly since the foundation of this scientific field in the mid-1990s (Jeffreys et al. 1985). The evolution is nicely documented on the polymorphic loci used at different times. The initial restriction fragment length polymorphism (RFLP) methodology (Bush 1984) was soon replaced by PCR-driven methods that used variable number of tandem repeats (VNTRs) (Budowle et al. 1990), short tandem repeats (STRs) (Fregeau and Fourney 1993), chromosomal single nucleotide polymorphisms (White et al. 1998), mtDNA typing (Butler and Levin 1998), or insertions-deletions (InDels) (Zhao et al. 2010). The majority of records in forensic DNA databases (Puri 2000; Khan 2021) consist of STR profiles; thus, we postulate that STRs are currently core polymorphisms used for DNA-based forensic identification. However, the set of STR loci has changed over the years toward a higher number of more informative loci (Hares 2015). Nonhuman DNA typing for forensic purposes was initially focused on species determination (Bataille et al. 1999) and identification of individual domestic animals, such as cats (Menotti-Raymond et al. 1997) and dogs (Hellmann et al. 2006), because animal hair serves as evidence in criminal cases (Pfeiffer et al. 2004). STR profiling of animal species has a wider range of applications, such as wildlife preservation (Sastre et al. 2009), veterinary public health protection (Workman et al. 2005), and food safety (Vallejo-Cordoba and González-Córdova 2010). The application of forensic genetics is specifically useful to track poaching and illegal wildlife trades. Methods of species identification are used to decipher the genetic composition of

traditional Chinese medicine products (Votrubova et al. 2017) or animal parts (Panday et al. 2014), and individual STR-based identification is used to track ivory (Potoczniak et al. 2020), pangolin shells (Singh et al. 2020), and rhino horns (Harper 2021). The aim of this technical note is to provide an overview of methodical approaches used to develop molecular systems for species determination/DNA quantification (*Ptig Qplex*) and individual identification (*Ptig STRplex*) of *Panthera tigris* samples. Similar systems have not been developed for DNA quantification and multiplex STR analysis of *Panthera tigris* samples.

## Material and Methods

### *Ptig Qplex* DNA quantitation system

Molecular system for species determination/DNA quantification *Ptig Qplex* uses quantitative RT-PCR with TaqMan probes and primers highly specific for *Panthera tigris*. The assay requires only a single tube/well reaction.

Real-time monitoring of the reaction, including the detection of possible inhibition, was performed using internal amplification of internal positive control (IPC) DNA. IPC DNA, an artificially prepared sequence of 366 oligonucleotides, eliminates false negative results. Nuclear DNA concentrations were measured using the STR locus *Pati01* (Wu et al. 2009). Newly designed primers are not *Panthera tigris* specific, but they also amplify the DNA of some other large cats, such as *Panthera leo* (see Fig. 1). The concentration of mitochondrial DNA was measured using primers and probes targeted to the D-loop of mtDNA *Panthera tigris*. The target sequence is highly specific for this species only. We detected no amplification products of related species. The composition of primers and probes in the *Ptig Qplex* amplification mix is shown in Table 1.

**Table 1** Primers and probes used in Ptig Qplex.

Primer/probe name	Final concentration ( $\mu\text{M}$ )	PCR product size (*bp)	Specificity	TaqMan probe fluorescent label
qPtigM_f	0.75	121	<i>D-loop</i> (mtDNA)	–
qPtigM_r	0.75			–
qPtigM_p	0.25		probe	VIC
qPtigN_f	0.5	~215–260	STR locus <i>Pati01</i> ** (nDNA)	–
qPtigN_r	0.5			–
qPtigN_p	0.25		probe	6-FAM
qPtigC_f	0.5	366	IPC (Internal Positive Control)	–
qPtigC_r	0.5			–
qPtigC_p	0.25		probe	NED

\* bp = base pairs; \*\* Wu et al. 2009.

### qPCR reaction set-up

The qPCR thermocycler QuantStudio 5 (Applied Biosystems) was employed in this study.

The following qPCR conditions were employed: initial denaturation at 95 °C followed by 50 cycles of 95 °C for 10 secs and 60 °C for 20 secs.

The qPCR reaction included the following: 2× *TaqMan* Multiplex Master Mix (Applied Biosystems); 20× qPtig Assay Mix for mtDNA, nuclear DNA and IPC DNA (Forensic DNA service, CZE). The final reaction volume was 10  $\mu\text{l}$  and was prepared as described in Table 2.

**Table 2** The qPCR reaction setup.

qPCR reaction composition	10 $\mu\text{l}$ reaction	Final concentration in qPCR
2× <i>TaqMan</i> Multiplex Master Mix	5 $\mu\text{l}$	1×
20× qPtig mtDNA Assay Mix (20× qPtigM)	0.5 $\mu\text{l}$	1×
20× qPtig nDNA Assay Mix (20× qPtigN)	0.5 $\mu\text{l}$	1×
20× qPtig IPC DNA Assay Mix (20× qPtigC)	0.5 $\mu\text{l}$	1×
IPC DNA (0.1 pg/ $\mu\text{l}$ )	1 $\mu\text{l}$	0.1 pg
Template DNA	1 $\mu\text{l}$	different
H <sub>2</sub> O	1.5 $\mu\text{l}$	

### Ptig STRplex DNA profiling system

The molecular system *Ptig STRplex* enables the determination of the DNA profile from the sample of *Panthera tigris* based on the analysis of short tandem repeats (STRs). STRs used in the presented multiplexes contain tri- or tetranucleotide repetitions or complex repetitions (see Tables 3 and 4). The number of repeats within loci is highly variable. Fluorescently labeled amplified STRs were subsequently analyzed using capillary electrophoresis.

*Ptig STRplex* contains 11 pairs of fluorescently labeled primers divided into 2 multiplexes: *PtigPlex1* and *PtigPlex2*. The primers used in multiplexes have similar melting points and do not create primer dimers. Gender determination was performed using primers targeting the

SRY gene (Zou et al. 2015). The ZFX/ZFY locus serves as a supplementary system for gender determination, where both X and Y chromosome sequences are amplified (inspired by (Pilgrim et al. 2005).

**Table 3** STR multiplex *PtigPlex1 Panthera tigris*.

STR repeat name	Repeat structure	Size (bp*)	Primer concentration ( $\mu\text{M}$ )	5' primer fluorescent label
Ptig3	(TATC) <sub>n</sub>	118–190	0.16	FAM
Ptig5	(TAGA) <sub>n</sub>	152–220	0.15	ATTO565
Ptig6	(TGGAA) <sub>n</sub>	129–180	0.082	ATTO550
Ptig15	(TGAGA) <sub>n</sub> (CGAGA) <sub>n</sub> (CAAGA) <sub>n</sub>	187–250	0.36	YAKYE
Ptig17	(TC) <sub>n</sub> (GATA) <sub>n</sub> (TAGAGA) <sub>n</sub>	266–410	1.2	FAM
Ptig18	(TGTC) <sub>n</sub>	110–140	0.073	ATTO565
Gender				
ZnfXY*	–	164, 167	0.4	YAKYE

\* ZnfXY – males (YX) 164 bp and 167 bp; females (XX) 167 bp (Pilgrim et al. 2005).

\* bp = base pairs

**Table 4** STR multiplex *PtigPlex2 Panthera tigris*.

STR repeat name	Repeat structure	Size (bp*)	Primer concentration ( $\mu\text{M}$ )	5' primer fluorescent label
Ptig8	(ATCTAT) <sub>n</sub> (ATC) <sub>n</sub>	109–220	0.12	ATTO550
Ptig9	(CTAT) <sub>n</sub>	131–200	1.3	YAKYE
Ptig10	(AC) <sub>n</sub> (GTAT) <sub>n</sub>	162–240	0.7	FAM
Ptig11	(TATC) <sub>n</sub> (ATC) <sub>n</sub>	119–200	0.15	ATTO565
Ptig16	(ATAA) <sub>n</sub> (ATAC) <sub>n</sub>	90–150	0.45	FAM
Gender				
SRY*	–	100	0.07	ATTO565

\* SRY – male, 100 bp; female, no product (Zou et al. 2015).

\* bp = base pairs

### PCR set-up

PCR mix: Gold Star 10× buffer (Promega), *AmpliTaq Gold* DNA Polymerase (Applied Biosystems), and 10× *PtigPlex1* Primer Mix or 10× *PtigPlex2* Primer Mix (Forensic DNA service, CZE). The total volume of the PCR reaction is 12.5  $\mu\text{l}$ . Details are provided in Table 5.

**Table 5** PCR set-up.

PCR	12.5 $\mu\text{l}$ reaction	Final concentration in PCR
Gold Star 10× buffer	1.25 $\mu\text{l}$	1×
10× <i>PtigPlex1</i> Primer Mix or 10× <i>PtigPlex2</i> Primer Mix	1.25 $\mu\text{l}$	1×
Template DNA	different	10 pg nuclear DNA
<i>AmpliTaq Gold</i> DNA polymerase	0.25 $\mu\text{l}$	2.5 U/PCR
H <sub>2</sub> O	to 12.5 $\mu\text{l}$	

PCR thermocycler MasterCycler Nexus gradient (Eppendorf) was employed in this study.

The following PCR conditions were employed: initial denaturation at 95 °C for 11 mins followed by 32 cycles of 94 °C for 30 secs, 60 °C for 1 min 10 secs, and 72 °C for 1 min and 10 secs; final extension at 72 °C for 60 min, followed by 60 °C at 60 min.

#### Fragment analysis set-up

**STR multiplex *PtigPlex1*:** 0.5 µl PCR product + 12 µl deionized formamide (Hi-Di formamide) + 0.3 µl size standard (Size Standard LIZ600, Applied Biosystems)

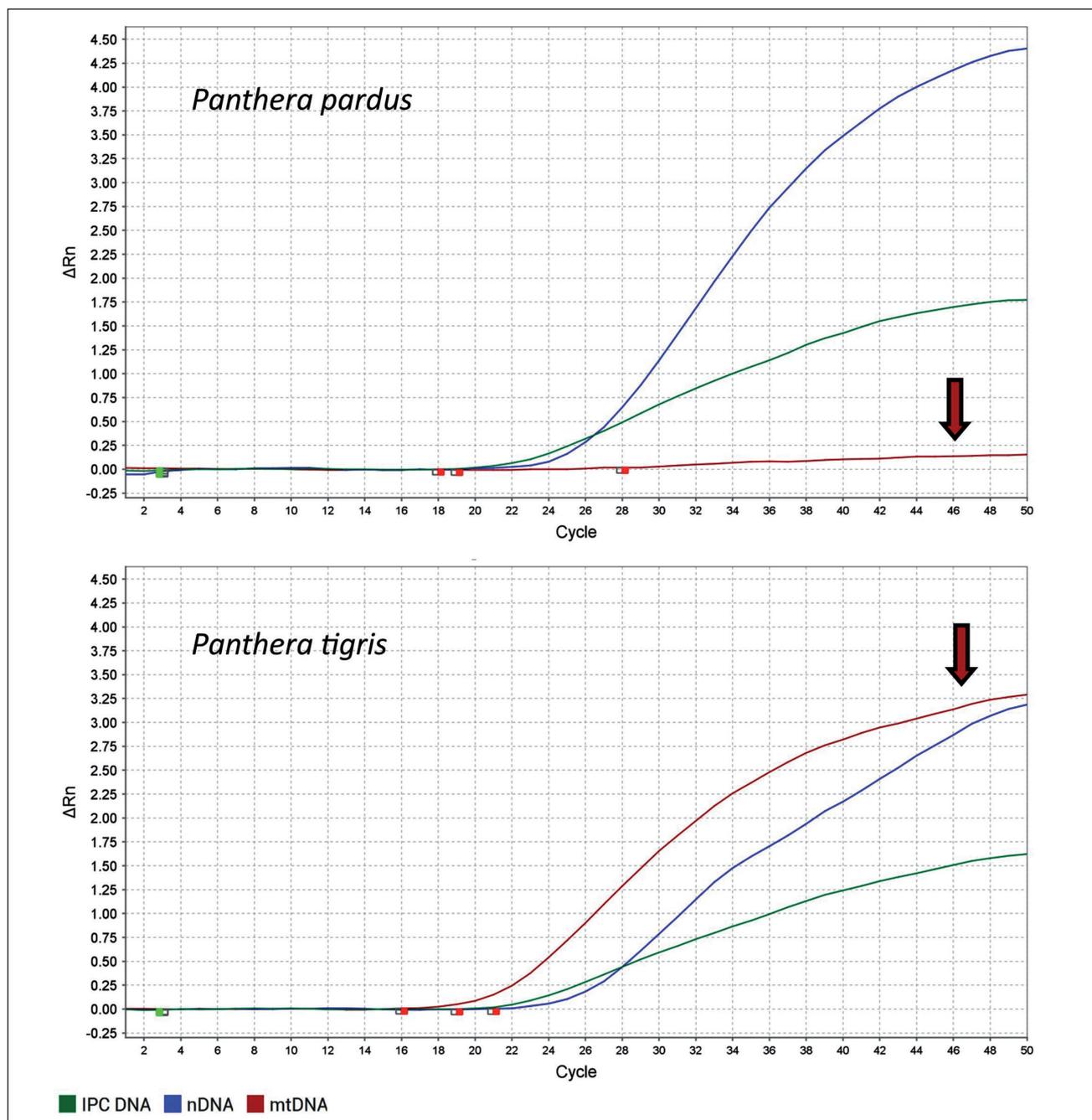
**STR multiplex *PtigPlex2*:** 0.8 µl PCR product + 12 µl deionized formamide (Hi-Di formamide) + 0.3 µl size standard (Size Standard LIZ600, Applied Biosystems).

Capillary electrophoresis SeqStudio 3200 Genetic Analyzer (Applied Biosystems) was employed in this study. Raw data processing was done by GeneMapper5 software (Applied Biosystems).

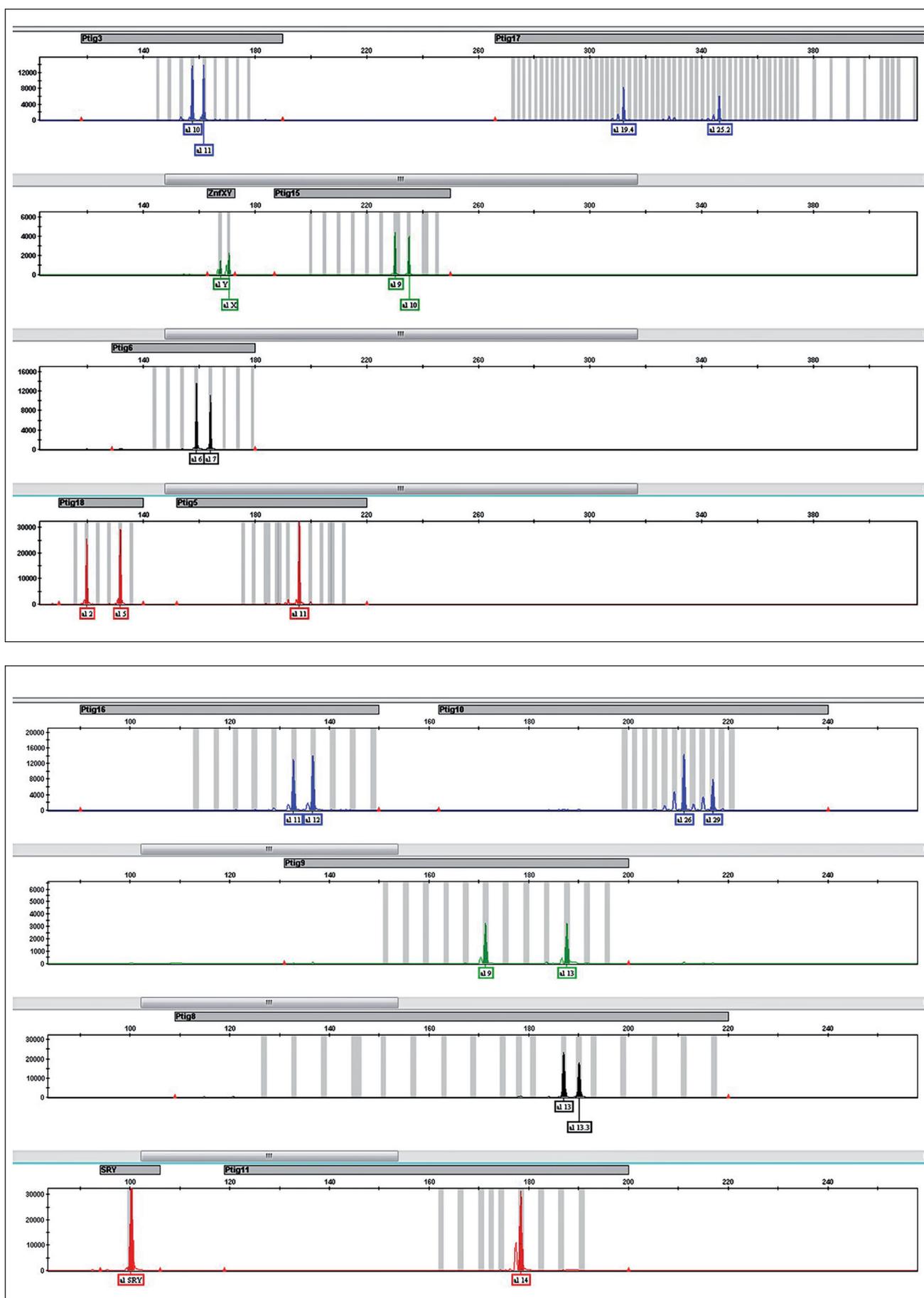
#### Results

Examples of QuantStudio 5 (Applied Biosystems) quantitation and species determination outputs are shown in Fig. 1.

Examples of the resulting DNA profiles with *PtigPlex*-es are shown in Fig. 2.



**Fig. 1** The resulting qPCR plots generated using *Ptig Qplex* – comparison of nuclear DNA quantitation (blue curve) and mitochondrial DNA (red curve) in different cats. IPC DNA (green curve) serves as an internal positive control for qPCR. An arrow points to *Panthera tigris*-specific mtDNA amplification.



**Fig. 2** Resulting electropherograms *PtigPlex1* (a) and *PtigPlex2* (b) with a DNA profile consisting of 11 STRs and sex (male) determination from samples obtained from *Panthera tigris*.

## Conclusions

In July 2018, Czech Republic authorities raided premises in Prague and other locations, revealing a tiger slaughterhouse at the center of an international criminal trade ring. The raids, under the name Operation Trophy, were the culmination of two-and-a-half years of work and employed more than 200 enforcement officers from customs, police and the Czech Environmental Inspectorate. In the illegal slaughterhouse, they found a freshly killed tiger that was shot through the skull to leave its skin undamaged; a boiler for preparation of tiger glue; numerous tiger claws, bones and skins; and dozens of dead animals that were often in a state of decay. DNA analyses focused on species identification and individual identification of *Panthera tigris* samples. Genetic analysis performed using the above-described *Ptig Qplex* and *Ptig STRplex* identification systems enabled the identification of biological material from 7 different individuals of *Panthera tigris* and other CITES protected species. The illegal tiger trade seems to be more extensive than originally estimated. It exists not only in Asia but also in Europe. The offenders trade less recognizable types of goods, such as bones, claws, teeth, broth, paste, wine, and powder. Our participation in the case provided evidence that tigers from private breeders are used as a source for traditional Chinese medicine. The idea of our research team is to provide testing capacity and/or testing kits meeting the ISFG recommendations for nonhuman DNA typing (Linacre et al. 2011) to combat the illegal trade of endangered species and create a worldwide shared database of DNA profiles.

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