

## Detection of *Ophidiomyces ophidiicola* in wild barred grass snakes (*Natrix helvetica*) in the Netherlands

*Detectie van Ophidiomyces ophidiicola bij wilde ringslangen (Natrix helvetica) in Nederland*

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### ABSTRACT

*Ophidiomyces ophidiicola* is the causative agent of ophidiomycosis (also called snake fungal disease) and has been associated with mycosis in individual snakes and population declines in several North American snake species. While cases of ophidiomycosis from North America have been numerous in the past two decades, reports from Europe remain sparse and are lacking from the Benelux. Between 2017 and 2023, sloughed skins, carcasses and swabs from 284 free-ranging, captive and introduced snakes belonging to 22 species were screened for the presence of *O. ophidiicola* in the Netherlands. Two free-ranging barred grass snakes' (*Natrix helvetica*) shed skin sloughs tested positive despite a lack of gross cutaneous signs of disease. These findings are presented in this article. To the authors' knowledge, this is the first report of the presence of *O. ophidiicola* in the Netherlands.

### SAMENVATTING

De schimmel *Ophidiomyces ophidiicola* veroorzaakt ophidiomycose (ook wel "snake fungal disease") bij individuele slangen en wordt geassocieerd met dalende populaties van diverse Noord-Amerikaanse slangensoorten. Alhoewel gevallen van ophidiomycose de laatste twee decennia in Noord-Amerika talrijk zijn, werden er in Europa nog maar enkele meldingen gerapporteerd. *O. ophidiicola* werd in de Benelux tot nu toe niet gedetecteerd. Tussen 2017 en 2023 zijn vervellingen, karkassen en huidswabs van 284 wilde, geïntroduceerde en van in gevangenschap gehouden slangen, behorende tot 22 soorten, in Nederland getest op de aanwezigheid van *O. ophidiicola*. Twee vervellingen van wilde ringslangen (*Natrix helvetica*) testten positief op de aanwezigheid van de schimmel, alhoewel beide stalen geen zichtbare afwijkingen consistent met de ziekte vertoonden. Volgens de auteurs is dit de eerste beschrijving van *O. ophidiicola* in Nederland.

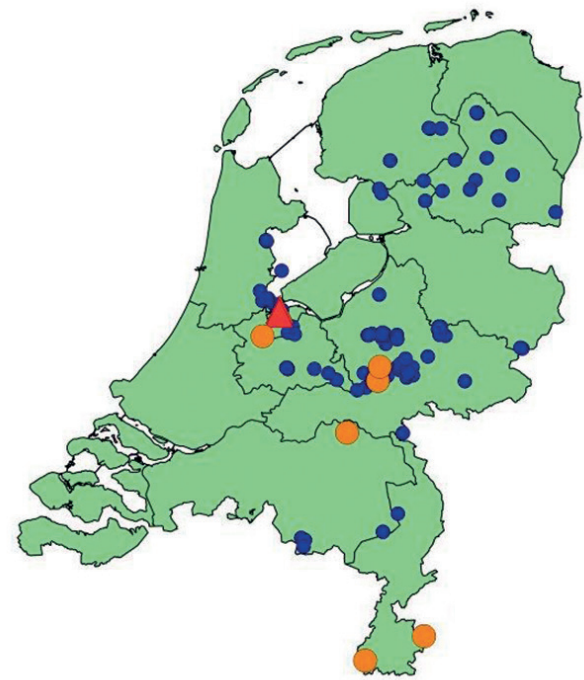
### INTRODUCTION

The fungal pathogen *Ophidiomyces ophidiicola* is the causative agent of ophidiomycosis, also known as snake fungal disease (Lorch et al., 2016; Lorch et al., 2015; Davy et al., 2021). Individuals infected by *O. ophidiicola* may display mild signs of disease, such as small (visible) cutaneous lesions, crusts and ulcera-

tions to more deleterious signs such as severe difficulties with ecdysis, impairment of movement including a lack of righting reflex, which affects foraging- and thermoregulatory behavior but also impairs predator evasion (Allender et al., 2015a; McKenzie et al., 2021). This in turn may lead to a reduction in fitness, which may affect reproductive output and increases the risk of predation in individual ophidians (Lorch et

al., 2015a; Tetzlaff et al., 2017). Currently, *O. ophidiicola* has been detected on three continents in 62 wild ophidian species as well as in various captive collections and (wild) specimens in trade (Takami et al., 2020; Blanvillain et al., 2022; Ladner et al., 2022). Most past research has focused on North America due to earlier detection of the pathogen on this continent, and due to a high prevalence in certain taxa and population declines in several species in association with ophidiomycosis (Clark et al., 2011; Burbink et al., 2017; Allender et al., 2018). The pathogen is present in Europe, and clinically sick ophidians have been found on this continent (Franklinos et al., 2017; Allain et al., 2024). Ophidiomycosis-induced population declines in Europe have not been detected yet (Blanvillain et al., 2022; Allain et al., 2024). To date, three distinct *O. ophidiicola* clades have been described. Clade I has only been detected in free-ranging snakes in Europe. Clade II has been reported in free-ranging snakes in North America, Taiwan and captive populations worldwide. Clade III has thus far only been found in two wild ophidians in Taiwan and captive populations (Sun et al., 2021; Ladner et al., 2022). Recent studies reveal that clade I and II have been present in Switzerland since at least 1959 and have been present in other sites in Europe as well (Blanvillain et al., 2022; Origgi et al., 2022). Clade II might be more virulent than clade I due to higher growth rates (Franklinos et al., 2017). Molecular dating suggests that clade I and II diverged approximately 2000 years ago, and indicates that the presence of clade II in North America might be the result of several recent introductions, followed by subsequent geographical expansion (Ladner et al., 2022). Due to the presence of clade II in many captive collections, spillover to wild populations is a risk, which may have occurred in North America (Ladner et al., 2022).

Until recently, knowledge on *O. ophidiicola* presence and distribution in free-ranging European ophidians has been limited, with initial records dating back to 2010 (Franklinos et al., 2017). These first reports hailed from the United Kingdom and the Czech Republic, followed by Switzerland, Italy and Germany (Franklinos et al., 2017; Meier et al., 2018; Origgi et al., 2022; Marini et al., 2023; Schöler et al., 2024). In a recent study, in which an updated distribution overview of *Ophidiomyces ophidiicola* in continental Europe is presented, the known occurrence of *O. ophidiicola* has been significantly extended, and tentative hotspots (in order of prevalence) have been identified in Switzerland (and adjacent France and Italy) (26.7%), Germany (12.5%) and 12.1% in Ukraine (Blanvillain et al., 2022). *O. ophidiicola* has also been detected in Poland and in the Slovak Republic (Blanvillain et al., 2022; Přibyl, et al., 2023). High prevalences may be found in populations of semi-aquatic ophidians and species with a preference for moist habitats like *Natrix helvetica*, *Natrix natrix*, *Natrix tessellata*, and *Zamenis longissimus*. Both clade I and clade II



- ▲ *Ophidiomyces ophidiicola* detected (n=2)
- *Ophidiomyces ophidiicola* not detected (n=249)
- Cutaneous gross signs consistent with Ophidiomycosis (n=6)

**Figure 1.** Map of the Netherlands showing where samples were collected to assess presence of the fungal pathogen *Ophidiomyces ophidiicola* in free-ranging snakes. (Map: Tariq Stark).

have been detected in Europe. European *O. ophidiicola* hotspots, including host assemblages with more severe infections, could be explained by the presence of a certain clade(s), presence of highly susceptible host species or a combination of both (Blanvillain et al., 2022; Ladner et al., 2022). Records of *O. ophidiicola* in free-ranging snakes from continental Western Europe are sparse, despite the presence of susceptible host species like *N. helvetica* and *N. natrix* (Blanvillain et al., 2022). Even with syndromic surveillance in place and opportunistic sampling in the Netherlands since 2017, positive samples remain absent (Stark et al., 2023). To the authors' knowledge, this is the first report of *O. ophidiicola* via two qPCR positive samples derived from two free-ranging *N. helvetica* in the Netherlands.

## METHODS

Samples were acquired via three routes. Firstly, volunteers of Reptile, Amphibian, Fish Conservation Netherlands (RAVON) were asked to report free-ranging snakes with suspected ophidiomycosis, but also to collect sloughed skin sheds and carcasses of both native and introduced (exotic) free-ranging snakes.

Secondly, a similar request was sent out to the general public, who were also asked to report snakes displaying signs of disease or collect skin sheds and carcasses of captive snakes (syndromic surveillance). The general public was targeted via (social) media campaigns, radio and TV. Finally, RAVON professionals collected samples haphazardly and also more targeted, when a ophidiomycosis suspected dead or diseased snake was reported. In this dataset, several introduced, free-ranging species were included. In the Netherlands, eastern grass snakes (*Natrix natrix*) but also native barred grass snakes (*Natrix helvetica*) have been illegally introduced in several areas (van Riemsdijk et al., 2020; Asztalos et al., 2021). Introduced, free-ranging *Pituophis* sp. in a non-disclosed location in the coastal dunes have also been screened (Struijk et al., 2018). Skin sheds were stored dry in paper envelopes and frozen at -23°C in paper until the time of analysis. Carcasses and skin swabs were also stored at -23°C.

In the present study, DNA was extracted from swabs, sheds and skin samples using Qiagen DNeasy Tissue kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions with an added lyticase (300 U, 1 hour, 37°C) step. Sheds were ground to powder before DNA extraction using stainless steel grinding balls (20 mm) for 1 to 2 minutes at 30 Hz. A total of 50 mg of powdered skin shed was weighed and placed in 1.5 mL Eppendorf tubes for further DNA extraction. Details with regard to primers, targeting the ITS1 segment between the 18S and 5.8S RNA gene (OphioITS-F, OphioITS-R), probe (Probe-FAM) and cycling parameters (1 cycle at 50°C for 2 minutes followed by 95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds, and a final cycle of 72°C for 10 minutes) of the qPCR assay to detect *O. ophidiicola* DNA (Allender et al., 2015b). The dynamic range of this assay is from  $1.05 \times 10^8$  to  $1.05 \times 10^1$  gene copies.

## RESULTS

Between 2017-2023, a total of 301 samples from 241 free-ranging snakes, 33 captive individuals and 10 introduced snakes in the Netherlands (n=284 individuals) were screened for the presence of *O. ophidiicola* (Figure 1, Table 1). In total, they comprised 22 species, representing 17 genera. Free-ranging *N. helvetica* were well represented in the dataset (44.4%), followed by *Vipera berus* (22.5%) and *Coronella austriaca* (18.0%). The remainder can be assigned to captive individuals of various families (11.6%) and introduced *Natrix helvetica* (1%) and *Pituophis* sp. (2.5%). Of the 301 samples, 231 were skin sloughs (76.8%), 54 carcasses (17.9%) and 16 swabs (5.3%). Three free-ranging *N. helvetica*, one introduced *N. helvetica* and two free-ranging *C. austriaca*, showed gross signs consistent with ophidiomycosis infection (Figures 1 and 2, Table 1). *O. ophidiicola* was not detected

in these individuals.

*O. ophidiicola* was detected in two skin sheds of free-ranging *N. helvetica*. Both skin sheds were found in September 2019 (Table 1). One skin shed belonged to an adult and one to a subadult (respectively found on September 10<sup>th</sup> and September 18<sup>th</sup>, 2019). Both samples originated from the Naardermeer, a nature reserve in the west of the Netherlands (52.295759 -5.116825, WSG84). Gross signs of ophidiomycosis were not found in either of these samples. *O. ophidiicola* was not detected on two *N. helvetica* skin sheds found in the same area in June and July of that same year.

## DISCUSSION

The detection of *O. ophidiicola* in Europe came rather late in comparison with North America. Targeted efforts to detect the pathogen thus started later than in North America (Franklinos et al., 2017). This lag in detection could be the result of several factors, including the still relatively limited awareness about ophidiomycosis among ecologists, nature volunteers, and the



**Figure 2.** Depictions of a grass snake (*Natrix helvetica*) with signs of suspected ophidiomycosis. The pathogen was not detected on individuals displaying such signs (Photographs: Richard Struijk and Tariq Stark).

**Table 1. An overview of snakes screened for the presence of *Ophidiomyces ophidiicola* between 2017-2023. The positive samples are displayed in bold and refer to two *N. helvetica* sloughs both found in September 2019.**

Species	Individuals sampled	Number of samples	Slough: carcass: swab	Adult:subadult: juvenile	Symptomatic individuals	Wild: captive :introduced	Oo positive samples (qPCR)
<i>Agkistrodon bilineatus</i>	1	1	1:0:0	1:0:0	0	0:1:0	0
<i>Aspidelaps cowlesi</i>	3	3	3:0:0	3:0:0	0	0:3:0	0
<i>Aspidelaps lubricus</i>	1	1	1:0:0	1:0:0	0	0:1:0	0
<i>Boa constrictor occidentalis</i>	2	2	2:0:0	2:0:0	0	0:2:0	0
<i>Coelognathus helena</i>	2	2	2:0:0	2:0:0	0	0:2:0	0
<i>Coronella austriaca</i>	52	53	22:28:3	18:14:20	2	51:1:0	0
<i>Daboia palaestinae</i>	1	1	1:0:0	1:0:0	0	0:1:0	0
<i>Eryx colubrinus loveridgei</i>	1	1	1:0:0	1:0:0	0	0:1:0	0
<i>Lampropeltis triangulum</i>	2	7	7:0:0	2:0:0	0	0:2:0	0
<i>Macrovipera lebetinus schweizeri</i>	1	1	1:0:0	1:0:0	0	0:1:0	0
<i>Montivipera raddei kurdistanica</i>	1	1	1:0:0	1:0:0	0	0:1:0	0
<i>Natrix helvetica</i>	<b>130</b>	<b>138</b>	<b>115:17:6</b>	<b>84:34:12</b>	<b>4</b>	<b>126:1:3</b>	<b>2</b>
<i>Natrix maura</i>	5	7	2:5:0	0:5:0	0	0:5:0	0
<i>Natrix natrix</i>	1	1	1:0:0	1:0:0	0	0:1:0	0
<i>Naja haje</i>	2	2	2:0:0	2:0:0	0	0:2:0	0
<i>Naja nigricincta</i>	2	2	2:0:0	2:0:0	0	0:2:0	0
<i>Naja nubiae</i>	2	2	2:0:0	0:2:0	0	0:2:0	0
<i>Pantherophis guttatus</i>	1	1	1:0:0	1:0:0	0	0:1:0	0
<i>Pituophis</i> sp.	7	8	1:0:7	7:0:0	0	0:0:7	0
<i>Pseudechis colletti</i>	1	1	1:0:0	1:0:0	0	0:1:0	0
<i>Trimeresurus trigonocephalus</i>	1	1	1:0:0	0:1:0	0	0:1:0	0
<i>Vipera berus</i>	65	65	61:4:0	58:7:0	0	64:1:0	0

general public. Hence, infections could go unnoticed, resulting in unclarity about the impact of ophidiomycosis in Europe (diNicola et al., 2022; Ladner et al., 2022; Allain et al., 2024). Since the first detection of *O. ophidiicola* in Europe, knowledge on the spatial and temporal presence of the fungus including strain (clade) types, but also host range has increased significantly. Uncertainty remains about the impact of the pathogen on European snakes (Blanvillain et al., 2022; Origgi et al., 2022). The outcome of the disease might be exacerbated by the type(s) of clades and host assemblages involved, in combination with environmental and anthropogenic factors (Allain et al., 2024; Joudrier et al., 2024). Cases of more severe infections are known from Switzerland and Italy, where clade I and II are present in host assemblages (*Natrix* sp.) whereas disease outcome may be less severe where only one clade (I) is involved (Allain et al., 2024; Joudrier et al., 2024). Susceptible hosts in the form of native *N. helvetica*, introduced *N. natrix*, and to a lesser extent *V. berus* populations, occur in the Netherlands (Franklinos et al., 2017). Individuals with signs consistent with ophidiomycosis were found in this study but despite the presence of cutaneous macro-

scopic lesions, *O. ophidiicola* was not detected on any of these ophidians (Figure 1). Carcasses, shed skin and dry skin swabs of such individuals, in one case all three sample types originating from one individual, have been screened without the detection of *O. ophidiicola* (Table 1). Other pathogens capable to cause skin disease in ophidians, like *Paranannizziopsis* spp., cannot be ruled out as causative agents for these ophidiomycosis-like signs (Dubey et al., 2022; Lorch et al., 2023). *N. helvetica* shed skin sloughs, which tested positive for the presence of *O. ophidiicola*, did not show signs of infection (Table 1). Based on the ophidiomycosis classification system by Baker et al. (2019) and Davy et al. (2021), these results are not abnormal. Based on the presence or absence of gross lesions and fungal hyphae, individual snakes can be assigned to one of the five ophidiomycosis categories: ophidiomycosis negative (no lesions and qPCR negative), ophidiomyces present (no lesions and qPCR positive), possible ophidiomycosis (lesions present and qPCR negative), apparent ophidiomycosis (lesions present and qPCR positive), and confirmed ophidiomycosis (histopathology showing arthroconidia and positive qPCR/culture). The lack of clear signs of

infection might be attributed to an early stage of infection in these particular hosts (diNicola et al., 2022; Ladner et al., 2022). Lesions might also be very small and are easy to miss on skin sloughs, as demonstrated in a study by Franklinos et al. (2017). The possible impact of disease outcome in relation to environmental factors, *O. ophidiicola* strain and a particular host species or species assemblages in Europe is largely unknown. The population where *O. ophidiicola* was detected in this study is part of the Dutch national reptile monitoring program, but no apparent population declines have been noted on this particular transect. At a national level, the population trend of *N. helvetica* shows a moderate decline in the Netherlands but it is unclear if the presence of *O. ophidiicola* plays any role in this decline (RAVON, 2023).

The involvement of citizens in this research in order to rapidly detect and limit the spread of deleterious pathogens can be highly effective (Lawson et al., 2015; Thomas et al., 2019). People can contribute to early detection both actively and passively via adhering to biosecurity measures, active monitoring, syndromic surveillance and passive surveillance (Lawson et al., 2015; Bird and Mazet, 2018). In this study, the involvement of citizen science proved crucial to collect samples in order to detect an apparently highly cryptic fungus and will be critical to gain a better understanding about the impact of ophidiomycosis in Europe in future research and conservation efforts.

## ACKNOWLEDGEMENTS

This study was performed under permit FF/75A/2016/015 issued by Reptile, Amphibian and Fish Conservation Netherlands. Dutch land owner organizations that provided field permits are Natuurmonumenten (Society for Preservation of Nature Monuments in the Netherlands) and Staatsbosbeheer (State Forestry). All involved volunteers and citizen scientists are thanked for their indispensable contribution to this study. Peter Schilperoord and Richard de Jong are thanked for samples of captive snakes. RAVON colleagues, Arnold van Rijsewijk, Jesper Berndsen, Raymond Creemers, Jelger Herder, Jöran Janse and Ronald Laan are thanked for their contributions to the study. The Ministry of Agriculture, Nature and Food Quality, the 'Van der Hucht de Beukelaar Stichting', the European Snake Society and Stichting Herpetofauna are thanked for their financial support of this study.

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