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Screening of *Ophidiomyces ophidiicola* in the free-ranging snake community annually harvested for the popular ritual of *San Domenico e dei Serpari* (Cocullo, AQ, Italy)

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Abstract. In the Abruzzi village of Cocullo (Italy), each year, on May 1st, local snake hunters (known as *Serpari*) display colubrids, captured in the wild, to commemorate the ancient ritual of San Domenico. The ascomycete *Ophidiomyces ophidiicola* (Oo) is the causative agent of ophidiomycosis, an emerging disease with sublethal effects. Skin lesions, such as dysecdysis, edematous, crusty or necrotic scales, swellings, nodules, and ulcers, are the most common clinical manifestation of the disease. The pathogen and its associated disease are well characterized in wild snakes in North America, whereas broad screenings of free ranging wild ophidians in Europe are rare. In 2019, as part of a multi-year snake health monitoring project, all the Cocullo ophidians were carefully examined for integumentary affections and those showing signs consistent with ophidiomycosis were dry swabbed on the skin and on any visible cutaneous lesions with a single applicator. The extracted DNA underwent a broad-range panfungal PCR targeting the D1-D2 region, as well as two conventional PCRs specific to the ITS2 and IGS regions of Oo DNA. Twenty-three out of 129 snakes (13/82 *Elaphe quatuorlineata*; 7/31 *Hierophis viridiflavus*; 3/15 *Zamenis longissumus*; 0/1 *Natrix helvetica*) resulted clinically affected, but no specific Oo genomic DNA was detected by PCR. The Cocullo ritual celebration provided a unique opportunity for the first systematic testing of a large sample size of a local snake community for the monitoring of this pathogen in Italy.

Keywords. Ophidiomycosis, Snake Fungal Disease, SFD, snakes, health monitoring, Cocullo, Abruzzi (Italy).

Ophidiomyces ophidiicola (Oo) is the etiological agent of ophidiomycosis (also known as Snake Fungal Disease – SFD), a fungal infection of snakes (Lorch et al., 2015). This onygenalean fungus is resistant to various physical and chemical agents (Allender et al., 2015b), and hibernacula may represent its environmental reservoir (Campbell et al. 2021). *Ophidiomyces* infection has been associated with sublethal effects on adults (Agugliaro et al., 2020; Lind et al. 2018a, b; Tetzlaff et al., 2017) and potentially lethal outcomes on newborns (e.g., Britton et al., 2019), translating into a potential impact on wild populations' fitness and a threat to conservation. This emerging

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infectious disease occurs with various cutaneous signs as dysecdysis, desquamation, scales abnormalities (e.g., displacing), local skin thickening, yellowish/brownish crusts, skin ulcerations, swelling and nodules (revised by Baker et al., 2019), whereas visceral lesions are less frequently recorded. Albeit impacts on different populations seem locally divergent or controversial, Oo has been detected in free-ranging ophidians in most part of North America (Di Nicola et al., 2022). In Europe, samples deriving from wild *Coronella austriaca, Hierophis viridiflavus, Natrix helvetica, N. maura, N. natrix, N. tessellata, Vipera berus, V. nikolskii* and *Zamenis longissimus* from UK, Czech Republic, Switzerland, Germany, France, Austria, Hungary, Poland, Ukraine, or Italy tested positive with molecular methods (Franklinos et al., 2017; Meier et al., 2018; Schüler et al., 2022; Blanvillain et al., 2022; Marini et al., 2023), and a retrospective analysis date back the presence of the fungus in Italy and Switzerland since 1959 (Origgi et al., 2022).

In this paper we report the results of an investigation aimed at testing the presence of Oo in a snake community from Central Italy. Data were obtained by snakes captured for a religious ritual (the Catholic cult of *San Domenico* – of pagan and pre-Christian origins) in the village of Cocullo (Abruzzi). This ceremony takes place in the first days of May, and has remained unchanged for several hundred years. The main feature of this occurrence is the presence of large numbers of wild-caught snakes by local snake hunters (*serpari*) during the weeks before the events. This ritual is well known and important in Abruzzi's (Italy) culture and history, and it is closely dependent on the local ophidiofauna. In recent years, the ceremony has been accompanied by some significant conservation actions by the local authorities of Cocullo, due to the increased awareness of the importance of environmental protection, in particular snake conservation. Although no decline of Cocullo's snakes' populations has been anecdotally detected in past years, some effects on the reproductive phenology of *E. quatuorlineata* and *Z. longissimus* has been observed (Filippi and Luiselli, 2003) and a few areas surrounding Cocullo are characterized by some disturbance factors for ophidiofauna, including a high density of wild boars (regarding this critical issue on snakes see Filippi and Luiselli, 2002). Various species of Colubridae are involved during the celebration, in particular *Elaphe quatuorlineata* (Pellegrini et al., 2017), one of the largest and more vulnerable species of snakes in Mediterranean central Italy (Filippi and Luiselli, 2000; Filippi, 2003; Capula and Filippi, 2011), but also *Zamenis longissimus* and *Hierophis viridiflavus*. Snakes are captured by snake hunters from the 19th of March till the 30th of April every year. Since 2010, all snakes captured by *serpari* are assessed during the 2-3 days preceding the event, that has a fixed date on May 1st (overall n = 1300 snakes were registered and checked from 2010 to 2023, Filippi and Montinaro, in prep.): a scientific committee (composed by EF and GM in collaboration with a veterinarian) records the captured species, biometric data (weight, snout-vent length [SVL]), sex, age class (juvenile, subadult, adult), site of capture of the ophidians brought by snake hunters. Moreover, PIT tags are checked or implanted, and a physical examination is carried out in addition to a swab for bacteriological analyses (processed by IZS - *Istituto Zooprofilattico Sperimentale* Abruzzo e Molise from 2015 – e.g., Filippi et al. 2010). Then, at the end of the rite, or in any case within seven days, snakes are released by *Serpari* in the same place where they were captured.

In 2019, on 29th and 30th of April, we also conducted a focused survey to assess the presence of Oo (in 2020 and 2021 the rite did not take place due to the Covid-19 pandemic). All snakes underwent an additional physical examination. During this investigation, a particular attention was given to macroscopic clinical signs consistent with ophidiomycosis (Fig. 1). Based on this, a binary value according to Hileman and colleagues (2018) was assigned to each snake: "0" (absence of signs consistent with ophidiomycosis); "1" (presence of signs consistent with ophidiomycosis). The clinical signs from the snakes categorised as "1" (clinically affected) were carefully documented, and the grade of infection severity was retrospectively calculated following the Infection Severity Score (ISS) proposed by Baker and colleagues (2019) (Table 1). A single sterile cotton-tipped applicator with wooden stick (Aptaca Spa, Canelli, Italy) was used to (dry) swab each individual belonging to the apparently affected group, and then placed in 20 ml plastic tubes (Sarstedt AG & Co. KG, Nümbrecht, Germany). The snakes were swabbed with moderate pressure 10 times along the entire dorsal surface, ventral surface, head, and, additionally, ≥ 10 times on each suspected lesion (see Di Nicola et al. 2022; Marini et al., 2023). In cases in which scales and/or lesions naturally exfoliated during swabbing (i.e., abnormal scales partially detached or scales adjacent to skin lesions) or pieces of exuviae detached (due to dysecdysis), these tissues were stored together with the swab from the same individual in the same 20 ml vial. The tubes were stored at +4 °C until shipment. Each snake was handled by the veterinarian carrying out the swab (DM) with new disposable nitrile or latex gloves and all the equipment eventually used (hooks, forceps, scale) was disinfected with 95% denatured ethanol or 5% sodium hypochlorite solution. The DNA has been extracted by placing each swab (with or without tissue) in a 1,5

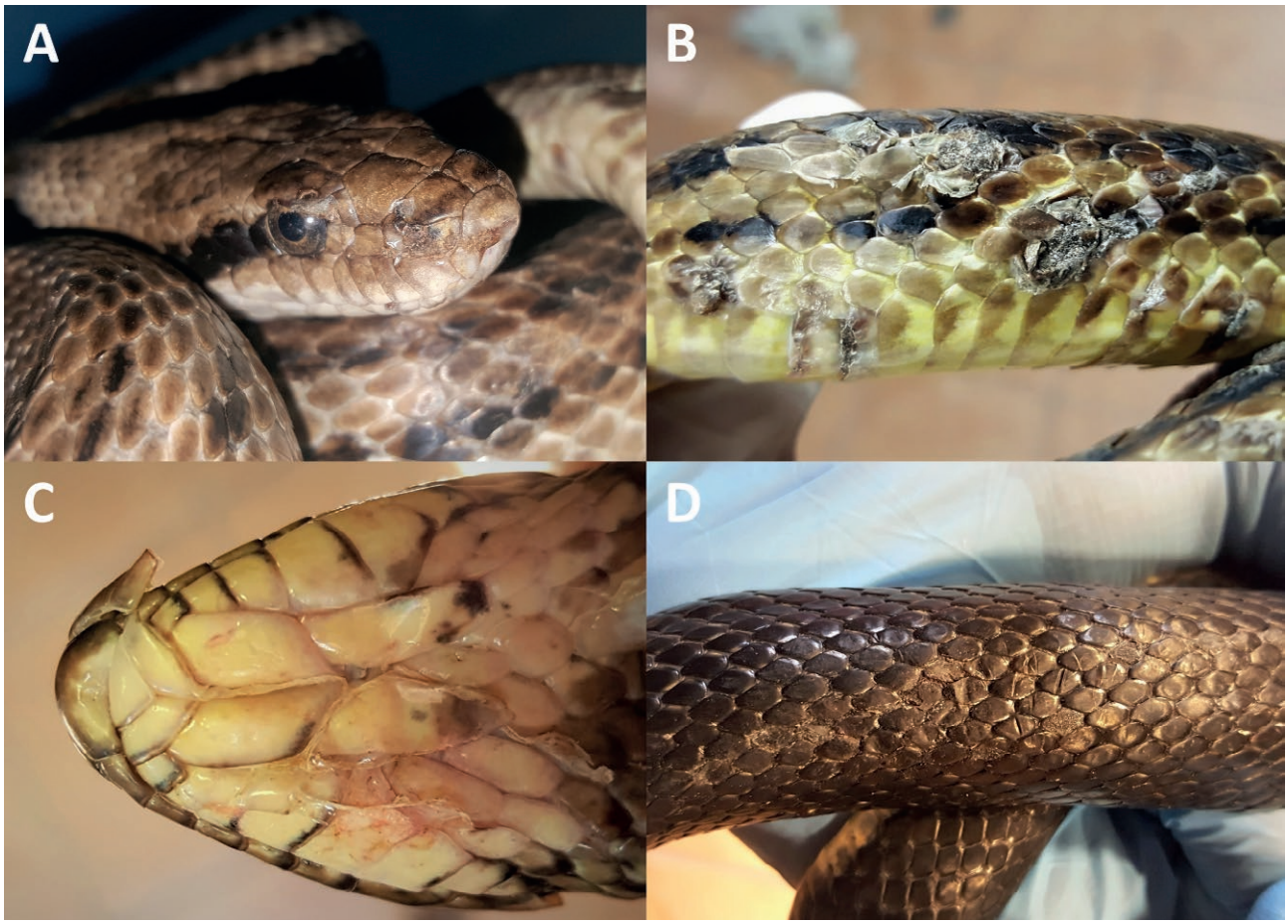

Fig. 1. Representative lesions of individuals grouped in category "1" (presence of signs consistent with ophidiomycosis): (A) EQ21, *Elaphe quatuorlineata* showing erosions of rostral, right internasal, nasal, preocular, ocular and supraocular scales and displaced dorsal scales; (B) EQ46, *Elaphe quatuorlineata* exhibiting multifocal swollen, eroded and hyperaemic (dorsal and ventral) scales associated with crusts and dysecdysis; (C) HV28, *Hierophis viridiflavus* with hyperaemia, erythematous skin, and retained exuvium in the gular region; (D) HV04, *Hierophis viridiflavus* showing wrinkled, depressed and crusty dorsal scales.

ml safe-seal tube with one aliquot (500 µl) of lysis buffer (0.1 M Tris–HCl, pH 8.5, 0.05% Tween 20, 0.24 mg/ml proteinase K), then placed in a heat block first at 60 °C for 1 hour and after 95 °C for 15 minutes. Afterward, 10 µl of each lysate were diluted in 90 µl of nuclease free water. A broad-range standard panfungal PCR (amplifying the D1-D2 region of the large subunit [LSU – 28S] of the ribosomal RNA [rRNA] gene complex – Borman et al., 2006) was performed following Frankinos et al. (2017). Moreover, two different set of primers described by Bohuski and colleagues (2015), targeting specific regions of Oo genome – ITS2 (internal transcribed spacer region 2) and IGS (intergenic spacer region) within the rRNA gene – were employed for conventional PCR assays (Origgi et al., 2022). Each conventional (qualitative) PCR assay has been run in 30 µl amplification mixture composed of 3 µl of PCR Buffer, 0.6 µl dNTP mix (10 mM each), 0.75 µl of each primer (100 mM), 0.3 µl of Taq Polymerase, 2.5 µl of DNA template (diluted lysates), 3.8 µl of $MgCl_2$ (25 mM), and 18.3 µl of water (Solys Biodyne, Luzerna Chem, Lucerne, CH). The reactions were carried out as follows: initial denaturation (95 °C for 3 min) followed by 35 cycles including 30 s at 95 °C (denaturation), 30 s at 52 °C for ITS and 50°C for IGS (annealing), 30 s at 72 °C (elongation). A final extension at 72 °C for 10 min followed. Lastly, 5µl of the PCR product were resolved on a 2% agarose gel by electrophoresis and visualized under UV light.

A total of 129 snakes (adults, subadults and juveniles) were brought by *serpari* and examined by the scientific committee. Ophidians belonged to the following species: *Elaphe quatuorlineata* (n = 82), *Hierophis viridiflavus* (n = 31); *Zamenis longissimus* (n = 15), *Natrix helvetica* (n = 1). Twenty-three snakes out of 129 (17.8%)

Table 1. Individuals categorised as clinically affected (category "1") and sampled at Cocullo's festival in 2019. For each snake the table shows the identification code, species, sex and age class, snout-vent length (SVL), weight, type of collected sample, lesion scores (type, location, number, coverage) and the relative Infection Severity Score (ISS), and the description of gross signs. M: adult male. F: adult female. SAF: subadult female. S: dry swab. T: tissue. BCS: body condition score.

Id	Species	Sex and age	SVL (cm)	Weight (g)	Sample type	Lesion type score	Lesion location score	Lesion number score	Lesion coverage score	ISS (Infection Severity Score)	Gross signs description
EQ21	*Elaphe quatuorlineata*	SAF	107	306	S	3	3	3	3	12	Crusty erosions of rostral, right internasal, nasal, preocular, ocular and supraocular scales. Crusty, dry, dusty, dislocated and (sometimes) eroded scales multifocally along the dorsum. Brownish and dusty crust of the tail (> 3x2cm).
HV04	*Hierophis viridiflavus*	M	89	264	S	2	1	3	3	9	Desquamations with dusty aspect. Concave, wrinkled or crusty dorsal scales. Traumatic and crusty lesions of ventral scales.
EQ24	*Elaphe quatuorlineata*	M	139	1086	S	2	2	3	2	9	Ectopic or crusty or dislocated dorsal and tail scales. Retained shed between supraocular, ocular and postocular scales. Focal discolorations. Low BCS.
HV11	*Hierophis viridiflavus*	M	95	260	S	2	2	2	2	8	Retained shed in the parietal and dorsal scales of head region. Crusty, dusty and/or raised dorsal scales. Light dehydration.
HV12	*Hierophis viridiflavus*	M	87	296	S	2	2	2	2	8	Coalescent desquamations of head and trunk scales. Concave or crusty dorsal scales.
EQ27	*Elaphe quatuorlineata*	F	142	854	S	2	2	2	3	9	Bilobed loose swelling (> 4x1,5cm - presumptively subcutaneous nodule) on the left lateral region of the trunk. Moderate number of ectopic and dislocated dorsal scales. Wrinkled scar between rostral and internasals scales.
ZL01	*Zamenis longissimus*	M	99	194	S	2	3	1	2	8	Tumefaction on the left side of the upper jaw. At buccal inspection, the mucosa was found hyperaemic, haemorrhagic and swelled.
ZL07	*Zamenis longissimus*	M	105	396	S	2	2	2	2	8	Desquamation with a slightly crusty and wrinkled aspect on the right loreal, preocular and supralabial scales. Dislocated, concave and ectopic dorsal scales.
HV15	*Hierophis viridiflavus*	M	96	320	S	1	2	2	2	7	Abrasion between rostral and internasals scales. Dislocated, ectopic, wrinkled or absent dorsal scales.
EQ39	*Elaphe quatuorlineata*	F	150	1000	S	2	2	2	1	7	Crusty lesion on right sopraocular scale. Dislocated dorsal scale.
EQ40	*Elaphe quatuorlineata*	M	124	682	S	1	1	2	2	6	Focal discolorations. Concave dorsal scales.
EQ46	*Elaphe quatuorlineata*	F	112	380	S, T	3	1	3	3	10	Dysecdysis and retained moults in many locations. Tumefacted, eroded and hyperaemic (dorsal and ventral) scales. Several yellowish-brownish crusts lesions in the dorsal and ventral region of the trunk, multifocal pattern. Some swollen scales or crusts underlying nodular formations. Low BCS and muscular weakness.
EQ50	*Elaphe quatuorlineata*	M	116	444	S	1	2	2	2	7	Multifocal discolorations. Wrinkled dorsal scales. Irregular caudal edges of ventral scales.
EQ54	*Elaphe quatuorlineata*	F	138	966	S	2	2	2	3	9	Crusty lesions on dorsal scales. Concave and dislocated dorsal scales. Erythematous and hyperaemic ventral scales. Nodular formation on the tail (> 1x1cm).

Id	Species	Sex and age	SVL (cm)	Weight (g)	Sample type	Lesion type score	Lesion location score	Lesion number score	Lesion coverage score	ISS (Infection Severity Score)	Gross signs description
EQ55	*Elaphe quatuorlineata*	M	117	556	S	2	1	3	3	9	Scattered dark scars. Dislocated, crusty, wrinkled or absent dorsal scales.
HV21	*Hierophis viridiflavus*	M	96	348	S	2	1	3	3	9	Brownish-yellowish moist fresh crusts dorsally and ventrally on the tail. Concave, crusty or absent dorsal scales.
HV23	*Hierophis viridiflavus*	M	93	288	S	3	1	2	2	8	Dislocated and concave dorsal scales. Patches of discolorations. Tumefaction (> 1x1 cm) on the left side of tail (adjacent erythematous and erosive ventral scales).
HV28	*Hierophis viridiflavus*	M	103	342	S, T	2	2	2	3	9	Dysecdysis. Retained exuvium at level of snout and chin. Hyperaemic and erythematous skin in the gular region. Desquamation and wrinkling of dorsal scales. Two nodular formations (0.5x0.5 cm) on the trunk. Irregular caudal edges of ventral scales.
ZL13	*Zamenis longissimus*	F	79	162	S, T	3	1	3	2	9	Crusty, raised or concave dorsal scales. Retained shed. Focal erosive fresh lesion on ventral scales. Multifocal crusts on caudal trunk and tail.
EQ58	*Elaphe quatuorlineata*	M	140	1040	S	2	3	3	2	10	Light multifocal discoloration. Crusty, dry or dislocated dorsal scales. Three crusty lesions on the ventral scales (one on the cloaca).
EQ62	*Elaphe quatuorlineata*	M	139	1088	S	3	1	2	3	9	Dry or dislocates dorsal scales. Swelling and hyperaemia or erosion and of ventral scales close to cloaca. One nodular formation at trunk level (1x1 cm), and one at tail level (0.5x0.5 cm).
EQ64	*Elaphe quatuorlineata*	M	134	790	S	2	1	2	2	7	Dislocated and ectopic dorsal scales. Desquamations. Crusty lesion of the tail.
EQ69	*Elaphe quatuorlineata*	M	136	858	S	2	1	2	2	7	Diffused dark dislocated and wrinkled dorsal scales. Three nodular formations on the trunk (< 1x1cm). Docked tail.

showed signs consistent with a fungal dermatitis and were assigned to the category "1" (apparently affected): *Elaphe quatuorlineata* (n = 13; 15.8%), *Hierophis viridiflavus* (n = 7; 22.6%); *Zamenis longissimus* (n = 3; 20%). Table 1 reports all the clinically affected ophidians and the macroscopic signs that allow ranking these individuals in category "1", as well as each category calculated for counting the individual ISS. Among all the individuals, the ISS varied between 6 and 12, being 9 the median score. No influence of the species on the ISS was found ($\chi^2 = 11.49$, P = 0.32, df = 10). Cluster analysis of the ISSs – with and without normalization of the lesion coverage on the individual weight – did not reveal any particular trend or clustering (data not shown). A total of 23 swabs (and 3 tissues linked to one of them – Table 1) have been collected. After DNA extraction, for every sample (with or without tissue) a conventional PCR was carried out for each of the three targeted region of Oo (D1-D2, ITS2 and IGS regions – 23 samples x 3 reactions = 69 results). No product of consistent size was observed on agarose gel from each PCR electrophoresis (0/69). The number of category "1" individuals versus the number of category "0" individuals of each species did not differ statistically ($\chi 2 = 0.89$, P = 0.64, df = 2). The presence of clinical signs in adult *E. quatuorlineata* appeared to be associated with weight ($n_{cat1} = 12$, x = 812.00 $\pm$ 247.01; $n_{cat0} = 63$, x = 689.02 $\pm$ 174.49; t-test = 2.09 P = 0.04) but not with sex (Fisher test P = 0.68) and SVL (n_{cat1}= 12, x = 131.42 $\pm$ 10.67; n_{cat0} =64, x = 127.88; t-test = 0.98, P = 0.33). SVL and weight were correlated in both apparently clinically healthy (r_{61} =0.74, P < 0.01) and clinically affected individuals (r_{10} =0.92, P < 0.01). No females with clinical signs were observed in *H. viridiflavus* and, among males, the number of category "1" adults did not appear to be related to SVL (n_{cat1}= 7, x = 94.14 $\pm$ 5.24; n_{cat0} = 13, x = 91.65 $\pm$ 6.14; t-test = 0.91, P = 0.38). SVL and weight did not positively correlate in clinically affected (r_5 =0.62, P > 0.05) while these morphometric parameters were correlated in unaffected individuals (r_{11} = 0.82, P < 0.05). Three adults of *Z. longissimus* (1 female and 2 males) out of 15 were grouped in category "1". Oo genomic DNA was not detected in any of our samples (n= 23, observed prevalence 0%, Bayesian 95% credible intervals: 0.00-0.14).

Considering the increasing number of ophidiomycosis reports in Europe, a standardised monitoring for snake communities is warranted. To the best of our knowledge, this is the first systematic testing of a large sample size of a local snake community for the monitoring of *O. ophidiicola* in Italy. We investigated only individuals with signs consistent with a fungal dermatitis because the swabs coming from these ophidians are more likely to result (true) positive compared to those showing no lesions (Hileman et al. 2018; Long et al. 2019). According to our data, none of the species studied shows an obvious high incidence of clinical signs compared to the other species and, within the same species, no particular trends emerged between clinically affected individuals versus clinically not affected ones and parameters as sex, SVL and weight. The used ISS was a helpful tool to characterize the severity of the infection of each individual, independently of the limitations associated with the lack of positive samples in our study. According to our experience, a normalization of the lesion coverage to the size of the animal is recommended (e.g., weight, surface – see Blanvillain et al., 2022). No evidence of the presence of Oo DNA was revealed by PCR. However, PCR negativity is consistent with either the actual absence of the target DNA sequence or its presence under the limit of detection. Furthermore, eventual inhibitors could also hamper the PCR results. Accordingly, we cannot rule out the occurrence of some false negative. Lastly, the snakes were sampled once, and repeated sampling of the same individuals was shown to significantly reduce the probability of a false-negative (Hileman et al. 2018). Hence, in order to detect eventual false negative and increase sensitivity such screening should be improved by performing multiple re-samplings (e.g., 3-5 swab applicators - Hileman et al. 2018; Marini et al., 2023). Also, the use of real-time (quantitative) PCRs instead of conventional (qualitative) ones would improve the detection of false negatives from swab samples (Allender et al., 2015a). This fungus might occur in all the temperate regions around the globe (Burbrink et al., 2017) and snake susceptibility may vary according to phylogenetic and ecological factors (Haynes et al., 2020). The actual natural history of the colonization of this fungus is still unclear. The possible introduction of the fungus into North America by pathogen pollution has been suggested (Ladner et al., 2022) along with evidenced of the presence of both the American (Switzerland) and the European clade (Italy) in the European continent for more than 60 years (Origgi et al., 2022). Therefore, it is essential to shed lights on the distribution of this fungus in European continent along with its associated (clade-specific). Accordingly, it is important to carry out screening in Italian territories, implementing what was started in Cocullo. On the other hand, the monitoring of the possible presence of Oo extends and enriches the health monitoring and conservation actions in place at Cocullo since 2010. In particular, to reduce the potential risk of disease and to ensure an excellent standard of handling and keeping of wild ophidians, a *vademecum* on snakes' management in terraria has been published and delivered to the *Serpari*. Additionally, professional terraria have been allo-

cated to properly house the snakes every year, and dedicated exhibit with numerous environmental education and training activities concerning snakes are carried out for the thousands of tourists attending the ritual every year.

The annual monitoring of the ophidians involved in the Cocullo ritual will provide a great opportunity for collecting baseline data critical to assess the population health of the local snake community, which goes beyond the specific Oo screening, and which represents a paradigmatic example of how cultural traditions, citizen science and conservation may come all together.

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