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# Exploring the eDNA dynamics of the host-pathogen pair *Pacifastacus leniusculus* (Decapoda) and *Aphanomyces astaci* (Saprolegniales) under experimental conditions

Johannes C. Rusch[1,2], David A. Strand[1], Charlotte Laurendz[2], Tom Andersen[2], Stein I. Johnsen[3], Lennart Edsman[4], Trude Vrålstad[1]

**1** *Norwegian Veterinary Institute, Ås, Norway* **2** *University of Oslo, Department of Biosciences, Oslo, Norway* **3** *Norwegian Institute for Nature Research, Lillehammer, Norway* **4** *Department of Aquatic Resources, Swedish University of Agricultural Sciences, Drottningholm, Sweden*

Corresponding author: Trude Vrålstad (trude.vralstad@vetinst.no)

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## Abstract

The oomycete *Aphanomyces astaci* causes crayfish plague, a disease threatening native European crayfish. It is carried and transmitted by American crayfish species, which are the original hosts of *A. astaci*. In recent years, environmental DNA (eDNA) methods have been successfully implemented to monitor the spread of both *A. astaci* and its hosts. However, still little is known about how population density and other environmental factors influence the detectability of this host-pathogen complex. In a mesocosm experiment, we tested the influence of crayfish density, temperature and food availability on the detectability of eDNA for *A. astaci* and its host, signal crayfish *Pacifastacus leniusculus*. We also compared eDNA results with crayfish population density measured by catch per unit effort (CPUE) from two lakes with varying crayfish density and *A. astaci* prevalence. The mesocosm experiment revealed that a limited set of controlled factors can substantially change the detectable amount of eDNA, even though the physical presence of the target organisms remains the same. In cold, clear water, eDNA quantities of both targets increased far more than in a linear fashion with increased crayfish density. However, the presence of food decreased the detectability of crayfish eDNA, presumably through increased microbial-induced eDNA degradation. For *A. astaci*, where eDNA typically represents living spores, food did not affect the detectability. However, high water temperature strongly reduced it. The increased complexity and variability of factors influencing eDNA concentration under natural conditions, compared to a controlled experimental environment, suggests that establishing a reliable relationship between eDNA quantities and crayfish density is difficult to achieve. This was also supported by field data, where we found minimal correspondence between

eDNA quantity and CPUE data. A comparison between quantitative real-time PCR (qPCR) analysis and droplet-digital PCR (ddPCR) analysis revealed higher detection success of the targets in field samples when using qPCR. Overall, our results support eDNA as an effective tool for presence-absence monitoring, but it seems less suited for biomass quantification and population density estimates. Detection of *A. astaci* and *P. leniusculus* is not influenced uniformly by respective environmental factors. Consequently, we recommend a strategy of monitoring both targets, where the detection of one may point towards the presence of the other.

**Keywords**
crayfish plague, ddPCR, environmental biomonitoring, environmental DNA, freshwater crayfish, mesocosm experiment, occupancy modelling

## Introduction

The oomycete *Aphanomyces astaci* is a fungal-like water mould that causes crayfish plague, a disease lethal to crayfish indigenous to Europe (Söderhäll and Cerenius 1999). It was first introduced into Europe around 1859 (Alderman 1996 and references therein) and is now widespread throughout Europe, mostly through the introduction of American non-indigenous crayfish species (Holdich et al. 2009). These are natural hosts of *A. astaci* that carry and transmit the disease but, unlike their European counterparts, they usually do not succumb to it as they have evolved natural defence mechanisms against the parasite infections (Söderhäll and Cerenius 1999). *Aphanomyces astaci* has contributed to the drastic decline of indigenous crayfish species throughout Europe and the disease can potentially cause the eradication of most if not all indigenous crayfish species populations in Europe (Holdich et al. 2009). Due to the severe impact on these populations, it is considered a listed disease by the World Organisation of Animal Health (OiE) (OIE 2019) and in Norway (Regulation on animal health requirements for aquaculture animals and products thereof and on the prevention and control of certain diseases in aquatic animals, FOR-2008-06-17-819) and features on the IUCN list of 100 of the world's worst invasive alien species (Lowe et al. 2004). In many European countries, legislation is in place and measures have been implemented to combat further spread of *A. astaci* (Regulation (EU) No 1143/2014) (Jussila and Edsman 2020). These measures include monitoring (Strand et al. 2019; Strand et al. 2020) of the spread of *A. astaci* accompanied by local bans on fishing and strict disinfection regulations. Other measures include eradication of *A. astaci* carrying American non-indigenous crayfish species (Sandodden and Johnsen 2010; Peay et al. 2019), as well as the creation of ark-sites (Brickland et al. 2009) where absence of the disease-agent and non-indigenous crayfish species has to be substantiated.

Environmental DNA (eDNA) monitoring is increasingly used for biomonitoring of species, including both macroorganisms and microorganisms (Leese et al. 2016). For macroorganisms, genetic material in the form of shed or abraded cells and cell-fragments or propagules, such as gametes, is captured on a filter, extracted and analysed (Taberlet et al. 2012; Thomsen and Willerslev 2015; Taberlet et al. 2018), while for microorganisms,

such as the zoospores of *A. astaci*, it can be captured on the filter as live cells (Strand et al. 2011). The eDNA dynamics of *A. astaci* have been studied both experimentally (Strand et al. 2012; Svoboda et al. 2013) and under natural conditions (Strand et al. 2014, 2019; Wittwer et al. 2018) and relative quantification of spores in eDNA samples is possible, enabling the detection of outbreak situations (Strand et al. 2019). In Norway, the surveillance of *A. astaci* has been carried out solely through the use of eDNA monitoring in recent years (Vrålstad et al. 2017; Strand et al. 2019). Additionally, presence-absence monitoring of the pathogen and both the susceptible host and the carrier have yielded good results (Agersnap et al. 2017; Strand et al. 2019; Rusch et al. 2020). However, even though some studies show correlation between population density and eDNA quantity for fish species (Takahara et al. 2012; Doi et al. 2015a; Lacoursière-Roussel et al. 2016; Capo et al. 2019, 2021), no clear correlation has yet been established between the detectable amount of eDNA and crayfish population density (Dougherty et al. 2016; Dunn et al. 2017; Johnsen et al. 2020; but see Chucholl et al. 2021 and Sint et al. 2021). The emission of eDNA seems to be influenced by numerous biotic and abiotic factors (Roussel et al. 2015; Stewart 2019), such as ambient water temperature, life-cycle and corresponding behaviour of crayfish (Dunn et al. 2017).

The host-pathogen pair *Pacifastacus leniusculus* and *A. astaci* are a particularly interesting model for studying eDNA dynamics as crayfish leave relatively low traces of eDNA in the water (Johnsen et al. 2020) compared to *A. astaci*, especially during an outbreak situation (Strand et al. 2019). The overarching hypothesis of our study is that eDNA emitted from *P. leniusculus* correlates with population density/number of individuals. However, we also expect that several factors affect both the emission and detectability of eDNA from *P. leniusculus* and its parasite *A. astaci* in the ambient water. The goal of this study was to test the influence of temperature, food availability and crayfish density on the measurable eDNA amount emitted from *P. leniusculus* and its obligate parasite *A. astaci* in a mesocosm experiment. We expected rising temperatures and access to food to cause increased crayfish activity (Flint 1977; Rusch and Füreder 2015) and more faecal matter and, thus, also increased shedding of eDNA from crayfish. For *A. astaci*, we expected that increased crayfish density would naturally lead to increased amounts of *A. astaci* eDNA, while food availability for the crayfish was not suspected to directly influence the pathogen amounts. Strand et al. (2012) showed that latent carrier signal crayfish released more *A. astaci* spores in temperate (18 °C) water than in cold (4 °C) water. We, therefore, expected that a water temperature close to the previously-described sporulation optimum of *A. astaci* near 20 °C (Alderman and Polglase 1986; Alderman et al. 1987; Diéguez-Uribeondo et al. 1995) would lead to the highest *A. astaci* sporulation and eDNA amounts.

To link the experimental data to a real-life situation, we also included a small field survey where water samples were obtained in parallel with catch per unit effort (CPUE) data from two lakes with varying crayfish density and varying infection load with *A. astaci*. The results from the experiments and field survey will hopefully provide more detailed understanding of eDNA dynamics of the host-pathogen pair and provide knowledge that can help in designing better monitoring programmes involving *A. astaci* and freshwater crayfish.

## Materials and methods

### Crayfish capture and husbandry

In total, 125 *P. leniusculus* specimens (71 female, 54 male, average total length 109.6 mm ±16.8 mm) were obtained by trapping from two Norwegian lakes (Rødenessjøen and Øymarksjøen) within the Halden watercourse in south-eastern Norway. Crayfish in both lakes have a well-documented history of infection with *A. astaci* (Vrålstad et al. 2011; Strand et al. 2014, 2019). The crayfish were marked both by writing numbers on the carapace and by pricking small holes into the tail-fan in a specific pattern as first described by Guan (1997). After measuring length and determining sex, crayfish were kept in a large communal tank at the aquarium facilities of the Norwegian University of Life Sciences (NMBU), Oslo. Shelters and food were provided. We used aerated tap water that was oxygenated with a large aquarium pump. Temperature and oxygen were measured daily.

Capture, transport and husbandry of crayfish were conducted with permits from the Norwegian Food Safety Authority, the County Governor of Østfold and the Norwegian Environment Agency. This, along with euthanasia at the end of the experiment, was conducted in accordance with the Norwegian Animal Welfare Act (LOV-2009-06-19-97) and EU regulations (EU Directive; 2010/63/EU).

### Mesocosm experiment

The experiment was designed as full-factorial to analyse the influence of crayfish density, availability of food and temperature on the detectability of eDNA from *P. leniusculus* and *A. astaci* (Fig. 1). For this purpose, four tanks containing 100 l of water were set up with two different densities of crayfish: 2 crayfish (low density) and 20 crayfish (high density) with two different treatments: 1) food/no food. This series of experiments was carried out over six weeks, with three replicates for each temperature (one week = one replicate, Fig. 1). The three first weeks (replicates) were conducted at high (20 °C) temperature representing summer and the three last weeks (replicates) at low (10 °C) temperature representing spring/autumn conditions in Norway. Food was provided in the beginning of the week to one tank of each density. The fed crayfish in the low and high density tanks were given 2 and 20 frozen peas and ½ and 10 frozen shrimps (*Pandalus borealis*), respectively.

For each week, crayfish were randomly picked from the communal tank, assigned to an experimental tank and their number-markings were recorded. All crayfish were kept in their respective tanks for one week. In all tanks, crayfish were provided with sufficient shelters made from PVC tubes. After seven days, triplicate water samples of 1 l (3 × 1 l) were taken from each tank using a peristaltic pump (Masterflex I/P, Cole-Parmer, Vermon Hills, USA), tygon tubing (Masterflex), an in-line filter holder (Millipore, Billerica, Massachusetts, USA) and glass fibre filters (47 mm AP25, Millipore) according to Strand et al. (2019). Before sampling at each tank, tap water was pumped

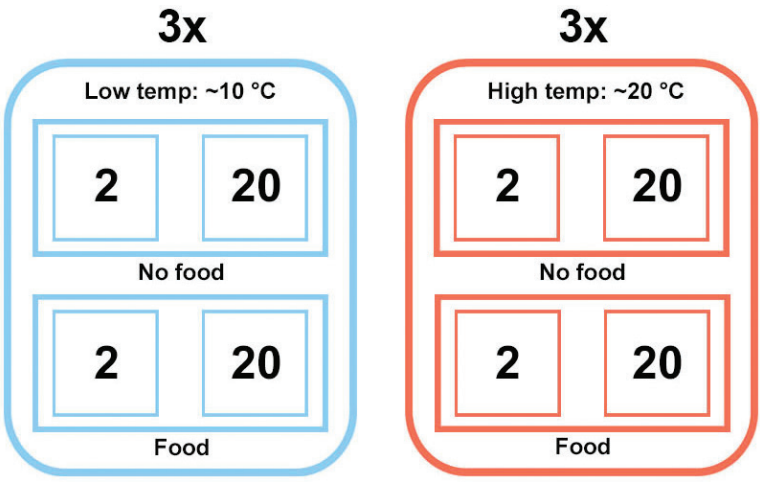


**Figure 1.** Schematic drawing of the experimental setup with *P. leniusculus* and *A. astaci*. The numbers 2 and 20 represent the number of crayfish present in the respective tanks with 100 l of water. One trial consisted of two tanks with 2 crayfish (low density) and two tanks with 20 crayfish (high density). Crayfish from one tank of each density group were fed, while the crayfish in the parallel tanks got no food. Three replicate trials were run at high (20 °C) water temperature and another three replicate trials at low (10 °C) water temperatures, in total for six weeks.

through the tubes for 5 minutes followed by 1 l of water from the respective tank. At the start of each experiment, negative control eDNA water samples (3 × 1 l) were taken from a clean bucket filled with water from the same source as used in the experiment to check for the presence or absence of eDNA of both *A. astaci* and *P. leniusculus*. Filters were placed in separate sterile 15 ml Falcon tubes and frozen at –20 °C until further analysis. After each sampling date, the tubes were submerged in a 10% chlorine bleach solution which was also pumped through the tubes for a minimum of 10 min. Subsequently, tap water was pumped through the tubes for another 10 minutes, followed by a 10% sodium thiosulphate ($Na_2S_2O_3$) solution to neutralise any residual chlorine. The tubes were then stored at –20 °C until the next sampling date. At the end of each week after sampling, the crayfish were returned to the communal tank, the experimental tanks were drained and the tanks and shelters were scrubbed with detergent and thoroughly cleaned. Using this setup, three replicate trials (always containing a random selection of crayfish individuals from the communal tank) were conducted (Fig. 1).

At the end of the experimental period, the crayfish were euthanised by placing them in ice slush for anaesthesia, followed by piercing of the brain using a scalpel. Tissue samples were taken from the tail-fan of 45 crayfish used in the experiment and analysed with species-specific *A. astaci* quantitative real-time PCR (qPCR) assay for determining the *A. astaci* prevalence and semi-quantitative agent levels, as described in Vrålstad et al. (2009).

## Field samples and lakes

Water samples were also obtained from two lakes with well-documented illegally introduced *P. leniusculus* populations (Table 1, Fig. 2). Lake Øymarksjøen (Viken County, eastern Norway) has a surface area of 14.13 km$^2$ and alien crayfish were first discovered there in 2008 (Vrålstad et al. 2011). Lake Stora Le (Värmland County and Västra Götaland County, western Sweden) has a surface area of 136.1 km$^2$. *Pacifastacus leniusculus* was first officially reported from three localities in 2002, although, by that time, they had already been in the lake for several years according to local fishermen (Jansson 2017).

In Lake Øymarksjøen, nine sampling sites were selected at which two water samples were collected per site (one in June 2016 and one in August 2016). In order to estimate *P. leniusculus* CPUE, a total of five foldable cylindrical crayfish traps (LiNi) with two entrances and a mesh size of 14 mm (Westman et al. 1978), were set at each site. The traps were baited with raw chicken (Johnsen et al. 2020) and set overnight on 1 September 2016. Thus, the trapping in Lake Øymarksjøen was conducted after eDNA sampling.

Three sampling sites with varying density of crayfish populations were chosen in Lake Stora Le based on previous monitoring (Jansson 2017; Bohman 2020). At each of these three sites, five LiNi traps (Westman et al. 1978; Bergqvist et al. 2016) baited with roach and attached to a line 10 m apart were laid out on 19 September 2016. The traps were lifted the following day and CPUE was estimated. A few hours later, five water samples (5 × 5 l) were taken at each location 10 metres apart, matching the position of the traps, using the filtering protocol described below. We aimed to filter water samples of five litres on site at each sampling location using the same equipment described for the mesocosm experiment, except that we used the Masterflex E/S portable sampler instead. When filters clogged up prior to reaching five litres, the volume of filtered water was recorded (see Table 1). Filters were placed in separate 15 ml Falcon tubes which were stored on ice directly after filtration. Upon arrival at the laboratory, the samples were stored at –20 °C until further analysis.

## DNA extraction and eDNA quantification

Before DNA extraction, the filters were frozen at –80 °C and then freeze-dried for 24 h, using a vacuum freeze dryer (Heto drywinner, Thermo Fisher Scientific, Waltham, USA). DNA was extracted from the filters according to a cetyltrimethyl ammonium bromide (CTAB) protocol described in Strand et al. (2019). During extraction, each filter was split into two subsamples (labelled A & B). A laboratory-environmental control and a blank extraction control were included, as described in Strand et al. (2019).

All qPCR analyses were run on a Mx3005P qPCR thermocycler (Agilent, Santa Clara, USA), using the assay for *A. astaci* developed by Vrålstad et al. (2009) and the assay for *P. leniusculus* developed by Agersnap et al. (2017) (Suppl. material 1). We used TaqMan Environmental Mastermix (Thermofisher Scientific, Waltham, US). The qPCR settings for *A. astaci* followed Vrålstad et al. (2009) with modifications to the annealing/extension cycle according to Strand et al. (2014). The qPCR programme for *P. leniusculus* followed the protocol described in Agersnap et al. (2017).

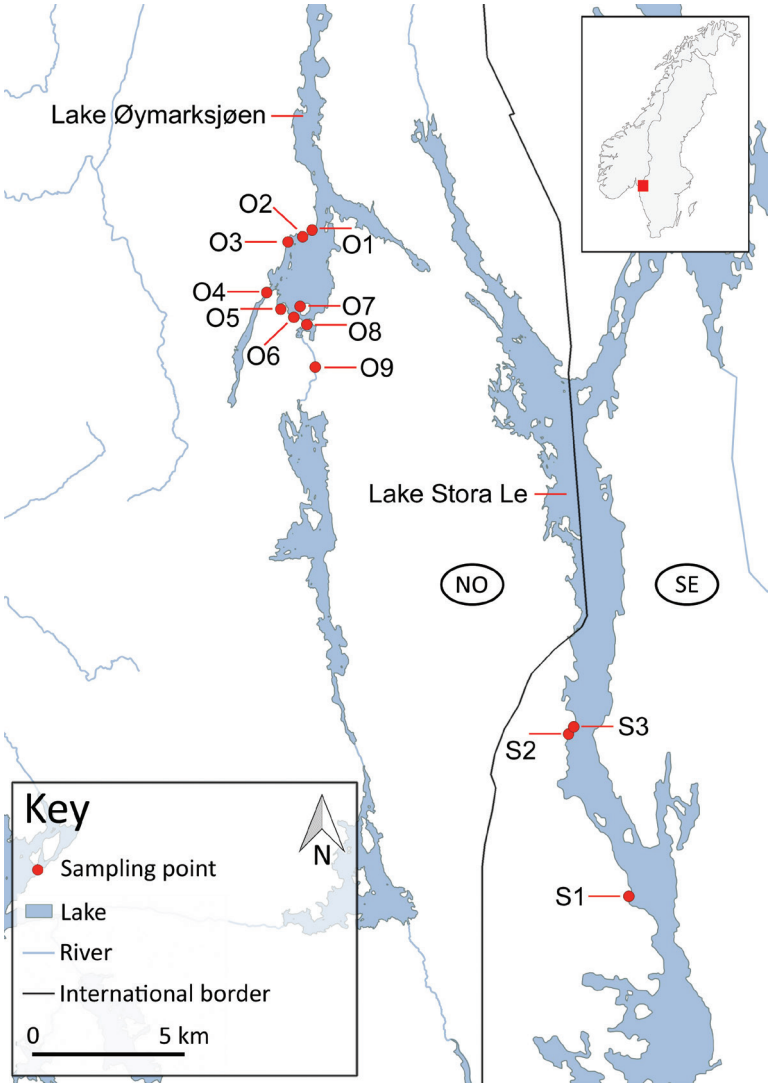


**Figure 2.** Sampling points in Lake Øymarksjøen in Norway and Lake Stora Le in Sweden. The countries are indicated by their two-letter ISO codes: NO and SE. Sampling points in Øymarksjøen are numbered O1–O9, the sampling points in Lake Stora Le are S1–S3. The respective sampling points are depicted as red dots, the international border is represented by the black line. The location of the map is illustrated by the red area in the inset map in the top right corner.

All DNA isolates were analysed both undiluted and 10-fold diluted to account for potential inhibition, in total four replicates per filter sample. The level of inhibition was determined by calculating the difference in Ct-values between the undiluted and diluted samples (ΔCt) following Kozubíková et al. (2011). In the absence of inhibition, ΔCt theoretically equals 3.32. To account for errors in pipetting, amplification efficiency and other inaccuracies, a variance of 15% was deemed acceptable (ΔCt range

**Table 1.** List of sampling sites including location, sampling date and amount of water filtered.

| Site code | Location | Date sampled | Sample volume (in l) | Coordinates |
|---|---|---|---|---|
| O1 | Øymarksjoen, west of Sandbøl | 08.06.2016 | 5 | 59.3522N, 11.6608E |
| | | 10.08.2016 | 5 | |
| O2 | Øymarksjoen, above Sandbøl | 08.06.2016 | 5 | 59.3501N, 11.6556E |
| | | 10.08.2016 | 4.5 | |
| O3 | Øymarksjoen, south of Sandbøl | 08.06.2016 | 5 | 59.3483N, 11.6472E |
| | | 10.08.2016 | 5 | |
| O4 | Øymarksjoen, Fossbekkbrua | 08.06.2016 | 4.5 | 59.3331N, 11.6364E |
| | | 10.08.2016 | 4 | |
| O5 | Øymarksjoen, hyttefelt | 08.06.2016 | 5 | 59.3283N, 11.6450E |
| | | 10.08.2016 | 4 | |
| O6 | Øymarksjoen, west of Bønesøya | 08.06.2016 | 5 | 56.3261N, 11.6528E |
| | | 10.08.2016 | 5 | |
| O7 | Øymarksjoen, Bønesøya | 08.06.2016 | 5 | 59.3294N, 11.6561E |
| O8 | Øymarksjoen, Blåsnuppen | 08.06.2016 | 5 | 59.3242N, 11.6601E |
| | | 10.08.2016 | 2.5 | |
| | | 10.08.2016 | 3.5 | |
| O9 | Mokallen, outlet to Strømselva | 08.06.2016 | 5 | 59.3117N, 11.6667E |
| | | 10.08.2016 | 3.5 | |
| S1 | Stora Le | 20.09.2016 | 5 (x5) | 59.1594N, 11.8625E |
| S2 | Stora Le | 20.09.2016 | 5 (x5) | 59.2067N, 11.8231E |
| S3 | Stora Le | 20.09.2016 | 5 (x5) | 59.2089N, 11.8261E |

2.82 to 3.82). In cases of observed inhibition, the result from the 10-fold diluted subsample was used alone for estimation of eDNA copy number. If the ΔCt range was larger than 3.82, the result from the undiluted subsample was used alone to calculate the eDNA copy number. For subsamples with an accepted range, the mean eDNA copy number per subsample was calculated from the undiluted and 10-fold diluted qPCR result. Reactions with a Ct of 41 or higher were treated as 0 (no detection; Kozubíková et al. 2011; Agersnap et al. 2017).

Genomic DNA from *P. leniusculus* and *A. astaci* with a known DNA copy number concentration was included in each run to create a standard curve for relative quantification of targeted DNA copies in each reaction (Strand et al. 2019) using the manufacturer's software (MXpro, Agilent, Santa Clara, USA). In the following comparisons between qPCR and ddPCR results, the copy numbers per reaction for both DNA extraction subsamples (A and B, listed in Suppl. material 2) were used in the linear regression model (see below).

Droplet digital PCR (ddPCR) was performed on a QX200 AutoDG Droplet Digital PCR System (Bio-Rad, Hercules, USA). For ddPCR analysis of the samples, we drew upon the qPCR assays developed for *A. astaci* (Vrålstad et al. 2009) and *P. leniusculus* (Rusch et al. 2020) (Suppl. material 1). The positive droplet count and total droplet count per sample are reported by the manufacturer's software (Quantasoft v.1.7.4.0917, Biorad, Hercules, USA). Calculation of eDNA copy numbers per reaction volume is performed by the same software and is estimated using the ratio between

positive and negative droplets within a sample, using Poisson-statistics. We defined a positive detection as ≥ 3 positive droplets in assays with > 8000 total droplets (Dobnik et al. 2015). While the ddPCR eDNA copy numbers for samples with < 3 positive droplets were included in the linear regression comparing ddPCR and qPCR, reactions with < 3 positive droplets were scored as negative in the following statistical analysis and reactions with < 8000 total droplets were scored as missing values (Suppl. material 2). In oversaturated samples, i.e. where the DNA content exceeded the dynamic range of ddPCR quantification with all droplets being positive, we set the maximum amount of DNA copies per reaction to 200,000 for the calculation of copies per litre.

## Statistical analysis

We used linear regression on log(x + 1)-transformed variables to investigate the overall consistency between ddPCR- and qPCR-based copy numbers (per reaction) and assessed "goodness of fit" from the Pearson correlation coefficient (r) between the two. We used generalised linear models (GLMs) to estimate effect sizes of the treatments in the laboratory experiments. Since the positive droplet count in a ddPCR assay conforms better to statistical distributions of the exponential families than the non-integer copy number estimates derived from this statistic, we decided to model the logarithm of positive droplets using the logarithm of total droplets as offset (i.e. including a "+ offset(log(tot. drp))" term in the model formula). Using this model construct, we essentially modelled the fraction of droplets that are positive with maintaining a dependent variable that is an integer count. Since this type of data often exhibits more zero counts than expected from a Poisson distribution (so-called over-dispersion), we fitted models of both the Poisson and negative binomial families and compared their performances by Akaike's Information Criterion (AIC). To investigate possible interactive effects between treatments, we fitted models with and without interactions and compared these also by AIC. To account for the pseudo-replication introduced by taking three samples from each tank at the end of each experimental run, we used Tank ID nested within experimental run as a random intercept effect (i.e. including a "+ (1 | Run / Tank)" term in the model formula). We also chose to sum the droplet counts from the A and B filter halves instead of having an additional hierarchical level in the models. We fitted the resulting generalised linear mixed models (GLMMs) with the glmmTMB package (Brooks et al. 2017) (See Suppl. material 3). The glmmTMB is likelihood-based with the same syntax as the older lme4 package, but is known to be faster and more computationally stable due to its use of the Template Model Builder (TMB) automatic differentiation engine (Brooks et al. 2017). All statistical analyses were carried out using R version 4.0.3 (R Core Team 2020).

For the field data, we used 3-level hierarchical occupancy models to represent the variation between sites, between replicated filter samples from the same site and between assays on separate halves of the same filter. In this analysis, we focused on presence of *P. leniusculus* and *A. astaci* eDNA. Here also, a positive detection was defined as ≥ 3 positive droplets in a reaction with > 8000 total droplets (reactions with < 8000 total droplets were flagged as missing values). We fitted the resulting 3-level binomial

models with a Bayesian approach using the msocc package for R (Stratton et al. 2020). We used msocc's default non-informative priors, but increased the number of Markov Chain Monte Carlo samples to 11000, with the first 1000 discarded as warm-up and the remainder thinned by 10 (See Suppl. material 4).

While all DNA, PCR and environmental laboratory controls remained negative in the ddPCR analysis, we experienced low positive signals in some of the inlet water controls in weeks 5 and 6. To test if these weak positive detections influenced the results, we used the same GLMM analysis as described above. All samples collected in the same week as the positive inlet controls that were equal to or lower than the positive control for that week were set to zero. Thus, we used the droplet count of the positive inlet control as the threshold for scoring samples positive. The statistical GLMM tests for the effect of the contamination showed no difference in the significant factors when adjusting for the positive inlets controls (see Suppl. material 5). We, therefore, included all samples from weeks 5 and 6 when analysing the results.

## Results

### Host & pathogen eDNA in the mesocosm experiment

From the 45 analysed crayfish, representing 36% of the total amount of crayfish used in the experimental population, the prevalence of *A. astaci* was 78% and the agent level varied from $A_0$ to $A_6$. According to this classification, agent levels $A_0$ and $A_1$ are considered negative, while agent levels $A_2$ to $A_6$ indicate presence of the pathogen with exponentially increasing amounts of detectable pathogen DNA (Vrålstad et al. 2009). Tail-fan samples, taken and analysed after the experiment had been concluded, confirmed that all but two tanks and replicates certainly included crayfish individuals with a positive *A. astaci* carrier status (See Suppl. material 6).

Suppl. material 2 summarises ddPCR and qPCR data from the mesocosm experiments, including eDNA copy numbers obtained by both methods. For qPCR analyses of *A. astaci* from the mesocosm experiment samples, we observed inhibition in only two samples ($\Delta Ct < 2.82$), both from tanks containing 2 crayfish with food added, one at low and one at high temperature. For *P. leniusculus* qPCR results, inhibition was observed in seven samples, both with and without food added and at both temperatures (Suppl. material 2). A comparison of the qPCR and ddPCR results from the mesocosm trials yielded a significant positive correlation between the methods for both *A. astaci* (Fig. 3A) and *P. leniusculus* (Fig. 3B). In the following, we use the ddPCR data in the further presentation of results. Corresponding results for qPCR are presented and discussed in Laurendz (2017).

Of the 72 water samples taken during the aquarium experiment and analysed with ddPCR, 46 were positive for *A. astaci* and 60 were positive for *P. leniusculus*. A total of 21 DNA extraction subsamples were excluded from the analysis due to the total droplet count in the reaction being below 8000. The number of positive droplets per ddPCR reaction ranged from 3 to > 19433 (See Suppl. material 2). In the text below, the number of positive droplets represents a proxy for eDNA quantity.

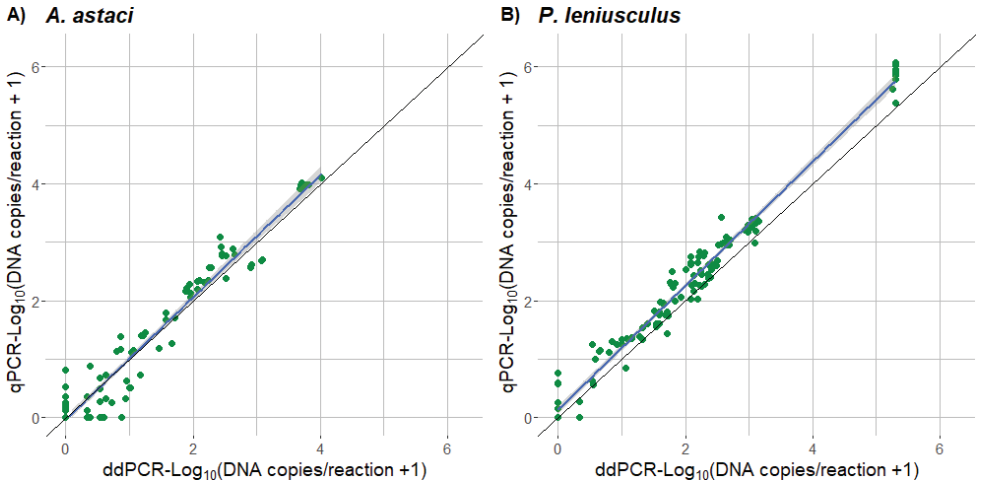


**Figure 3.** Scatterplot of the estimated DNA copies per reaction of both qPCR and ddPCR analysis for *A. astaci* (**A**) and *P. leniusculus* (**B**) from the mesocosm trial. A significant positive correlation between the methods was observed. A) *A. astaci*: Pearson's r = 0.98, $p < 2.2\times10^{-6}$. B) *P. leniusculus*: Pearsons's r = 0.99, $p < 2.2\times10^{-16}$. Black line represents 1:1 correspondence between ddPCR and qPCR.

For *A. astaci*, the median eDNA copy number per litre was much lower at 20 °C than at 10 °C, irrespective of any other factor/influence (Table 2, Fig. 4). The highest median copy numbers per litre were observed in the 10 °C tanks at high crayfish density, both when food was provided (median eDNA copies per litre = 44556) and when food was missing (median eDNA copies per litre = 28622).

These observations were reflected by the statistical modelling. For *A. astaci*, the two-way interaction model had the lowest AIC value. High crayfish density had a significant positive effect on eDNA quantity (positive droplets), whereas high temperature had a significant negative effect on eDNA quantity of *A. astaci*. The combination of high temperature and high density also had a significant negative effect on the amount of detectable *A. astaci* eDNA (See Suppl. material 3, Fig. 5A).

For *P. leniusculus*, the highest median number of eDNA copies per litre (> $8.4\times10^6$) was observed in the treatment group with high crayfish density and no food at 10 °C. However, the treatment group with high crayfish density and no food at 20 °C had a median of 17467 eDNA copies per litre, lower in fact than the treatment group with low crayfish density and no food at 20 °C (median eDNA copies per litre = 20667) (Table 2, Fig. 4).

The results of the model matched the results of the detected eDNA copy numbers per litre of *P. leniusculus*. Here, the three-way interaction model had the lowest AIC value. High density and the combination of no food at low temperature and high density were determined to have a significant positive effect on the amount of eDNA quantity (positive droplets) by the GLMM model. The two combinations of high temperature with food and high temperature with high density had a significant negative effect (Fig. 5B, Suppl. material 3).

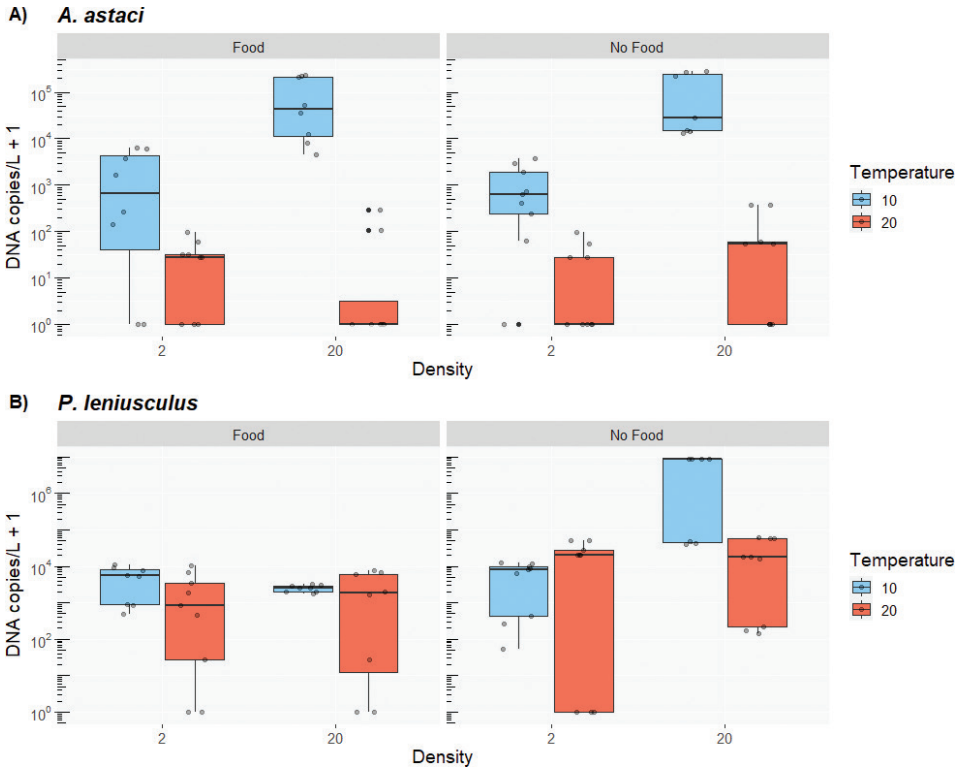


**Figure 4.** Boxplot of detectable eDNA copies per litre for *A. astaci* (**A**) and *P. leniusculus* (**B**), as detected by ddPCR. For temperature, the blue box indicates the interquartile range at 10 °C, while the red box indicates the interquartile range at 20 °C. Density is indicated by 2 (crayfish per tank) and 20 (crayfish per tank) and the median is represented by the thick black horizontal bar within the boxes. **A** for *A. astaci*, the median copy number/l was generally very low at 20 °C, while high median copy numbers/l were observed at 10 °C and high crayfish density. Food had no apparent effect **B** for *P. leniusculus*, the highest median eDNA copy number/l was observed at high crayfish density at 10 °C, with no food. The median copy number/l was generally substantially lower at 20 °C and, in particular, in the tanks where crayfish were fed. Food had a negative effect on eDNA copy numbers both at 10 °C and 20 °C.

**Table 2.** Summary of the median eDNA copies per litre with coefficient of variation in brackets of *P. leniusculus eDNA* and *A. astaci* eDNA for the combinations of test conditions: density, food availability and temperature. Fold change indicates the relative increase (x : 1) or decrease (1 : ×) in eDNA copy numbers per litre of water from low density (2 crayfish) to high density (20 crayfish).

| Temp | Target | Food | | | No food | | |
|---|---|---|---|---|---|---|---|
| | | 2 crayfish | 20 crayfish | Fold change | 2 crayfish | 20 crayfish | Fold change |
| 10 °C | *P. len* | 5378 (92%) | 2533 (21%) | 1 : 2.1 | 8089 (78%) | 8488889 (93%) | 1049 : 1 |
| 20 °C | *P. len* | 844 (139%) | 1689 (120%) | 2 : 1 | 20667 (94%) | 17467 (75%) | 1 : 1.2 |
| 10 °C | *A. ast* | 262 (132%) | 44556 (107%) | 170 : 1 | 622 (115%) | 28622 (108%) | 46 : 1 |
| 20 °C | *A. ast* | 27 (103%) | 0 (210%) | NA | 0 (147%) | 53 (170%) | NA |

The effect of crayfish density on the amount of detected eDNA copies per litre, both for *P. leniusculus* and *A. astaci* eDNA, varied considerably. At 10 °C, we observed a 170-fold increase of the median eDNA quantity (represented by DNA copies per litre) of *A. astaci* from tanks with 2 crayfish to tanks with 20 crayfish provided with food. In the absence of food, a 46-fold increase was observed. At 20 °C, almost no *A. astaci* eDNA was detected in any of the tanks, only trace levels close to or below LOD (3 positive droplets) were observed (Table 2, Fig. 5, Suppl. material 2).

For *P. leniusculus* at 20 °C, we found only a two-fold increase of the median eDNA quantity between the tanks with 2 and 20 crayfish provided with food and even a minor (1.2-fold) decrease when food was missing. At 10 °C, the median eDNA quantity was 2.1 fold lower in the tanks with 20 crayfish compared to 2 crayfish, when food was provided. However, in the absence of food, the median eDNA quantity was as much as 1049-fold higher in the tanks with 20 crayfish compared to 2 crayfish (Table 2, Fig. 5).

## Host and pathogen eDNA in natural environments

Of the 15 samples analysed from Lake Stora Le, 10 (66.7%) were positive for *A. astaci* eDNA and 7 (46.7%) were positive for *P. leniusculus* eDNA using ddPCR. Of the 18 samples analysed from Øymarksjøen, 11 (61.1%) were positive for *A. astaci* eDNA, while none was positive for *P. leniusculus* eDNA with ddPCR. For qPCR, 13 (72.2%) samples were positive for *A. astaci* and 12 (66.7%) were positive for *P. leniusculus* eDNA (Table 3).

While there was relatively good correlation between the qPCR and ddPCR results from Lake Øymarksjøen for *A. astaci* (Fig. 6A), the correlation between qPCR and ddPCR results for *P. leniusculus* was weak (Fig. 6B). Here, eight samples that were positive for *P. leniusculus* eDNA using qPCR were recorded as negative with ddPCR.

Using the msocc package, we calculated the statistical probability of detecting *A. astaci* and *P. leniusculus* at crayfish densities ranging from 0 to 20 CPUE, based on the detection rates from field samples (Fig. 7). The probability of presence at site ($\psi$) is stated for each location and organism respectively in Table 3. The probability of occurrence in the sample ($\theta$), conditional upon presence at site, was 0.69 for *P. leniusculus* and 0.72 for *A. astaci*, respectively. The probability of detection in the filter replicate, conditional upon occurrence in the sample, was 0.86 for *P. leniusculus* and 0.7 for *A. astaci*, respectively.

The probability of detecting eDNA of *A. astaci* using the sampling method described above reached 100% at a crayfish density of 2 CPUE in both Stora Le and Øymarksjøen. For *P. leniusculus*, we calculated a 100% eDNA detection probability above a crayfish density of 5 CPUE in Stora Le. The lack of positive detections in Lake Øymarksjøen using ddPCR provided us with insufficient data points to calculate the detection probability for Lake Øymarksjøen accurately. In the subsequent analysis using qPCR data, we calculated a 100% eDNA detection probability above 3 CPUE in Øymarksjøen. The eDNA concentration in the samples obtained from the field was consistently lower than in the aquarium samples, even in locations with high CPUE.

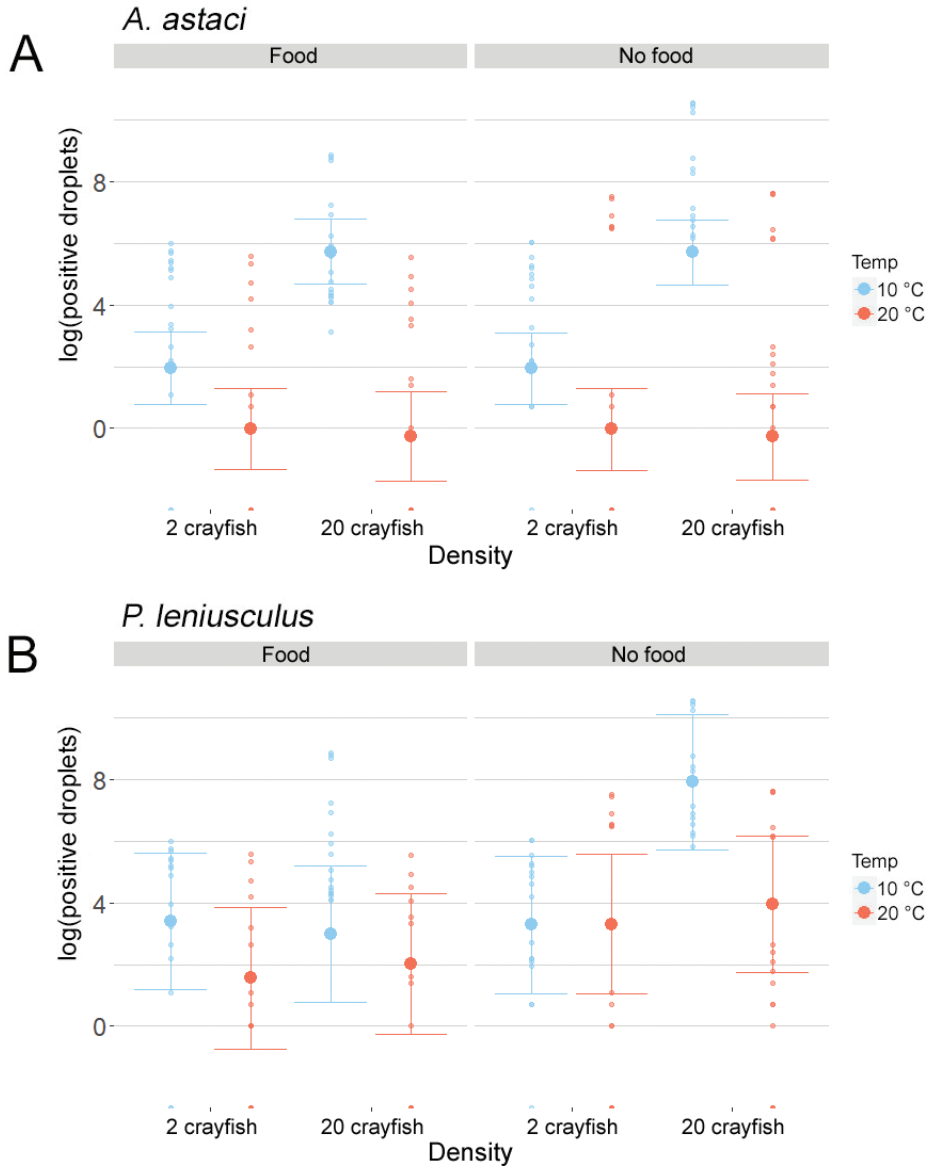


**Figure 5.** Generalised mixed effect model analysis of the influence of temperature, density and food availability on the amount of detectable eDNA of *A. astaci* (**A**) and *P. leniusculus* (**B**) in the mesocosm experiment. The amount of detectable eDNA is represented as positive droplets per sample (log scale). **A** for *A. astaci*, the eDNA quantity (positive droplets) was significantly higher in tanks with high crayfish density (20 crayfish) at 10 °C, while high temperature (20 °C) had a significant negative effect on the eDNA quantity for all combinations **B** for *P. leniusculus*, the eDNA quantity (positive droplets) was significantly higher for the combination "no food" for 20 crayfish at 10 °C, while the combination 20 crayfish provided with food at 20 °C had a significant negative effect on the eDNA quantity.

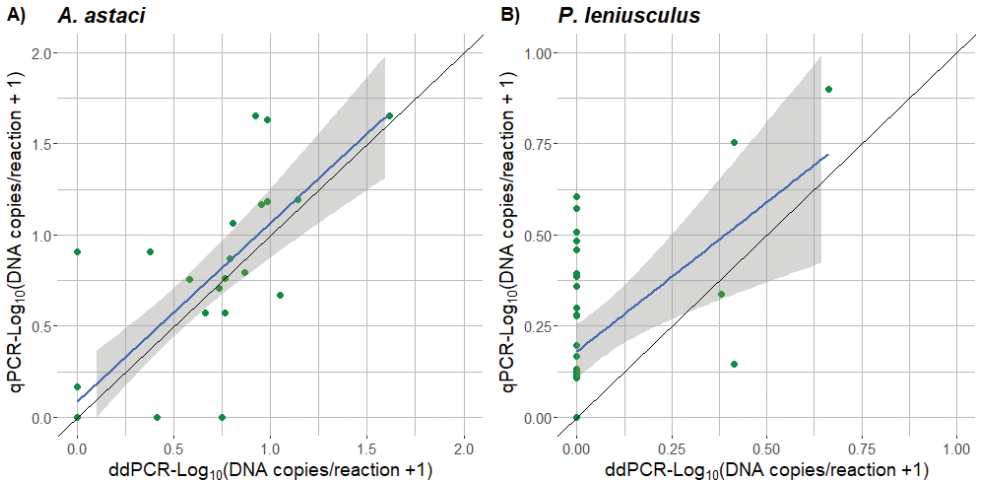


**Figure 6.** Scatterplot of the estimated DNA copies per reaction of both qPCR and ddPCR analysis for *A. astaci* (**A**) and *P. leniusculus* (**B**) from Lake Øymarksjøen. **A** for *A. astaci*, the correlation between qPCR and ddPCR results is relatively good (Pearson's r = 0.81, p = $2.4\times10^{-10}$) **B** for *P. leniusculus*, the correlation between qPCR and ddPCR results is poor (Pearson's r = 0.53, p = 0.0011). Black line represents 1:1 correspondence between ddPCR and qPCR.

**Table 3.** Summary of results from field samples at Lake Stora Le and Lake Øymarksjøen for eDNA detection of *A. astaci* and *P. leniusculus*. The dates of sampling are provided together with the location and sample replicate in Table 1. The volume of water is stated in litres (l) and the catch per unit effort (CPUE) for each respective site is presented. Observed detection-frequency (ω) for both *A. astaci* (*A. ast*) and *P. leniusculus* (*P. len*) is stated. A sample was scored positive for detection if one or both of the two filter subsamples yielded positive amplification of target DNA. The detection probability per site (ψ) as calculated using msocc occupancy modelling is also stated for both organisms.

| Lake | Location | # samples | Volume (l) | CPUE | ω *ddPCR / qPCR* | | detection probability (ψ) | |
|---|---|---|---|---|---|---|---|---|
| | | | | | *A. ast* | *P. len* | *A. ast* | *P. len* |
| **Stora Le** | | | | | | | | |
| | S1 | 5 | 25 | 20 | 0.8 / NA | 0.8 / NA | 1 | 1 |
| | S2 | 5 | 25 | 3.6 | 1 / NA | 0.6 / NA | 0.99 | 0.96 |
| | S3 | 5 | 25 | 0.6 | 0.2 / NA | 0 / NA | 0.94 | 0.29 |
| **Øymarksjøen** | | | | | | | | |
| | O1 | 2 | 10 | 4 | 0.5 / 0 | 0 / 0.5 | 0.99 | 0.00 |
| | O2 | 2 | 9.5 | 9.6 | 1 / 1 | 0 / 0 | 1 | 0.00 |
| | O3 | 2 | 10 | 13.2 | 0.5 / 0.5 | 0 / 0.5 | 1 | 0.00 |
| | O4 | 2 | 8.5 | 17.6 | 0 / 0.5 | 0 / 0.5 | 1 | 0.00 |
| | O5 | 2 | 9 | 25.4 | 1 / 1 | 0 / 1 | 1 | 0.00 |
| | O6 | 2 | 10 | 12.2 | 1 / 1 | 0 / 1 | 1 | 0.00 |
| | O7 | 1 | 5 | 25.8 | 0 / 0 | 0 / 0 | 1 | 0.00 |
| | O8 | 3 | 11 | 13.2 | 1 / 1 | 0 / 1 | 1 | 0.00 |
| | O9 | 2 | 8.5 | 6 | 0 / 1 | 0 / 1 | 0.99 | 0.00 |

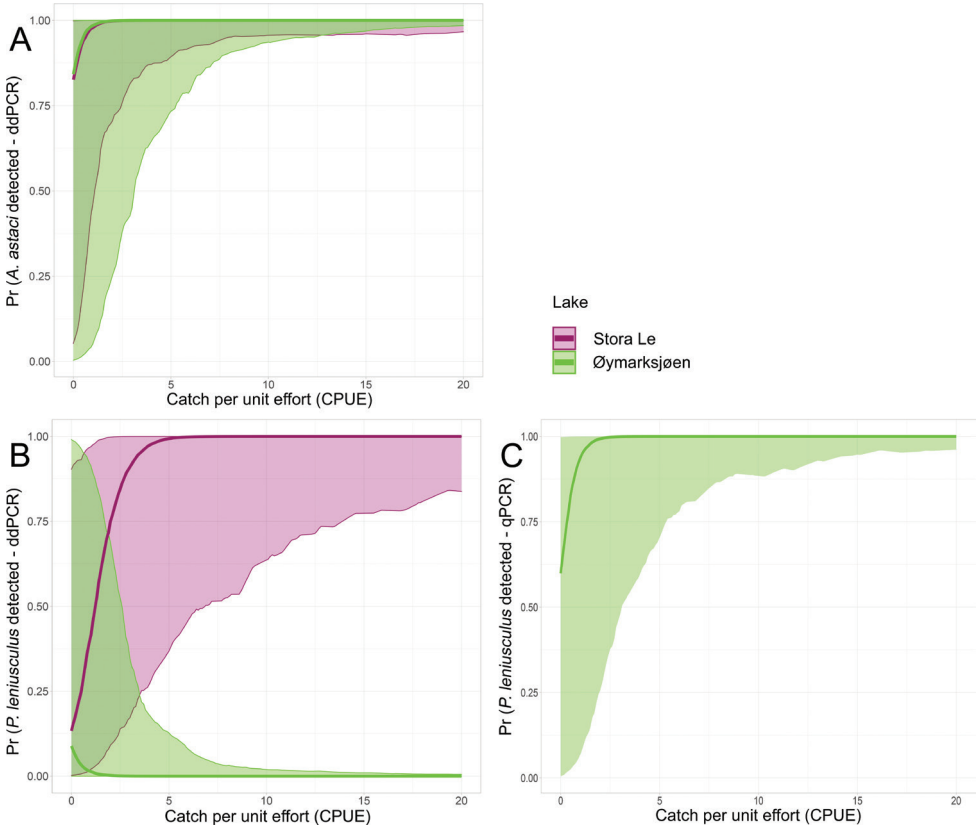


**Figure 7.** Modelling of probability of detection for *A. astaci* (**A**) and *P. leniusculus* (**B**) with respect to catch per unit effort (CPUE) in lakes Øymarksjøen (green line) and Stora Le (purple line) using msocc, based on ddPCR results. The thick lines represent the median detection probability, while the thin lines represent the upper and lower quantile. The figures are based on 11000 iterations, the first 1000 as warm-up and the rest thinned by 10. Figure 7C depicts the probability of *P. leniusculus* detection in relation to CPUE in Lake Øymarksjøen, based on qPCR results.

## Discussion

The mesocosm experiment conducted in our study demonstrates that environmental factors might drastically change the detectable amount of eDNA from *A. astaci* and *P. leniusculus*. In the cold and clear water in the experimental tanks, i.e. in the absence of food supplies, eDNA quantities of *P. leniusculus* and *A. astaci* increased far more than in a linear fashion with crayfish density. However, food availability seemed to contribute to a faster degradation of *P. leniusculus* eDNA. *A. astaci*, on the other hand, was unaffected by the presence of food in the cold water, while a water temperature of 20 °C had a surprisingly huge negative impact on *A. astaci* detectability from eDNA, regardless of food availability.

We found little support for our hypothesis that eDNA emitted from *P. leniusculus* scales directly with the number of individuals. Instead, we observed that small changes to the experimental environment led to large changes – both positive and negative – in

the quantity of detectable eDNA. This indicates that the complexity and variability of influencing factors under field conditions obstructs predictable correlations between eDNA quantities and crayfish density. This is supported by our field data with no clear correlation between eDNA detectability and crayfish population density (as estimated by CPUE). A study on another crustacean, the green crab (*Carcinus maenas*), recently concluded that eDNA cannot be used to rigorously predict the biomass of the target species under controlled conditions (Danziger et al. 2022). The conclusion that eDNA is seemingly not a well-suited tool for the quantification of biomass and population density of *P. leniusculus* is also in concordance with the recently-published study by Johnsen et al. (2020). They demonstrated that high crayfish density was associated with a high detection probability, but not with increased amounts of eDNA.

For surveillance purposes, our study supports a strategy of detecting both the host and the pathogen. As the eDNA detectability of this alien host-pathogen couple seems to be affected differently, eDNA surveillance of both targets will increase the total detection probability, since detection of one may also suggest the presence of the other. This will, of course, only apply in habitats or regions where *A. astaci* is prevalent in alien crayfish hosts and not for American crayfish populations with very low or even missing pathogen prevalence (Schrimpf et al. 2013; Tilmans et al. 2014; Mojžišová et al. 2022).

Under field conditions, eDNA itself and the detectability of eDNA is subjected to a multitude of factors, such as UV radiation, dilution, inhibition through humic acids, retention in substrate and transport that expedite its degradation or disappearance from the system (Jerde et al. 2016; Shogren et al. 2017; Stewart 2019; Wang et al. 2021). In a mesocosm experiment, many of these environmental factors that contribute to fast degradation, masking or disappearance of eDNA are reduced or eliminated. At 10 °C and in the absence of food, we observed an over 1000-fold increase in *P. leniusculus* eDNA and 50-fold increase in *A. astaci* eDNA from a 10-fold increase of crayfish density. While planning the experiment, we expected the availability of food to increase the eDNA concentrations through an increased activity level (Danziger et al. 2022) and faeces production (Ghosal et al. 2018). However, when crayfish were fed, we detected less *P. leniusculus* eDNA in tanks with high crayfish density than in low-density tanks. The water in the high-density tanks with fed crayfish became murkier than in the other tanks and this most likely triggered a much higher microbial activity which can lead to faster degradation of eDNA (Barnes et al. 2014; Barnes and Turner 2016; Salter 2018; Saito and Doi 2021). When measuring eDNA content after a week in this water, a higher degradation of eDNA from *P. leniusculus* might be expected. In contrast to the live *A. astaci* zoospores and even encysted spores (Söderhäll and Cerenius 1999) where the DNA is protected in living cells, the eDNA sources from *P. leniusculus* are more vulnerable to rapid degradation.

Even though sporulation of *A. astaci* has been described as most efficient below 20 °C (Alderman and Polglase 1986; Alderman et al. 1987; Diéguez-Uribeondo et al. 1995) and also observed to decrease above 18 °C (Strand et al. 2012), the drastic reduction of detectable eDNA of *A. astaci* at 20 °C compared to 10 °C was surprising. Strand et al. (2012) observed a negative correlation between temperatures rising from 17 °C to 23 °C and the number of spores produced from infected *P. leniusculus*. A temperature of 10 °C might, therefore, be more conducive to sporulation than temperatures around

20 °C, which seem to be beyond the temperature optimum of the *A. astaci* strain that infected our experimental crayfish. However, this does not fully explain the apparent failure of *A. astaci* spore production in our experiment at 20 °C. Factors both regarding different temperature optimum of different *A. astaci* strains, as well as host differences in the immunity performance at different temperatures, could also have played a role.

The huge increase (> 1000 fold) in eDNA concentrations in the high-density tanks with non-fed crayfish at 10 °C might be explained by injuries from aggressive interactions (Sint et al. 2021) combined with the relatively clean water with assumingly low microbiological activity. In a similar tank experiment, Dunn et al. (2017) successfully detected eDNA of *P. leniusculus*, but only established a significant relationship between eDNA concentration and crayfish biomass when female crayfish were ovigerous. In their study, samples were taken after 11 days. Contrary to our findings and those of Dunn et al. (2017), Harper et al. (2018) observed an increase in eDNA concentration when comparing tanks with one and three *P. leniusculus.* Additionally, Sint et al. (2021) report a clear correlation between eDNA signal strengths and crayfish densities. However, Harper et al. (2018) observed a decrease of eDNA over time, whereas Sint et al. (2021) observed a linear increase during the first three days. While Harper et al. (2018) sampled one, three and seven days after adding crayfish to the tanks and Sint et al. (2021) took multiple samples up to 59 hours after the crayfish had been added to the tanks, the sampling in our experiment and that of Dunn et al. (2017) was conducted after seven and eleven days, respectively. This could have led to a state of saturation or equilibrium where eDNA is emitted from crayfish at a similar rate to its degradation by microbial activity (Barnes et al. 2014; Salter 2018; Saito and Doi 2021), thus obscuring any differences between the tested factors. The short persistence of crayfish eDNA is reflected in the study by Harper et al. (2018). Here, seven days after removal of crayfish, eDNA was detected only in the tanks that had contained three crayfish. Therefore, daily sampling might have revealed more interaction between crayfish density and eDNA concentrations in our study.

When using ddPCR, we observed a relatively good detectability of eDNA from both targets in the field samples in Lake Stora Le and also good detectability of *A. astaci* in Lake Øymarksjøen. Surprisingly, we did not detect *P. leniusculus* in any of the samples from Lake Øymarksjøen with ddPCR, but in 66.7% of the samples when using qPCR. It is unlikely that this was caused by insufficient assay specificity as we obtained satisfactory results from the mesocosm experiment using the same assay on *P. leniusculus* originating from the interconnected lakes Øymarksjøen and Rødenessjøen. However, these results are similar to those in the study by Johnsen et al. (2020) where reduced detection frequency was observed for noble crayfish (*Astacus astacus*) eDNA when using ddPCR compared to qPCR. Inhibition has been reported in another study that screened samples for *P. leniusculus* using ddPCR (Porco et al. 2022). This is in stark contrast to other studies focusing on other organisms than crayfish, that report on higher sensitivity when analysing eDNA samples with ddPCR compared to qPCR (Doi et al. 2015b; Mauvisseau et al. 2019; Wood et al. 2019; Brys et al. 2021). With the exception of the samples from Lake Øymarksjøen analysed for *P. leniusculus*, we found a good correlation between qPCR and ddPCR results, both in field samples and in the mesocosm experiment, but the correlation was unquestionably much bet-

ter in the mesocosm experiment, pointing towards environmental factors in lakes that might impact negatively on the ddPCR results.

The overall detection rate for both organisms was higher in Lake Stora Le than in Lake Øymarksjøen. A speculative explanation is that this may result from trapping (for logistical reasons) prior to sampling in Lake Stora Le. Ideally, eDNA sampling should be carried out before trapping, as crayfish are drawn to the bait from their shelters and feeding activity combined with increased interactions may lead to higher rates of eDNA shedding. Nonetheless, we also observed higher turbidity in Lake Øymarksjøen than in Lake Stora Le. In Stora Le, non-detection of both *A. astaci* and *P. leniusculus* occurred only at locations with low CPUE (0.6 and 3.6) and the detected eDNA quantity corresponded well to the crayfish density. This also may be attributed to trapping prior to sampling as the data suggest from Øymarksjøen and other recent studies where no clear or only weak correlations were found between crayfish density and eDNA concentration (Dougherty et al. 2016; Cai et al. 2017; Larson et al. 2017; Rice et al. 2018; Johnsen et al. 2020). Generally, we detected eDNA of *A. astaci* at a higher frequency than that of *P. leniusculus*. One possible explanation for this may lie within the nature of the eDNA sampled. While eDNA from crayfish most likely consists of (dead) cell shedding in the water column, *A. astaci* eDNA is likely to be captured in the form of living zoospores and encysted spores which are less susceptible to immediate degradation caused by chemical and biological processes. Furthermore, compared to other aquatic organisms such as fish, crayfish seem to emit very low amounts of eDNA (Forsström and Vasemägi 2016; Fossøy et al. 2020; Johnsen et al. 2020).

Through the mesocosm experiment and the comparison with additional field data, we demonstrated that the detectability of both *P. leniusculus* and *A. astaci* eDNA is influenced by much more than mere population density. When sampling to monitor the presence of *A. astaci*, it is advisable to analyse the samples for eDNA of both the host and the pathogen for optimal detection efficiency. The crayfish plague agent *A. astaci* requires a crayfish host (or another freshwater decapod crustacean, see Schrimpf et al. 2014; Svoboda et al. 2014; Putra et al. 2018) for long term survival (OIE 2019). Moreover, only few studies report on NICS populations that are free of infections with *A. astaci* or below the level of detection (Schrimpf et al. 2013; Tilmans et al. 2014; Mojžišová et al. 2022). An efficient sampling strategy requires both a robust knowledge of the biology of the target species (Rusch et al. 2020) as well as taking spatio-temporal considerations into account (Thalinger et al. 2021). Furthermore, the number of samples heavily impacts the success of detection. Through our statistical modelling, we show that a high detection probability is dependent on crayfish density (CPUE). For *P. leniusculus*, the required density was 5 CPUE in Stora Le (based on ddPCR results, Fig. 7B) and 3 CPUE in Øymarksjøen (based on qPCR results, Fig. 7C) for a near 100% detection probability in only one sample. It is not uncommon to find crayfish populations with markedly lower population densities (Johnsen et al. 2020) where one sample would be insufficient for a positive detection. Other studies report similar results where crayfish are detected at low densities, but with only infrequent positive detection (Dougherty et al. 2016; Larson et al. 2017; Johnsen et al. 2020). For *A. astaci* in the two lakes studied by us, required crayfish density for a near 100% detection

probability was 2 CPUE. This number may be subject to variation, depending on the infection status, *A. astaci* prevalence and agent level (Vrålstad et al. 2009; Strand et al. 2014). Statistical modelling of the required sampling effort is, therefore, highly advisable (Dougherty et al. 2016; Johnsen et al. 2020; Sieber et al. 2020).

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## Supplementary material 1

**Primers and probes used in the present study**

Authors: Johannes C. Rusch, David A. Strand, Charlotte Laurendz, Tom Andersen, Stein I. Johnsen, Lennart Edsman, Trude Vrålstad

Data type: table (pdf file)

Explanation note: Primers and probes for *Aphanomyces astaci* and signal crayfish (*Pacifastacus leniusculus*) used in the present study.



Link: https://doi.org/10.3897/neobiota.79.82793.suppl1

## Supplementary material 2

**ddPCR and qPCR data from the mesocosm experiments**

Authors: Johannes C. Rusch, David A. Strand, Charlotte Laurendz, Tom Andersen, Stein I. Johnsen, Lennart Edsman, Trude Vrålstad

Data type: table (excel file)

Explanation note: ddPCR and qPCR data from the mesocosm experiments at 20° C ("warm") (EXP1-EXP3). ddPCR and qPCR data from the mesocosm experiments at 10° C ("low temp") (EXP4-EXP6).



Link: https://doi.org/10.3897/neobiota.79.82793.suppl2

## Supplementary material 3

**R-Script of GLM analysis**
Authors: Johannes Rusch, David Strand, Tom Andersen
Data type: statistics
Explanation note: R-Script of GLM analysis of data from the mesocosm experiments.

Link: https://doi.org/10.3897/neobiota.79.82793.suppl3

## Supplementary material 4

**R-script of MCOCC occupancy analysis**
Authors: Johannes Rusch, David Strand, Tom Andersen
Data type: statistics
Explanation note: R-script of the MCOCC occupancy analysis for field data.

Link: https://doi.org/10.3897/neobiota.79.82793.suppl4

## Supplementary material 5

**Results of the GLMM model**
Authors: Johannes C. Rusch, David A. Strand, Charlotte Laurendz, Tom Andersen, Stein I. Johnsen, Lennart Edsman, Trude Vrålstad
Data type: statistics
Explanation note: Results of the GLMM model for *A. astaci* (*A. ast*) and signal crayfish (*P. len*) determining statistical significance of three factors (food, temperature, density) on quantity of eDNA represented by positive droplets.

Link: https://doi.org/10.3897/neobiota.79.82793.suppl5

## Supplementary material 6

**Agent levels of *Aphanomyces astaci* in individuals of *Pacifastacus leniusculus* used in the experiment**

Authors: Johannes C. Rusch, David A. Strand, Charlotte Laurendz, Tom Andersen, Stein I. Johnsen, Lennart Edsman, Trude Vrålstad

Data type: table (pdf file)

Explanation note: Agent levels of *Aphanomyces astaci* in individuals of *Pacifastacus leniusculus* used in the experiment. The agent level categories (A0-A5; Vrålstad et al. 2009) are based on DNA copy numbers or PFU (PCR forming units) obtained from qPCR analysis of signal crayfish tissue samples.



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RESEARCH ARTICLE

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# Low genetic diversity in a widespread whistling alien: A comparison of *Eleutherodactylus johnstonei* Barbour, 1914 (Eleutherodactylidae) and congeners in native and introduced ranges

Franziska Leonhardt[1,2*], Clara Arranz Aveces[3], Anke Müller[1], Baptiste Angin[4], Mathieu Jegu[5], Pius Haynes[6], Raffael Ernst[1,2*]

**1** *Museum of Zoology, Senckenberg Natural History Collections Dresden, A. B. Meyer Building, Königsbrücker Landstraße 159, 01109 Dresden, Germany* **2** *Technical University of Dresden, Faculty of Biology, 01062 Dresden, Germany* **3** *Staatliches Museum für Naturkunde, Rosenstein 1, 70191 Stuttgart, Germany* **4** *Ardops Environment, Les Abymes, Guadeloupe, France* **5** *Office Français de la Biodiversité, Pérols, Occitanie, France* **6** *Department of Forestry, Ministry of Agriculture, Fisheries, Physical Planning, Natural Resources and Co-operatives, Castries, Saint Lucia*

Corresponding authors: Raffael Ernst (raffael.ernst@senckenberg.de), Franziska Leonhardt (franziska.leonhardt@senckenberg.de)

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## Abstract

There is no clear empirical evidence to support the general assumption that genetic diversity favours successful invasions. Many invading species disperse and establish successfully despite low genetic diversity, a phenomenon known as the genetic paradox of biological invasion. Model systems that allow comparison of genetic patterns between exotic and native source populations are still scarce. This is particularly true for amphibians. Here we compare genetic patterns of the widely introduced Johnstone's Whistling Frog, *Eleutherodactylus johnstonei*, with its successful alien congener *E. antillensis* and the single island endemic *E. portoricensis*. Genetic diversity and population differentiation in native and introduced populations of the three taxa were inferred from mitochondrial D-loop sequences (235 bp). Our results reveal that exotic populations of the two alien taxa, *E. johnstonei* and *E. antillensis*, are not only genetically impoverished due to founder effects, but that, moreover, their native range source-populations exhibit low genetic diversity and inter-population differentiation in the first place. Populations of the endemic *E. portoricensis*, on the other hand, are genetically more diverse and show marked inter-population differentiation. These observed

* These authors contributed equally to the manuscript.

genetic patterns are consistent with geological processes and invasion histories. We argue that the establishment success of the alien taxa in our model system is better explained by ecological factors and anthropogenic drivers than by genetic diversity. As these factors provide more parsimonious explanations, they should be given priority in management decisions. However, molecular studies with higher resolution are needed to fully test possible genetic and epigenetic components that could promote the invasion process.

**Keywords**
Alien amphibians, Anura, D-loop, genetic paradox, Lesser Antilles, population genetics

## Introduction

Understanding the mechanisms of successful invasions is at the heart of invasion biology. More recently, the field has turned to molecular approaches that address their genetic basis (Bock et al. 2015). Introduced populations are often genetically impoverished as a result of strong founder effects that drive populations through genetic bottlenecks (Nei et al. 1975; Dlugosch and Parker 2008). Although intra-population genetic diversity is thought to be essential for successful invasion and establishment (Booy et al. 2000), several taxa with low genetic diversity have successfully established themselves in non-native areas (Allendorf and Lundquist 2003). This phenomenon is known as the genetic paradox of biological invasions (Allendorf and Lundquist 2003; Pérez et al. 2006). Yet, a genetic paradox is often simply assumed (Estoup et al. 2016) and differences in the genetic makeup between native and introduced populations are rarely tested systematically (e.g. Bradshaw et al. 2007; Stipoljev et al. 2021). However, this information is a prerequisite for tackling more complex questions with respect to the assumed correlation between genetic diversity, adaptive potential and invasion success.

Evidence from studies that compared genetic diversity of invasive taxa in their native and introduced ranges is ambiguous (Dlugosch and Parker 2008). Many successful invaders show few signs of genetic impoverishment in introduced populations (Tonione et al. 2011; Wellband et al. 2017, 2018; Negri et al. 2018), while others show very low genetic diversity across a wide exotic range (Harrison and Mondor 2011; Edelaar et al. 2015; Castillo et al. 2018). However, to investigate the true interaction between genetic diversity and successful invasions, comparisons are needed not only between exotic and native populations, but also between invasive and non-invasive congeners. Studies following this framework allow us to address the importance of the original genetic makeup of the source population in determining invasion success (Rollins et al. 2013; Romiguier et al. 2014; Trucchi et al. 2016; Baltazar-Soares et al. 2017). Here, we introduce a novel amphibian model system to test correlative patterns of genetic diversity and invasion success.

Robber Frogs of the genus *Eleutherodactylus* Duméril & Bibron, 1841 are a very diverse and species rich (206 recognised species) group of small to medium-sized direct developing frogs that have their distribution centre in the Antilles (Dugo-Cota et al. 2019; Frost 2021). Most species have very restricted ranges and can be considered single-

island or even micro-endemics restricted to small habitat patches on particular islands. However, a few species have succeeded in establishing themselves outside their native range (e.g. *E. antillensis*, *E. coqui*, *E. planirostris* and *E. martinicensis*). The most widely and successfully expanding species in the genus, and one of the most successful alien amphibians, is Johnstone's Whistling frog, *Eleutherodactylus johnstonei* Barbour, 1914. Today, it occurs on the majority of Caribbean islands and in many countries on the South American mainland (Kaiser et al. 2002; Ernst et al. 2011) as well as in Europe, where it is restricted to confined populations in greenhouses (Leonhardt et al. 2019; Moravec et al. 2020). Due to a lack of historic distribution data, it is difficult to unambiguously trace back the geographic origin of the species. Based on the cumulative historical and molecular evidence (Kaiser 1997; Censky and Kaiser 1999; Yuan et al. 2022), we here assume St. Lucia to be the most likely origin of exotic populations outside the Lesser Antilles.

In the present study we investigate the genetic diversity and haplotype distribution of *Eleutherodactylus johnstonei* across its assumed native range and in selected exotic populations. We compare these data with two congeneric species, *E. antillensis* (successful alien, native to Puerto Rico) and *E. portoricensis* (Puerto Rican endemic). We integrate extensive field and laboratory data sets for our focus taxon *E. johnstonei* with previously published data for *E. antillensis* and *E. portoricensis* to test the following assumptions. (1) *E. johnstonei* goes through genetic bottlenecks resulting in reduced genetic diversity in introduced populations compared to native populations. (2) Successful alien species in our model system (*E. johnstonei* and *E. antillensis*) are *a priori* genetically more diverse with respect to their non-expanding congener (*E. portoricensis*). We discuss the results in the light of the genetic paradox of biological invasions and with respect to the invasion history and ecology of the species considering previously proposed expansion scenarios.

## Methods

Within our analytical framework, we integrated three taxon-based data sets including *Eleutherodactylus johnstonei* (this study, Leonhardt et al. 2019), *Eleutherodactylus portoricensis* (Barker et al. 2011) and *Eleutherodactylus antillensis* (Barker et al. 2012; Barker and Rodríguez-Robles 2017). In a first step, we compared molecular patterns (genetic diversity and differentiation, haplotype distribution) in the native and three introduced occurrence regions of the focus species *E. johnstonei* and reconstructed the invasion history based on mitochondrial D-loop sequences. For the two sister taxa we analysed genetic diversity, differentiation and haplotype distribution of the same mitochondrial D-loop fragment and compared them to the patterns uncovered in *E. johnstonei*.

### Field sampling

Field sampling was carried out in the assumed native range of St Lucia (LCA, Feb – Mar 2020) and exotic ranges in Guadeloupe (GLP, Feb – Mar 2020) and in greenhouses

of European botanical gardens in Germany, Switzerland and the Netherlands (EUR, May – Aug 2018). Data sets for Colombia (COL) were established in a previous study (Leonhardt et al. 2019; field sampling between 2016 and 2018). We aimed at sampling a minimum of five individuals per sampling site in each of the four regions, covering a wide range of habitats (see Fig. 1 and Suppl. material 1 for details on sampling sites). Tissue samples for genetic analyses were acquired using minimally invasive toe clipping (Vences et al. 2012). After clipping the external phalanx, toes were disinfected with cotton pads soaked in 70% ethanol to prevent subsequent infections and individuals were immediately released afterwards. Samples were stored in 95% Ethanol and deposited in the tissue collection of the Museum of Zoology, Senckenberg Natural History Collections Dresden (MTD). As part of the respective national biocontrol procedures, individuals from Guadeloupe were not released but collected as scientific vouchers.

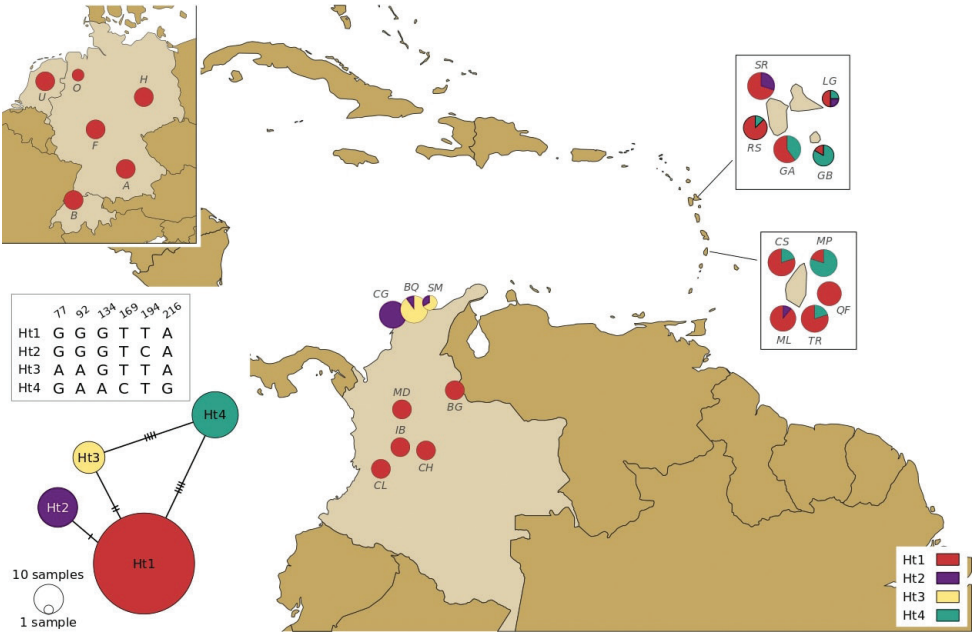


**Figure 1.** Haplotype distribution and network for *E. johnstonei* across native and exotic ranges. Bubble diagram of minimum spanning tree in the lower left shows interrelation between the four recovered haplotypes (Ht1, Ht2, Ht3, Ht4), circle size corresponds to sample size for respective Hts across the four regions, number of crossbars on connecting lines denote the number of polymorphic sites separating these haplotypes. Polymorphic sites are illustrated in the box above the haplotype network, numbers refer to positions in the alignment of the 235 bp D-loop fragment. The maps show the proportions of detected haplotypes at each population site, colours represent the haplotypes, circle size represents no. of samples; Europe: U – Utrecht, O – Osnabrück, H – Halle, F – Frankfurt, A – Augsburg, B – Basel, Colombia: CG – Cartagena, BQ – Barranquilla, SM – Santa Marta, MD – Medellin, BG – Bucaramanga, IB – Ibagué, CH – Chinauta, CA – Cali; Guadeloupe: SR – Saint Rose, RS – Rivière-Sens, LG – Le Gosier, GA – Grande Anse, GB – Grande Bourg; Saint Lucia: CS – Castries, MP – Morne Panache, QF - Quilesse Forest, ML – Morne Le Blanc, TR – Forest Ti Rocher.

These individuals were euthanized using commercially available toothache pain relief gel containing 20% Benzocaine and subsequently preserved in 70% Ethanol. Specimens are deposited in the collection of the Muséum national d'Histoire naturelle, Paris (MNHN) under collection numbers MNHN-RA-2021.0013 to MNHN-RA-2021.0062. For Saint Lucia, two specimens of each population were collected as reference vouchers and deposited at the Forestry Department of Saint Lucia.

## Molecular data sets

The D-loop of the mitochondrial control region was chosen as a marker because it is the most polymorphic mitochondrial region (Stoneking et al. 1991; McMillan and Palumbi 1997; Bronstein et al. 2018) and mtDNA is more sensitive for the detection of population structure and history than nuDNA due to its higher mutation rate (Allio et al. 2017). Moreover, this marker has proven to yield robust patterns in previous studies on genetic structure in our target taxa (Leonhardt et al. 2019; Barker et al. 2011, 2012; Barker and Rodríguez-Robles 2017). For *E. johnstonei*, a total of 113 independent tissue samples from Saint Lucia (N = 48), Guadeloupe (N = 38) and Europe (N = 27) were used to generate mitochondrial (mt) haplotypes from partial sequences of the D-loop region (235 bp). These were complemented with 48 previously established sequences from Colombia using the same marker (Leonhardt et al. 2019). DNA isolation, PCR amplification of the D-loop fragment and sequencing were performed as described in Leonhardt et al. (2019). All sequences are deposited in NCBI GenBank under accession numbers OW993929–OW994041.

We performed a systematic NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) search for D-loop sequences of taxa that are congeneric with *E. johnstonei* and fulfil the following criteria: a) sufficient sample size (minimum N = 48, matching sample size for native range samples of *E. johnstonei*) and covering both native and exotic range in case of invasive taxa, b) available meta data (localities, etc.) provided in associated publications. Two datasets of *Eleutherodactylus portoricensis* (Barker et al. 2011) and *Eleutherodactylus antillensis* (Barker et al. 2012; Barker and Rodríguez-Robles 2017) met these criteria. Associated data are summarized in Table 1 (see Suppl. material 1 for more detailed information). For each species one sequence was used for a pairwise alignment with one *E. johnstonei* sequence, using BioEdit (Hall 1999), in order to define the respective partial sequence. Thus, the same partial D-loop sequence was used for all four species and in all subsequent analyses.

## Molecular diversity and population genetic analyses

Sequence sets of each species (*E. johnstonei*, *E. antillensis*, *E. portoricensis*) were aligned using ClustalW multiple alignment within BIOEDIT Sequence Alignment Editor 7.2.5 (Hall 1999). Sites containing gaps were not considered for all subsequent analyses (assignment of haplotypes, parameters of molecular diversity and differentiation). All sequences were grouped by their respective sampling sites and regions as specified

**Table 1.** Molecular data sets of the three congeneric taxa.

| Species | NCBI Genbank Accession no. & date | Distribution | Region | $N_{samples}$ / $N_{sites}$ | Source |
|---|---|---|---|---|---|
| *E. johnstonei* | OW993929–OW994041 | native | Saint Lucia (LCA) | 48 / 5 | this study, Leonhardt et al. 2019 |
| | | exotic | Guadeloupe (GLP) | 38 / 5 | |
| | | exotic | Colombia (COL) | 48 / 8 | |
| | | exotic | Europe (EUR) | 27 / 6 | |
| *E. antillensis* | JN385299–JN385583, KY636451–KY636487 (03/12/2020) | native | Western Puerto Rico (WPR) | 139 / 28 | Barker et al. 2012; Barker and Rodríguez-Robles 2017 |
| | | native | Eastern Puerto Rico (EPR) | 64 / 13 | |
| | | native | Eastern Islands (EI) | 67 / 14 | |
| | | exotic | Saint Croix (SCX) | 37 / 5 | |
| | | exotic | Panama (PAN) | 15 / 3 | |
| *E. portoricensis* | HM229815–HM229958 (03/12/2020) | endemic | Puerto Rico – Cayey Mountains: | 32 / 3 | Barker et al. 2011 |
| | | | Cerro de la Tabla (CAY-CT) | 39 / 3 | |
| | | | Carite State Forest (CAY-CS) | 32 / 4 | |
| | | | Puerto Rico – Luquillo Mountains: | 15 / 1 | |
| | | | El Yunque (LUQ-EY) | 26 / 5 | |
| | | | Pico del Este (LUQ-PE) | | |
| | | | El Torro (LUQ-ET) | | |

in the source publications (see Table 1 and Suppl. material 1). To compare the genetic setup in the native vs. the exotic range of *E. johnstonei* and between *E. johnstonei* and congeneric sister taxa, the distribution and relatedness of haplotypes, as well as parameters of genetic diversity and population differentiation were analysed. For each of the three species, data on haplotype distribution within sites and regions was exported from DnaSP v6 (Rozas et al. 2017). Haplotype networks were generated with POPART (Leigh and Bryant 2015), using the Minimum Spanning network inference method. Haplotype networks were colour-coded by region to visualise the geographic distribution of haplotypes. Additionally, the distribution of haplotypes within native and exotic regions for the focal species *E. johnstonei* was mapped using QGIS 3.16.11 (QGIS Development Team 2021).

To address our hypothesis 1 (genetic bottlenecks) we estimated levels of molecular diversity within *E. johnstonei* across the four study regions (LCA, GLP, COL, EUR) and to address hypothesis 2 (genetic diversity differences between invasive and non-invasive species) additionally within native localities of all three sister taxa (*E. johnstonei*, *E. antillensis* and *E. portoricensis*). The following molecular diversity parameters were estimated based on pooled samples for an entire region, as well as for each locality within a region. The number of variable sites (s), the number of haplotypes (nHap) and how equally they are distributed (haplotype diversity, $H_D$), the average number of nucleotide differences between two sequences per site (nucleotide diversity, $\pi$) and the mean number of alleles per site (A) were analysed. All parameters were calculated with DnaSP v6 (Rozas et al. 2017), except for A, which was calculated with Arlequin v3.5.2.2 (Excoffier and Lischer 2010). Genetic differentiation of populations (i.e. localities) was assessed by pairwise $F_{ST}$ values (fixation indices), calculated in DnaSP. Pairwise $F_{ST}$ values estimate

the proportion of total genetic variation of two populations (γ diversity) between the two populations (β diversity) as opposed to the variation within the two populations (α diversity). Mann-Whitney-Wilcoxon tests were performed in R 4.0.2 (R Core Team 2021) to compare genetic diversity and differentiation of (1) populations in native regions vs. exotic regions of *E. johnstonei* and in (2) native populations of *E. johnstonei* vs. the two congeners. For Mann-Whitney-Wilcoxon tests we assumed that each locality represents one population, which means regions are compared by their average population-wide molecular diversity and pairwise population differentiation, respectively.

## Results

### Genetic makeup of *Eleutherodactylus johnstonei* in the native and introduced range

Populations of *E. johnstonei* show low molecular diversity and population differentiation across both native and exotic regions. Partial D-loop sequences (235 bp, 161 samples) across the whole sampled range feature only four haplotypes and six variable sites. Moreover, overall nucleotide diversity (0.0059) and haplotype diversity (0.5) are very low. A comparison of the three exotic (GLP: N = 38, COL: N = 48, EUR: N = 27) regions with the assumed native origin (LCA: N = 48) revealed that the latter did not exhibit the highest genetic diversity as originally hypothesised. In fact, molecular diversity within Guadeloupean populations was similar and even higher than in populations from Saint Lucia for all analysed parameters (see Table 2). European greenhouse populations, on the other hand, show clear signs of reduced molecular diversity as all analysed individuals show identical D-loop sequences corresponding to the haplotype Ht1. Considering the number of haplotypes (nHap), the number of variable sites (s), haplotype richness ($H_R$), haplotype diversity ($H_D$) and the mean number of alleles per locus (A), Saint Lucian populations are significantly more diverse than those from Colombia, while nucleotide diversity ($\pi$) is not significantly reduced in Colombia. Colombian populations are also more differentiated ($F_{ST}$ = 0.443) than those from Saint Lucia ($F_{ST}$ = 0.279) and Guadeloupe ($F_{ST}$ = 0.206).

Geographic distribution of the four detected haplotypes across native and exotic ranges of *E. johnstonei*, as well as the haplotype network and variable sites defining the haplotypes, are illustrated in Fig. 1. The dominant haplotype Ht1 is present in 110 out of 161 samples (68%) and in all four regions. In all European localities and inland localities in Colombia, Ht1 is the only haplotype that was detected. Three additional haplotypes (Ht2, 17%; Ht3, 11%; Ht4, 23%) were detected with lower abundance. Ht2 is present both at the Colombian coast and on the two Caribbean islands, while it is much more common in the former. Ht3 is the only geographically unique haplotype, which was exclusively detected along the coast of Colombia. It is also the least abundant of all four haplotypes recorded. Ht4 is widespread across St. Lucia and Guadeloupe. Both islands share the same three haplotypes, while there is a clearer differentiation between haplotypes in exotic ranges outside the Caribbean.

**Table 2.** Parameters of molecular diversity and population differentiation for *E. johnstonei* in native and exotic regions. For each region no. of samples ($N_{sam}$) and no. of populations ($N_{pop}$) are given in brackets. nHap: no. of haplotypes (DNAsp), s: no. of variable sites (DNAsp), $H_D$: haplotype diversity (DNAsp), A: mean number of alleles per locus (Arlequin), π: nucleotide diversity (DNAsp), $F_{ST}$: average pairwise $F_{ST}$ (DNAsp); for each region average values per population and total values for all samples (in brackets) are given; p-values of Mann-Withney-Wilcoxon tests testing for greater diversity and differentiation in St Lucia against the other regions are illustrated (p < 0.05*, p < 0.01**, p < 0.001***), Mann-Withney-Wilcoxon tests were based on population averages.

| Range ($N_{sam}$ / $N_{pop}$) | nHap | S | $H_D$ | A | π | $F_{ST}$ |
|---|---|---|---|---|---|---|
| St Lucia (48 / 5) native | 1.8 (3) | 2.6 (5) | 0.26 (0.414) | 1.011 (1.021) | 0.004 (0.0067) | 0.279 |
| Guadeloupe (38 / 5) exotic | 2.2 (3) | 3.6 (5) | 0.48 (0.553) | 1.015 (1.002) | 0.006 (0.008) | 0.206 |
| Colombia (48 / 8) exotic | 1.25* (3) | 0.75* (3) | 0.11* (0.627) | 1.003* (1.023) | 0.001 (0.0047) | 0.443 |
| Europe (27 / 6) exotic | 1** (1) | 0** (0) | 0** (0) | 1** (1) | 0** (0) | 0 |

**Table 3.** Parameters of molecular diversity and population differentiation for native populations of *E. johnstonei* and sister taxa. For each species no. of samples ($N_{sam}$) and no. of populations ($N_{pop}$) are given in brackets. nHap: no. of haplotypes (DNAsp), s: no. of variable sites (DNAsp), $H_D$: haplotype diversity (DNAsp), A: mean number of alleles per locus (Arlequin), π: nucleotide diversity (DNAsp), $F_{ST}$: average pairwise $F_{ST}$ (DNAsp); for each taxa average values per population and total values for all samples (in brackets) are given; Mann-Withney-Wilcoxon tests were based on population averages.

| Species ($N_{sam}$/$N_{pop}$) | nHap | S | $H_D$ | A | Π | $F_{ST}$ |
|---|---|---|---|---|---|---|
| *E. johnstonei* (48/5) | 1.8 | 2.6 | 0.258 | 1.011 | 0.0038 | 0.279 |
| successful alien | (3) | (5) | (0.414) | (1.021) | (0.0067) | |
| *E. antillensis* (270/55) | 1.3 | 0.33 | 0.149 | 1.002 | 0.0007 | 0.438 |
| successful alien | (15) | (12) | (0.546) | (1.055) | (0.0027) | |
| *E. portoricensis* (144/16) non-invasive, single-island endemic | 5 (54) | 6.1 (33) | 0.806 (0.964) | 1.028 (1.176) | 0.0102 (0.0277) | 0.457 |
| | p(Ej < Ep) = 0.002** | p(Ej < Ep) = 0.015* | p(Ej < Ep) = 0.002** | p(Ej < Ep) = 0.008** | p(Ej < Ep) = 0.008** | p(Ej < Ep) = 0.04* |
| | p(Ea < Ep) = 5.58e-10*** | p(Ea < Ep) = 3.88e-10*** | p(Ea < Ep) = 1.05e-9*** | p(Ea < Ep) = 7.34e-10*** | p(Ea < Ep) = 4.09e-10*** | p(Ea < Ep) = 0.18 |

## Genetic diversity and population differentiation within and among species of the *Eleutherodactylus* model system

We found molecular diversity and population differentiation to be lowest in successfully colonising alien species. On average, all parameters estimated per native population (nHap, s, $H_D$, A, π, $F_{ST}$) are higher in *E. portoricensis* as compared to *E. johnstonei* and *E. antillensis*. This was also confirmed by Mann-Withney-Wilcoxon tests for all parameters except of $F_{ST}$, which indicate significantly lower population differentiation of *E. johnstonei*, but not of *E. antillensis*, as compared to *E. portoricensis* (see Table 3). Native populations of *E. johnstonei* and *E. antillensis* show similar diversity estimates, while populations of *E. antillensis* are slightly more differentiated ($F_{ST}$(Ej) = 0.279, $F_{ST}$(Ea) = 0.438).

Haplotype distribution and networks for all model taxa are visualised in Fig. 2. We found no significant spatial clustering of haplotypes in *E. johnstonei*. While Ht2 is the only haplotype that exclusively occurs in one region (COL), the most dominant haplotype Ht1 occurs across the entire range of investigation. A similar pattern emerged in the second successful alien, *E. antillensis*. Here, two dominant haplotypes comprise 78% of all samples. Geographic clusters are largely missing. However, a few less abundant haplotypes exclusively occur in a single region and the second most abundant haplotype clearly dominates western Puerto Rico. The single-island endemic *E. portoricensis* shows a markedly different pattern with clear geographic clustering. Populations of *E. portoricensis* are clearly divided into the two subregions, Luquillo and Cayey, which do not share any haplotype. Within those two subregions there are several private haplotypes (Hts exclusively occurring in a single population) and fewer haplotypes that are shared between populations (reflected by the lower $F_{ST}$ value 0.478, see Table 3).

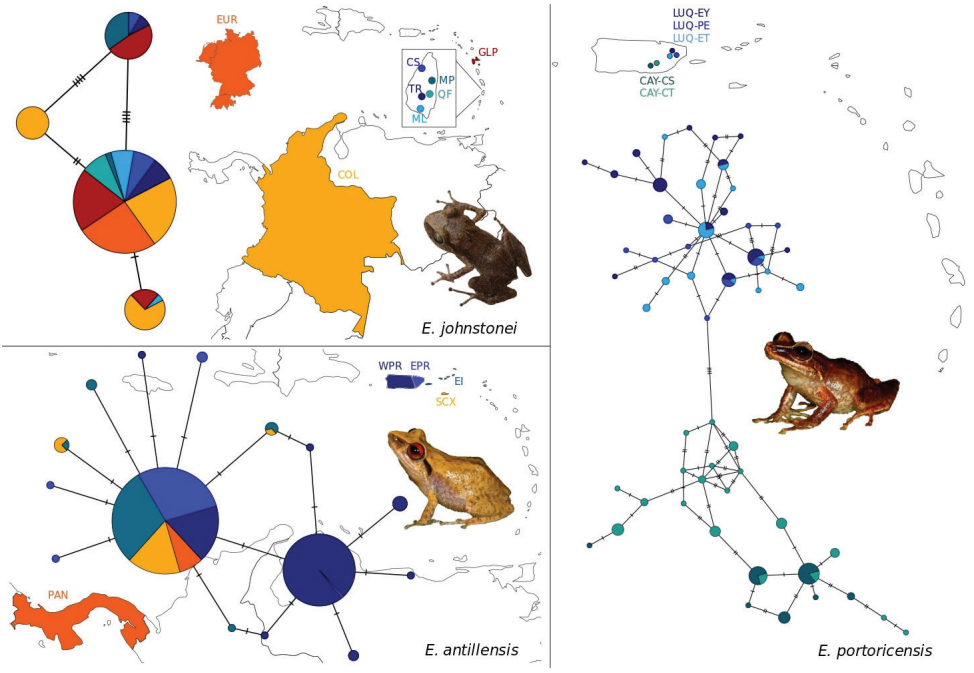


**Figure 2.** Comparative haplotype distribution and networks for all species of the *Eleutherodactylus* model system. Circle sizes correspond to respective sample sizes; pie chart colours correspond to respective populations. Yellow, orange and red correspond to exotic range populations in: EUR – Europe, COL – Colombia, GLP – Guadeloupe for *E. johnstonei*; PAN – Panama, SCX – Saint Croix for E. antillensis, blue and greenish colours represent native range populations (CS, QF, TR, MP, ML on Saint Lucia for *E. johnstonei*; WPR – Western Puerto Rico, EPR – Eastern Puerto Rico, EI – Eastern Islands for *E. antillensis*; LUQ-EY, LUQ-PE, LUQ-ET in the Luquillo Mountains and CAY-CS, CAY-CT in the Cayey Mountains on Puerto Rico for *E. portoricensis*). Photo sources: *E. johnstonei* - F. Leonhardt, *E. antillensis* - A. Lopéz, https://mir-s3-cdncf.behance.net/project_modules/max_1200/c1290514066123.5627cd91e1baf.jpg, *E. portoricensis* - A.D. Colón Archilla, https://alfredocolon.zenfolio.com/p973584972/h21cf17fc#h21cf17fc.

## Discussion

The Caribbean features America's most extensive Cretaceous and Cenozoic oceanic-continental tectonic zone and it has the majority of the active volcanic centres of the New World (Donelly 1989). Therefore, the region represents an ideal model to test (island)biogeographic theories and their molecular basis (e.g. Hedges et al. 1992; Losos and Schluter 2000; Vellend 2003; Dugo-Cota et al. 2019). However, the role of alien taxa in shaping biogeographic patterns in this region has only recently been studied (Helmus et al. 2014). Here we established the first comprehensive molecular data set covering both the native and exotic range of the most widespread amphibian species with a Caribbean origin, *Eleutherodactylus johnstonei*. In contrast to what we expected, we detected comparatively low levels of genetic diversity and population differentiation in the species' assumed native range, St. Lucia. We observed similar genetic patterns in introduced populations on the islands of Guadeloupe. Exotic populations outside the Caribbean, however, were genetically impoverished, indicating marked founder effects. As in *E. johnstonei*, the invasive congeneric *E. antillensis* showed comparably low genetic diversity in its native range. In stark contrast to this pattern, we found marked inter-population differentiation and higher overall molecular diversity in the non-invasive congener *E. portoricensis*.

The genetic patterns observed in exotic populations of *E. johnstonei* (see Fig. 1) mirror respective introduction histories in the three regions. Only a single haplotype (Ht1 *sensu* Leonhardt et al. 2019) is present in European greenhouse populations. Since Ht1 is also the dominant haplotype in populations from Guadeloupe, our results support a single introduction event in 1993, when the Botanical Garden of Basel received a plant shipment from Guadeloupe (H. Schneider pers. comm.) that likely contained the founder individuals. Additional populations were subsequently established through deliberate exchange between the European botanical gardens. Colombian populations of *E. johnstonei* show higher levels of both genetic diversity and inter-population differentiation, which supports the previously proposed two to three independent introduction events (Leonhardt et al. 2019). All Colombian inland populations exhibit the dominant haplotype Ht1 and were likely derived from a single introduction to Bucaramanga (Ortega et al. 2001; Leonhardt et al. 2019). The native range populations from St. Lucia, as well as populations from Guadeloupe, are possible sources of this introduction. For coastal populations in Colombia, two scenarios are possible: (1) In two independent introductions, as previously hypothesised in Leonhardt et al. (2019), Ht3 was introduced to Barranquilla and Ht2 to Cartagena. Individuals subsequently spread along the coast via jump dispersal, as described in Ernst et al. (2011), thereby establishing the Santa Marta population and introducing Ht2 into the Barranquilla populations. In this scenario, St Lucia or Guadeloupe are possible sources of the introduction to Cartagena (Ht2) and Ht3, introduced to Barranquilla, either originates from an un-sampled Caribbean island or was missed in our Caribbean samples due to its rarity. (2) A single introduction to Barranquilla from a Lesser Antillean source population containing both Ht2 and Ht3, and subsequent distribution

to Santa Marta and Cartagena. Disentangling these competing scenarios would require additional sampling in yet un-sampled Caribbean localities, as well as the use of higher resolution molecular markers. Guadeloupean populations of *E. johnstonei* do not only show higher genetic diversity, but also higher connectivity between populations revealed by spatial haplotype distributions. These differences in genetic patterns between Caribbean (GLP) and non-Caribbean (COL, EUR) introduced populations are mirrored in distribution patterns. While Colombian and European populations are spatially confined to urban and peri-urban habitats and greenhouses, Guadeloupean populations occupy a wider range of (mainly disturbed) habitats, resulting in a less patchy distribution (pers. obs., Kaiser 1997; Breuil 2002). These differences are likely caused by two main factors: (1) Guadeloupe's proximity to native range populations that allowed for several, possibly still ongoing, independent introductions and (2) the general ecosystem resemblance among the Caribbean islands as compared to introduction localities in non-Caribbean regions. Although observed genetic patterns revealed by the analyses of the mitochondrial D-loop fragment corroborate previously assumed invasion histories in the three exotic regions, additional marker systems, such as SNPs or microsatellites are desirable to add more power to the analytical framework (e.g. Guillemaud et al. 2010).

Genetic diversity and inter-population differentiation in *E. johnstonei*'s assumed origin St. Lucia (Censky and Kaiser 1999) was not higher than in Guadeloupe. Although novel molecular evidence was recently provided (Yuan et al. 2022), the actual origin has remained speculative to date (Kaiser 1997; Lescure 2000; Yuan et al. 2022). Our data allow two possible scenarios: (1) The native range of *E. johnstonei* is larger than previously assumed and includes several Caribbean islands. This would be in line with Yuan et al. (2022) who identified two mitochondrial lineages that are restricted to the eastern and western Lesser Antillean islands, respectively. The authors identified Montserrat as the most likely origin of the western clade and this may also be the source of our unique Colombian coastal haplotype (Ht3). The eastern clade, including the islands of St Lucia and Guadeloupe, was proposed to be the source of introduced populations on Jamaica, Curaçao, Trinidad and the Venezuelan mainland. While Yuan et al. (2022) consider *E. johnstonei* to be introduced to St Lucia, their sampling in this locality was limited and persuasive alternative origins of the eastern clade, are missing. Therefore, we propose an alternative scenario to be tested: (2) Rapid human-induced environmental change on St. Lucia resulted in habitat loss (Mycoo et al. 2017) and led to the extinction of local populations and therefore the loss of unique haplotypes that still persist in the introduced range (e.g. Ht3 in the coastal Colombian populations). If this scenario is true, exotic range populations safeguard genetic diversity that was lost in the native range. This raises the question of the role of these non-native populations in diversity conservation (compare Jones 2003; Osborne et al. 2013). A combination of advanced molecular approaches (McCartney et al. 2019; North et al. 2021) and assembly of existing mitochondrial and nuclear markers (this study, Yuan et al. 2022) across the entire (native and exotic) range of the species, as well as comparisons of the historical and current distribution of *E. johnstonei* and detailed niche models (Leonhardt et

al. in prep.) would allow to further test this assumption. We can also not fully rule out the possibility that we missed unique and rare haplotypes in our sampling scheme and that intensified sampling would eventually yield these "missing" haplotypes.

Despite the differences between native and introduced populations, overall genetic diversity in *E. johnstonei* is comparatively low and matches that of the congeneric *E. antillensis* (Barker et al. 2012; Barker and Rodríguez-Robles 2017). Investigated populations of *E. antillensis* feature only two dominant haplotypes and their distribution suggests a high connectivity among native populations in Puerto Rico. The native ranges of both *E. johnstonei* and *E. antillensis* are comparatively small and this has previously been suggested to explain low levels of molecular diversity in the latter (Barker et al. 2012; Barker and Rodríguez-Robles 2017). Our analyses of the restricted (235 bp) D-loop fragment seem to corroborate this assumption at first sight. However, the recovered patterns in the range-restricted and endemic *E. portoricensis* are in stark contrast to this observation. We found significantly higher levels of genetic diversity and spatial differentiation (geographic clusters), despite its small native range (this study, Barker et al. 2011). This may partially be explained by the habitat preferences and the spatial configuration of the habitat template occupied by *E. portoricensis*. The single island endemic is restricted to two mountain ranges (Luquillo and Cayey) that are separated by the Caguas river basin. The basin represents a barrier for the montane rainforest specialist and likely promoted the differentiation of two mitochondrial lineages (Velo-Antón et al. 2007; Barker et al. 2011). *E. antillensis*, on the other hand, is broadly distributed throughout the lowland, up to middle elevation habitats on Puerto Rico. Accordingly, the genetic structure is far less fine-scaled and mainly marks an east-west clade (Barker et al. 2012). The small (616 $km^2$) island of St. Lucia features only one central volcanic ridge (Mount Gimie, 958 m a.s.l.) and is thus geographically far less structured than Puerto Rico. This likely promotes gene flow that explains the observed genetic patterns in the generalist *E. johnstonei*, one of the most ubiquitous taxa in the herpetofauna of the island (Daltry 2009). Geological processes in the native ranges and the mode and timing of introductions in non-native localities are likely the main drivers shaping the genetic patterns detected in our *Eleutherodactylus* model system. While this is not unexpected, it cannot explain the invasion success of our alien amphibian model taxa.

Although it is commonly assumed that high intra-population genetic diversity promotes the adaptive capacity of a species and therefore correlates with invasion success, empirical data does not seem to support this notion (Harrison and Mondor 2011; Rollins et al. 2013; Trucchi et al. 2016). Successful alien amphibians investigated in our study (*E. johnstonei* and *E. antillensis*) show low genetic diversity in their native ranges as compared to an endemic congener. Although the single mitochondrial marker used here unarguably yields robust and ecologically interpretable results, we acknowledge the fact that employing molecular approaches with a higher resolution and coverage of several genomic regions, e.g. genotyping-by-sequencing approaches (Forsström et al. 2017; McCartney et al. 2019) or whole-genome re-sequencing (North et al. 2021) may provide slightly deviating results. However, this requires systematic testing, ideally within an identical framework.

The existing data strongly support the relevance of ecological and anthropogenic factors that drive the invasion process in our target taxa and explain the establishment success of our focus taxon *E. johnstonei.* These include: (1) Increased continuous propagule pressure (Simberloff 2009), i.e. reoccurring introduction events increase the statistical probability of a successful establishment (Leonhardt et al. 2019). (2) Exotic populations establish in specific microhabitats that resemble conditions in the native range habitats (greenhouses, urban and peri-urban gardens and tree nurseries) and therefore restrict the expansion potential (Ernst et al. 2011; Leonhardt et al. 2019). (3) Pre-adaptations that have been shown to favour successful invasions, such as direct development and therefore independence of aquatic reproduction habitats (van Wilgen and Richardson 2012; Allen et al. 2017) and the occurrence in human-altered habitats in the native range (Hufbauer et al. 2012), which further facilitates trans-location. Together, these factors may override potential impacts of genetic diversity and explain why genetic diversity *per se* does not translate into higher invasion success (Harrison and Mondor 2011; Rollins et al. 2013; Trucchi et al. 2016).

Frequent environmental disturbance causes a decrease of genetic diversity in various taxa (Banks et al. 2013), but selects for increased environmental tolerance (Leidinger et al. 2021) and phenotypic plasticity (Meyers et al. 2005), thereby hampering local adaptation (Kawecki and Ebert 2004). These conditions are met on many of the Caribbean islands, including St. Lucia that has been exposed to volcanic activity, frequent and reoccurring hurricane events and sea level changes (Government of Saint Lucia 2002; Mycoo et al. 2017). This likely contributed to the observed genetic patterns in the native populations and resulted in phenotypic plasticity, which is reportedly high in the entire genus *Eleutherodactylus* (Hoffman and Blouin 2000; Woolbright and Stewart 2008) including *E. johnstonei* (Ovaska 1991; Kaiser 2002). At the same time, populations on small islands, such as those of *E. johnstonei* on St Lucia, may have contributed to persistent inbreeding spanning generations. Thus deleterious alleles can be excluded from the gene pool, resulting in reduced genetic diversity and increased resistance to continuous inbreeding (Crnokrak and Barrett 2002). The detected genetic patterns reflect these assumptions and provide support for the pre-adaptation hypothesis explaining the establishment success of *E. johnstonei* despite low genetic diversity.

## Conclusion

Our empirical results add to an increasing body of evidence showing that successfully invasive species are not genetically more diverse or structured than their non-invasive congeners (Gaither et al. 2013; Rollins et al. 2013; Trucchi et al. 2016; Baltazar-Soares et al. 2017; Wellband et al. 2017). Genetic variation, assessed by standard molecular markers, rarely affects invasion success (reviewed in Dlugosch et al. 2015) and rapid adaptation is not limited by low genetic variation (Bock et al. 2015). If molecular processes alter the invasion process, it is likely to be through mediating response plasticity under epigenetic control (DNA methylation, Hawes et al. 2018) or through functional pre-adaptations detectable only through functional genomics (McCartney et al. 2019).

Focusing on anthropogenic drivers and ecological factors that provide simpler explanations is likely more relevant from a practitioner's point of view and will be more effective in guiding control and management decisions.

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## Supplementary material 1

**Detailed information on all populations of the three congeneric taxa used in the molecular data sets of this study**

Authors: Franziska Leonhardt, Clara Arranz Aveces, Anke Müller, Baptiste Angin, Mathieu Jegu, Pius Haynes, Raffael Ernst

Data type: Occurences.

Explanation note: For each population of the three taxa, the following details are given: region, site, coordinates (if available), NCBI GenBank Accession numbers, source publication.



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# Application of genetic and Spatially Explicit Capture-Recapture analyses to design adaptive feral cat control in a large inhabited island

Claire-Cécile Juhasz[1], Naïs Avargues[1], Laurence Humeau[2], David Ringler[3], Patrick Pinet[4], Clémence Hollinger[4], Richard Beaulieu[5], Lucie Faulquier[1], Arthur Choeur[1], Sophie Bureau[1], Denis Da Silva[2], Jérôme Dubos[1], Yahaia Soulaimana-Mattoir[1], Matthieu Le Corre[1]

**1** *UMR ENTROPIE (Université de La Réunion, IRD, CNRS, IFREMER, Université de Nouvelle-Calédonie), 15 Avenue René Cassin, CS 92003, 97744 Saint Denis Cedex 9, Ile de La Réunion, France* **2** *UMR PVBMT, Université de La Réunion, CIRAD, 15 Avenue René Cassin, CS 92003, 97744 Saint Denis Cedex 9, Ile de La Réunion, France* **3** *Kiore Services, La Castillerie, 44360 St Etienne de Montluc, France* **4** *Parc National de La Réunion, 258 rue de la République, 97431 La Plaine des Palmistes, Ile de la Réunion, France* **5** *Association pour la Valorisation de l'Entre deux Monde (AVE2M), 13 rue Josémont Lauret, 97418 La Plaine des Cafres, Ile de La Réunion, France*

Corresponding author: Claire-Cécile Juhasz (clrccljuhasz@gmail.com)

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## Abstract

Faunas of oceanic islands have a high proportion of endemic species which contribute to the uniqueness of island communities. Island species are particularly naïve and vulnerable to alien predators, such as cats (*Felis catus*). On large, inhabited islands, where the complete eradication of feral cat populations is not considered feasible, control represents the best management option to lower their detrimental effects on native fauna. The first objective of our study was to investigate population genetics of feral cats of Réunion Island. The second objective was to understand the space use of feral cats established near the breeding colonies of the two endemic and endangered seabirds of Réunion Island, the Barau's Petrel (*Pterodroma baraui*) and the Mascarene Petrel (*Pseudobulweria aterrima*). We evaluated genetic diversity, population structure and gene flow amongst six groups of feral cats located at a maximum of 10 km from known petrel colonies. We also analysed the behaviour and space use of one of these feral cat groups using camera-trap data and Spatially Explicit Capture-Recapture (SECR) models. Genetic analyses revealed that feral cats were structured in three genetic clusters explained mostly by the island topography. Two clusters were observed at five sampled sites, suggesting high connectivity amongst these sites. The last cluster was found

in only one site, suggesting high isolation. This site was a remote mountain area located in the vicinity of one of the main Barau's Petrel colonies. The behavioural study was conducted on this isolated feral cat population. Mark recapture analysis suggested that feral cats were present at low density and had large home ranges, which is probably explained by reduced food availability. Finally, we make several recommendations for refining feral cat management programmes on inhabited islands.

**Keywords**
camera trapping, endemic seabird conservation, *Felis catus*, genetic tools, invasive species control, oceanic island, SECR model

## Introduction

One third of the terrestrial biodiversity hot-spots includes oceanic islands and most of them are in the tropics (Myers et al. 2000). Oceanic islands are characterised by a high proportion of endemic species (Carlquist 1974; Myers et al. 2000; Kier et al. 2009) contributing to the uniqueness of island communities (see Burlakova et al. 2011). Insular species are particularly naive and vulnerable to the introduction of exotic predators (Moors and Atkinson 1984; Medina et al. 2011; Legge et al. 2017), which are known to be the main drivers of species extinction and biodiversity loss on islands (Moors and Atkinson 1984; Courchamp et al. 2003; Leclerc et al. 2018; Russell and Kueffer 2019). Domestic cats (*Felis catus*) have established feral populations on many islands worldwide (hereafter referred to as feral cats; Nogales et al. 2013). They present a high invasive ability (Van Aarde 1986) and are one of the most damaging species introduced on islands (Fitzgerald 1988; Courchamp et al. 1999b; 2003, Medina et al. 2011; Nogales et al. 2013; Jones et al. 2016). This generalist and opportunist predator has caused numerous extinctions of insular species and particularly of endemic vertebrates (Nogales et al. 2004; Nogales and Medina 2009; Doherty et al. 2016). There is an urgent need to counteract the biodiversity loss due to feral cat predation by optimising methods to eradicate or regulate this invasive predator (Myers et al. 2000; Kier et al. 2009; Burlakova et al. 2011).

Feral cat eradications, which consist of a complete and definitive removal of the feral cats, have been frequently conducted on islands (see Medina et al. 2011). However, their implementation on large, inhabited islands remains challenging. The main difficulties to eradicate feral cats from large inhabited islands are low social acceptability, inappropriate legislation, lack of long-term political commitment, important financial cost and reduced technical feasibility of such large-scale operations (Oppel et al. 2010; Glen et al. 2013; Russell et al. 2018). The situation is even more complicated by the presence of domestic cats which can be accidentally culled and which permanently supplement the feral cat population through breeding (Choeur et al. 2022). One alternative to eradication is long-term control of feral cats in key areas, in order to maintain the population below a threshold that results in a low and acceptable impact on biodiversity (Doherty et al. 2017; Palmas et al. 2020). However, in most cases, controlled

areas are not isolated from nearby uncontrolled areas and are continuously re-invaded by cats (Lazenby et al. 2015; Palmas et al. 2020). The re-invasion rate depends on various factors, such as the density of cats in uncontrolled nearby areas, the connectivity between controlled and uncontrolled areas and the dispersive behaviour of the cats (Palmas et al. 2020; Choeur et al. 2022). When cat control is implemented in key areas, there is a strong need to understand the individual dispersion (Pulliam 1988; Hanski 1999) and space use at global and local scales to estimate the rate of re-invasion and to optimise the cost-effectiveness of control campaigns (Palmas et al. 2020).

Population genetics is an efficient tool for informing the management of invasive mammalian species (Browett et al. 2020). Genetic-based techniques can be used to identify the origin of the invaders, to trace the invasion pathways and to appropriately target a population of manageable size with low re-colonisation risk (Robertson and Gemmell 2004; Abdelkrim et al. 2005; Rollins et al. 2006, 2009). This information can be used to design the best strategy for successful control campaign. For instance, introduced feral cats on the main island of the Kerguelen Archipelago are now well established over the entire island, suggesting that a complete eradication would be extremely difficult (Simberloff 2003; Pontier et al. 2005; Barbraud et al. 2021). However genetic analyses highlighted limited connections between sites, indicating that local control may have long-term benefits (Pontier et al. 2005). On the island of Hawai'i, the genetics of feral cat populations indicated high genetic diversity, population expansion and weak, but significant structure amongst three sites (Hansen et al. 2007). These results indicated that the most isolated site could be targeted for control (Hansen et al. 2007). On Christmas Island (Indian Ocean), no genetic structure was detected amongst feral cat populations, suggesting high connectivity and higher risk of re-invasion after local control. This indicates that, in this case, feral cats of the entire island should be removed or simultaneously controlled (Koch et al. 2020).

Camera trapping and Spatially Explicit Capture-Recapture (SECR) are effective tools to understand species behaviour and spatial ecology (Bridges and Noss 2011; Rovero et al. 2013; Royle 2015). SECR models are hierarchical models that account for both the spatial organisation and movement of individuals in relation to the placement and effectiveness of the detection devices (Kane et al. 2015). This method provides key information for designing effective feral cat control and for optimising management techniques (Robley et al. 2010; Lazenby et al. 2015; McGregor et al. 2015). Palmas et al. (2020) tested the feasibility and efficiency of an intensive control of feral cats in a semi-isolated peninsula in New Caledonia. SECR modelling suggested that feral cats recolonised the controlled area in three months, recovering to the same density as the one determined before the culling (Palmas et al. 2020). Recolonisation by feral cats was faster than expected despite the favourable geographical situation of the peninsula. In such cases, genetic studies may offer a strong benefit to management actions by highlighting the connectivity between controlled and uncontrolled populations.

Réunion Island (21°00'S, 55°39'E) is a large (2512 km$^2$), young (about two million years) inhabited (861,200 people in 2021) volcanic island of the Mascarene Archipelago, western Indian Ocean. The topography is extremely rough with a maximum

elevation of 3,071 m a.s.l., several summits above 2,500 m and three massive eroded calderas surrounded by huge vertical cliffs (of 1 km high). This topography has generated an important ecological heterogeneity (Strasberg et al. 2005). Réunion Island is part of the biodiversity hotspot of Madagascar and surrounding islands (Myers et al. 2000; Roberts et al. 2002; Mittermeier et al. 2005; Kier et al. 2009). Since the colonisation of the Island by humans in the late 1600s, this biodiversity has been strongly impacted by habitat loss, unregulated hunting and invasive species. A total of 57% (17 of the 30 species) of the native vertebrates of the Island are now extinct (Gargominy et al. 2020). One of the most problematic alien predators is the feral cat. This species was introduced in 1702 (Cheke and Hume 2010) and is known to prey upon several endemic species including the endangered Barau's Petrel (*Pterodroma baraui*; Faulquier et al. 2009) and the critically endangered Mascarene Petrel (*Pseudobulweria aterrima*; Riethmuller et al. 2012).

The objectives of our study were to estimate the genetic connectivity and space use of feral cat populations near the breeding colonies of the two endemic petrels. Based on 10 polymorphic microsatellite markers, we evaluated genetic diversity, population structure and gene flow amongst six groups of feral cats located at a maximum of 10 km from known petrel colonies. We used feral cat capture-mark-recapture (CMR) data and SECR models to estimate the density and the home range of feral cats near the well-studied Barau's Petrel breeding colony of Grand Bénare. We determined their density and general activity (movements and detection probabilities) in relation to the types of habitat used (trail vs. vegetation cover). Finally, genetic data and behavioural data were combined to propose an adapted feral cat control strategy.

## Materials and methods

### The endemic petrels of Réunion Island

The population size of Barau's Petrel is estimated between 10,000 and 30,000 breeding pairs (Virion et al. 2020). The first breeding colony was discovered in 1995 (Probst 1995; Probst et al. 2000). Several breeding sites have been discovered since then and two of them are monitored annually: Bras des Etangs (west side of Piton des Neiges, 2,400 m a.s.l.) and Grand Bénare (2,600 m a.s.l.). The breeding habitat is characterised by steep cliffs between 2,200 and 2,800 m a.s.l. mainly covered by endemic shrubs, such as *Erica reunionensis*, *Stoebe passerinoides* and *Sophora denudata* (Cadet 1977; Probst et al. 2000; Strasberg et al. 2005). Barau's Petrels breed seasonally between September and April. They completely leave their colonies after breeding. Feral cats have been reported at all known colonies. Faulquier et al. (2009) showed that feral cats established at Barau's Petrel colonies prey intensively upon adults, inducing a strong negative impact on populations, as this long-lived species is particularly sensitive to any additive mortality on adults (Russell et al. 2009; Dumont et al. 2010). Rats and mice are also present at Barau's Petrel colonies, at low density, probably because these altitudinal habitats are suboptimal for these species (authors' unpubl. data).

The population size of Mascarene Petrel is estimated at 250 breeding pairs (Attie et al. 1997). Two breeding colonies were discovered in 2016 and 2017 (Virion et al. 2020; Juhasz et al., in press) and have been monitored annually since. Burrows are located on steep cliffs from 650 to 1,200 m a.s.l. where the habitat is dominated by shrubs, such as the indigenous *Olea lancea* and the endemic *Monimia rotundifolia* (Juhasz et al., in press). The Mascarene Petrel breeds seasonally between August and March. They completely leave their colonies after breeding. Preliminary studies conducted at the breeding colonies suggest that predation by exotic mammals (rats and cats) and habitat loss constitute the main threats for this critically-endangered seabird species (Juhasz et al. in press; authors' unpubl. data).

## Genetic sampling of feral cats

We sampled feral cats from July 2015 to December 2016 at six sites. Five of these sites are included in the National Park of Réunion Island and located in native mountain forests (Fig. 1A, B). The site of Cilaos is in the southernmost massive caldera of the Piton des Neiges volcano (called "Cirque de Cilaos"). The site of Les Makes is located 9 km to the southwest of Cilaos. Dimitile and Grand Bassin are located respectively at 6 and 8 km to the southeast of Cilaos. These four sites are located between 1,200 and

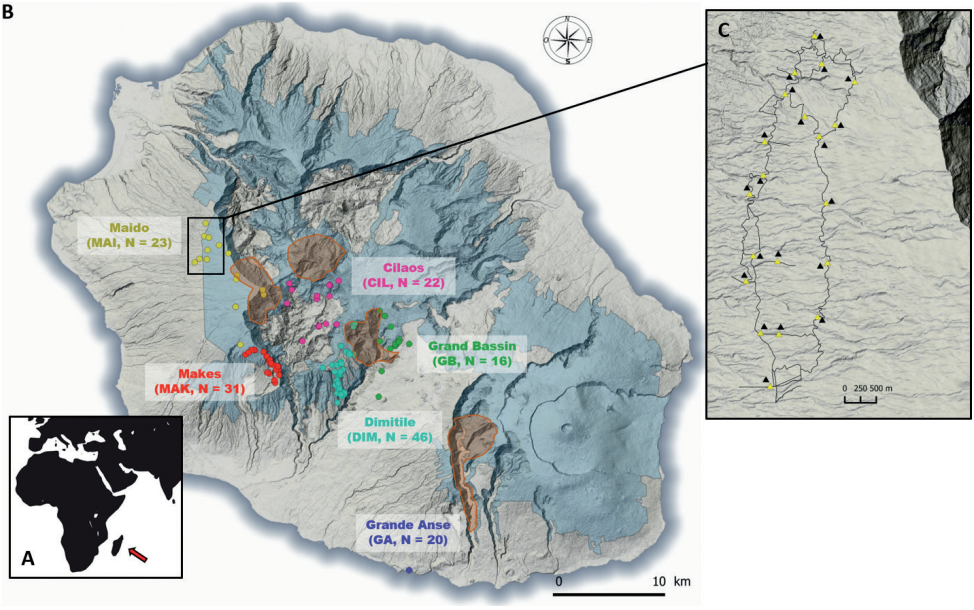


**Figure 1.** Maps illustrating **A** the locality of Réunion Island **B** genetic sampling sites, in 2015–2016 and **C** camera trapping sites in 2016. Each colour of dots corresponds to a different geographical area. Area codes and sample sizes are indicated in parentheses. The grey area corresponds to the National Park and the orange areas correspond to the presence of Barau's and Mascarene Petrels. Triangles correspond to the localities of camera traps on trails (yellow triangles) and under vegetation cover (black triangles). The black lines show the trails.

**Table 1.** Estimates ± standard errors of genetic diversity for 10 microsatellite loci of feral cats (total $N_{sample}$ = 158 individuals) in six geographical areas in Réunion Island, 2015–2016.

| Area | Code | $N_{sample}$ | $N$ | $AL$ | $PA$ | $AR$ | $H_O$ | $H_E$ | $F_{IS}$ |
|---|---|---|---|---|---|---|---|---|---|
| Maïdo | MAI | 23 | 22.80±0.20 | 5.20±0.53 | 1 | 4.68±0.45 a | 0.58±0.05 | 0.58±0.04 | -0.02±0.03 |
| Makes | MAK | 31 | 30.30±0.37 | 7.20±0.47 | 4 | 6.11±0.35 b | 0.65±0.04 | 0.70±0.02 | 0.06±0.04 |
| Cilaos | CIL | 22 | 21.60±0.31 | 6.90±0.41 | 3 | 6.35±0.38 b | 0.68±0.03 | 0.70±0.03 | 0.01±0.03 |
| Dimitile | DIM | 46 | 45.30±0.40 | 6.90±0.53 | 4 | 5.49±0.36 ab | 0.68±0.04 | 0.66±0.04 | -0.05±0.04 |
| Grand Bassin | GB | 16 | 15.70±0.15 | 6.00±0.47 | 3 | 5.90±0.45 ab | 0.58±0.06 | 0.65±0.04 | 0.08±0.05 |
| Grande Anse | GA | 20 | 19.80±0.13 | 6.50±0.27 | 2 | 6.20±0.24 b | 0.74±0.03 | 0.71±0.03 | -0.07±0.03 |

For each area, we give the mean number of genotyped individuals ($N$), the mean number of alleles per locus ($AL$), the mean allelic richness per locus, based on minimum sample size of 15 diploid individuals ($AR$; means followed by the same lower case letter are not significantly different [i.e. $P > 0.05$] according to the pairwise Wilcoxon's signed rank tests with Bonferroni correction), the private allelic richness ($AP$), the observed heterozygosity over all loci ($H_O$), the unbiased expected heterozygosity ($H_E$) and the fixation index ($F_{IS}$; Weir and Cockerham 1984).

1,400 m a.s.l. The fifth site, named Maïdo, is a volcanic plateau sloping to the west and located 9 km to the northwest of Cilaos. Maïdo lies between 1,500 and 2,850 m a.s.l. and is characterised by subalpine shrubland. The sixth study site, Grande Anse, is a coastal peri-urban area located 20 km to the south of all other sites at 0 to 110 m a.s.l. (Fig. 1B). All sampled sites are located at less than 10 km from a breeding colony of Barau's Petrel or Mascarene Petrel (Fig. 1B) and four of them (Cilaos, Dimitile, Grand Bassin and Maïdo) are located less than 3 km from the nearest petrel colony.

Cats were live-trapped with Tomahawk cage traps baited with sardines and brought to the veterinary clinic for sanitary inspection. The veterinarian checked for individual pit-tags and tattoos to identify potential owned cats. The behavioural profile of the cat was then evaluated to estimate the possibility to adopt it. If adoption was impossible because the cat was too wild, the cat was euthanised after the legal guard period of four days. The euthanasia was made by intra-venial injection of pentobarbital. Kidney tissue samples were collected from each euthanised cat and stored in 70% ethanol at -80 °C until laboratory analysis. The protocol was approved by the CYROI institutional ethical committee, certified by the French Ministry of Higher Education and Research (NoAPAFIS#6916-20151 00213267087 v.6). A total of 158 feral cats were trapped including 87 males, 67 females and 4 indeterminate (Table 1). None of them was identified as an owned or adoptable cat.

## Microsatellite genotyping

Total DNA was extracted from a small piece of kidney tissue using the QIAmp Blood and Tissue kit (Qiagen, Hilden, Germany). Genotyping was conducted for 10 polymorphic microsatellite loci (described in Menotti-Raymond and O'Brien 1995 for Fca43 and Fca96, Menotti-Raymond et al. 1999 for Fca31, Fca69, Fca76, Fca173, Fca275, Fca441 and Fca531 and Menotti-Raymond et al. 2003 for Fca1027) on DNA extracts from 158 individuals. A 3-primer PCR approach, using a M13 tail for the forward primer, was used for microsatellite loci amplification following Schuelke (2000).

Four different dyes (6-FAM, PET, VIC, NED) were used for the universal M13 forward primer to enable fragment analysis multiplexing. Simple PCR amplifications were performed using a GeneAmp PCR System 9700 (Applied Biosystems, Waltham, Massachusetts, USA) in 10 µl reaction volume containing 5 µl of MasterMix Applied 2× (Applied Biosystems, Waltham, Massachusetts, USA), 0.3 µl of the forward primer with M13 5'-tail (1 µM), 0.3 µl of the reverse primer (10 µM), 0.3 µl of dyes (10 µM), 2.1 µl of sterile deionised water and 2 µl of genomic DNA (20–40 ng/µl). PCR amplifications were carried out under the following conditions: an initial denaturing step at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 sec, 56 °C for 30 sec and 72 °C for 30 sec and a final elongation at 72 °C for 20 min. Up to four different simplex PCR plates, each with a different dye, were mixed and PCR product sizes were determined, using a 3730XL DNA analyser (Applied Biosystems, Waltham, Massachusetts, USA) at the Gentyane platform (Clermont-Ferrand, France) and were sized with LIZ(500) standard using GeneMapper (Applied Biosystems, Waltham, Massachusetts, USA).

## Genetic diversity

Evidence of null alleles, large-allele dropout and stutter peaks in all microsatellites was examined using MicroChecker 2.2.3 (Van Oosterhout et al. 2004). Each locus-pair combination was tested for linkage disequilibrium with GenePop 4.7.5 (Rousset 2008). The *P*-values were corrected using the Benjamini and Yekutieli (2001) method for multiple comparisons (Narum 2006). The mean observed number of alleles per locus (AL) and the number of private alleles per area (AP) were computed using GenAlEx 6.5 (Peakall and Smouse 2012). Allelic richness (AR; El Mousadik and Petit 1996), adjusted for discrepancies in sample size by incorporating a rarefaction method as implemented using FSTAT 2.9.3 (Goudet 2001), was used to make comparisons of the mean number of alleles amongst areas. The means of allelic richness amongst areas were compared using pairwise Wilcoxon's signed rank tests with Bonferroni correction. Observed heterozygosity ($H_O$), unbiased expected heterozygosity estimated according to Nei (1978) ($H_E$) and Wright's F-statistics ($F_{IS}$) according to the method of Weir and Cockerham (1984) were calculated for all and each population using GenAlEx 6.5 (Peakall and Smouse 2012). Deviations from Hardy-Weinberg equilibrium (*HWE*) were tested for each of the six areas using the package pegas 0.13 (Paradis 2010) using R 3.2.0 (R Core Team 2021), with the exact test based on $10^3$ Monte Carlo permutations.

## Genetic differentiation and structuring

Assignment tests, based on multi-locus microsatellite genotypes, were evaluated using two different clustering approaches. First, we used a Bayesian genotype clustering procedure in STRUCTURE 2.3.3 (Pritchard et al. 2000). The admixture model was used with the LOCPRIOR setting, which considers sample location and allows structure to be detected when genetic structure is weak or when the number of loci is small (< 20; Hubisz et al. 2009). The r-index was also used to determine the relevance

of the sampling location (LOCPRIOR), with low values of *r* indicating that sampling locations are informative to the overall model (Falush et al. 2003). Correlated allele frequencies were assumed (Pritchard et al. 2000). For each value (1–10) of the number of independent genetic clusters (K), we ran $10^6$ iterations 10 times (after a burn-in of $10^5$ steps). For choosing the optimal number of clusters, two criteria were used; the log likelihood given K (L(K); Pritchard et al. 2000) and the second-order rate of change of mean log-likelihood (ΔK; Evanno et al. 2005). Both criteria were calculated using STRUCTURE HARVESTER online Web server (Earl and vonHoldt 2012). CLUMPAK software (Kopelman et al. 2015) was used to find the optimal individual alignments of replicated cluster analyses and to visualise the results.

Population structure was also explored by integrating spatial coordinates of samples using a Bayesian model executed in a Markov Chain Monte Carlo, as implemented in the R package Geneland 4.0.5 (Guillot et al. 2005, 2008). The geographical information was used to detect spatial delineation of genetic discontinuities, where the number of area units is treated as an unknown parameter. We ran the MCMC ten times independently to verify the consistency of the results. We used K from 1 to 10, $10^5$ iterations with 100 burn-in generations, an uncertainty attached to spatial coordinates fixed to 200 m (i.e. the precision of our sample locations) and the maximum number of nuclei in the Poisson–Voronoi tessellation fixed to 300. The analysis was run with correlated allele frequency models, true spatial and no null allele models. Finally, all runs were examined for consistency.

Genetic differentiation amongst all pairs of areas was assessed by calculating pairwise $F_{ST}$ values following Meirmans (2006) and pairwise Nei's $G'_{ST}$ distances (Nei 1978). Statistical significance was tested by $10^4$ permutations of genotypes amongst areas under Bonferroni's correction, using GenoDive 3.04 (Meirmans and Van Tienderen 2004).

Pairwise genetic and geographic distances amongst sampling locations were used to test patterns of isolation by distance (IBD) using a Mantel test (Mantel 1967). We used the scaleGen function in adegenet 2.1.3 package (Jombart 2008) to calculate the Euclidean genetic distances amongst samples. Euclidian geographical distances between each pair of samples were calculated. The significance of the correlation coefficient between sample pairs was estimated using a Mantel test with 10,000 randomisations in R. In addition, we repeated IBD analyses using only the subset of natural areas to investigate the effect of geographic distance of Grande Anse peri-urban area from other areas, which might disproportionately contribute to IBD patterns.

## Estimates of recent gene flow

To determine possible source populations that could be targeted for control (Rollins et al. 2006), we estimated recent migration rates amongst areas using two methods. First, we used Bayesian assignment tests with BIMr 1.0 (Bayesian Inference of Migration rates, Faubet and Gaggiotti 2008). BIMr infers the proportion of recent immigrants in a population from their genotypes and calculates corresponding asymmetrical

migration rates amongst pairs of populations. BIMr determines recent migration even amongst weakly-differentiated populations (i.e. $F_{ST} > 0.01$) with unequal sample sizes (Faubet and Gaggiotti 2008). Due to overlapping generations in feral cats, BIMr estimates were interpreted as a relative index of recent gene flow rather than a precise estimate of gene flow in the previous generation. A burn-in period of 20,000 iterations followed by $10^5$ iterations for each run was used. The default values were used for all other parameter settings. Migration rate estimates were obtained by choosing the run with lowest Bayesian deviance, measured by the assignment values (Dassign; Faubet and Gaggiotti 2008). Posterior mean and mode migration rates and 95% high density predictive interval (HDPI) were estimated using the package HDInterval (Kruschke 2011) in R.

Estimates of recent migration rates and approximate 95% confidence interval (CI) were also explored by the Bayesian approach as implemented in BayesAss 3.0.4 (Wilson and Rannala 2003). Five runs were first performed by changing the number of seeds (s = 10, 100, 500, 750 and 1000) to obtain a suitable convergence. The number of iterations was $10^6$, of which $10^5$ were burn-in and the sampling frequency was 100. Mixing parameters were 0.6 for allele frequencies, 0.9 for inbreeding coefficients and 0.5 for migration rates. The final run consisted of the same mixing parameters and 100 numbers of seeds.

## Spatially explicit capture-recapture study

CMR data of feral cats were obtained during a single season of camera trap survey at Maïdo (Fig. 1C). This site was selected for: (i) the presence of scats and direct observations of feral cats, (ii) its proximity to a monitored Barau's Petrel colony (about 5 km) where feral cat predation is known to occur (Faulquier et al. 2009), (iii) the proximity of trails which were supposed to maximise the feral cat detection probability (Meek et al. 2014; McGregor et al. 2015) and (iv) the technical feasibility. We deployed and geo-referenced with GPS (Garmin 64 s; 5 m accuracy) 20 camera-traps (9 Scoutguard-MG882K-12mHD, 10 Bushnell Trophy Cam HD and 1 Reconyx HC600 Hyperfire) from 17 February to 25 April 2016 (68 days). Camera-traps were first placed along trails (17 Feb – 23 March), then under vegetation cover (23 March – 25 April). This study period encompassed the second half of the chick rearing and the beginning of the fledging period of Barau's Petrels. The mean distance (± sd) between cameras was 2,114.0 ± 1,273.0 m (min = 49 m, max = 5,626 m). Neither bait nor lure was used, to maintain homogeneous detection probabilities. Devices were set using a high-sensitivity trigger to capture three images per event at rapid-fire interval (0.13 s), with no delay between trigger events, to maximise feral cat identification. During the first week, half of the capture stations were equipped with two cameras placed on the opposing side of trails to capture both flanks of passing animals. Each observed feral cat was identified, based on natural marking such as spots, stripes and ocelli on both sides when possible (Bengsen et al. 2012; McGregor et al. 2015; Lavery et al. 2020; Palmas et al. 2020). A sampling occasion lasted one day (24 h, hereafter named "trap-night";

Otis et al. 1978; Wang and Macdonald 2009). For each sampling occasion, individuals were photographed ("captured"), identified ("marked") and "released". All feral cats previously identified and re-sighted were considered as a recapture. A capture event was defined as all pictures of unique individuals within a 30-min time period (Di Bitetti et al. 2006). Cameras were checked every 10 days to download data from memory cards and replace batteries. No feral cats were captured for the genetic study in the CMR study area during and 6 months before the behavioural study. However, from September to December 2015, four feral cats were captured at 5.9 to 7.8 km from the nearest camera trap.

The trapping effort (in trap-nights) was calculated by adding for each camera the number of days where each camera was active over the study period. The capture efficiency (in number of capture events/100 trap-nights) was calculated by dividing the number of feral cat capture events for all cameras divided by the total trapping effort and multiplied by 100 (Palmas et al. 2020).

Since we designed a short study period (to avoid emigration, immigration or mortality) and we did not consider kitten pictures in the dataset (to avoid recruitment), we applied SECR models that require a demographically closed population. These models assume no emigration or immigration, no mortality and no recruitment during each trap session (Otis et al. 1978; Efford 2004). The matrix of spatially explicit histories of capture-recaptures was constructed for each feral cat by linking each capture of each individual with the coordinates of the camera and with the occasion. Each camera was associated with a spatial covariate (trail vs. under vegetation) to check if trap location affects the detection probability. Data analyses were performed using the SECR package (Borchers and Efford 2008; Efford et al. 2009) in R 4.0.3. First, we estimated the mean maximum distance moved (MMDM) by the individuals between captures. Then, we implemented SECR models. The trap detector type « count » was chosen for the analysis, allowing more than one detection per animal. A habitat mask was used with a buffer width of 3,000 m around each camera-trap (determined with the SECR package; Efford 2021), but excluding the deep cliffs considered as inaccessible for feral cats. This resulted in a sampling area of 60.55 $km^2$. We assumed that home ranges of feral cats were distributed following a homogeneous spatial Poisson process during the trapping period (Efford 2004; Borchers and Efford 2008; Efford et al. 2009). The half-normal detection function was selected as the most appropriate for our models. This detection function is defined by two parameters: the animal detection probability considering that the camera-trap is located at its home range centre (g0) and the movement parameter, i.e. the distance scale of the detection function ($\sigma$). Models were used to investigate the effects of camera locations (on trail vs. under vegetation) on g0 and $\sigma$. Model performances were compared using the difference in Akaike Information Criterion modified for small sample size (AICc). Each model presenting a $\Delta$AICc < 2 was considered a competing best model. Finally, based on the estimates of the best model, we determined: (1) the site-specific population density *D*; in number of cats/$km^2$ and (2) the home range ($HR_{95}$) and core area ($HR_{50}$) in $km^2$ of feral cats (see Ringler et al. 2014).

## Results

### Genetic diversity

No null alleles, large-allele dropout nor stutter peaks were detected for the 10 microsatellite loci. The percentage of missing data was 1.58%. Linkage disequilibrium amongst loci was detected for four of the 45 loci pairs ($P < 0.05$), but no significant linkage disequilibrium was observed amongst any of the loci after the Benjamini and Yekutieli (2001) correction for multiple tests, suggesting that all loci were independent. The mean allelic richness (*AR*), based on a minimum sample size of 15 individuals, ranged from 4.7 (Maïdo) to 6.4 (Cilaos) alleles per locus and was relatively similar amongst areas, except for the less variable Maïdo area (Table 1). All areas contained one (Maïdo) to four (Makes, Dimitile) private alleles (Table 1). Observed heterozygosity for Maïdo ($H_O$ = 0.58) was similar to Grand Bassin, but it was lower than all other areas (*Ho* range 0.65–0.74; Table 1). Deviations from *HWE* were not significant for all areas (all $Ps > 0.05$). The raw microsatellite genotypes of the 158 individual feral cats are available in the supporting information (Appendix 1).

### Genetic structuration and gene flow

Clustering of microsatellite genotypes using STRUCTURE analysis indicated that mean values of the r-index used to determine the relevance of the sampling location in the clustering analysis was low (0.31 ± 0.14), suggesting that sampling locations are informative to the model. Analysis clearly showed that the best-supported model contained three genetic clusters (maximum value of Evanno's likelihood at K = 3, maximum value of L(K) and minimum standard deviation of L(K) at K = 3, Appendix 2). The first genetic cluster was almost exclusively detected for samples from Maïdo (91% of the genetic pool from Maïdo samples). The second cluster was detected amongst samples from Dimitile (77% of Dimitile's samples), Grand Bassin (38%) and Cilaos (25%). The third cluster was shared between Makes and Grand Bassin (more than 90% of samples), then Cilaos and Grand Bassin (about 70% of samples) and finally Dimitile (about 18% of samples; Fig. 2, Appendix 2).

Analysis using Geneland yielded a modal number of populations with a higher proportion of three putative genetic groups (K = 3; Appendix 3: Fig. A2F). The run with the highest average posterior density was selected. Sampled feral cats were clustered into five groups. Two inferred groups (part of Cilaos and Grande Anse) had very low posterior probabilities (Appendix 3: Fig. A2D and E, respectively) and the areas of these groups were already represented in groups with strong posterior probabilities (Appendix 3: Fig. A2A–C). The last three putative groups roughly corresponded to the areas defined using the topography of Réunion Island (Appendix 3: Fig. A2A–C); Maïdo, Dimitile, and the other areas, and corresponded to the results obtained using non-spatial analysis with STRUCTURE.

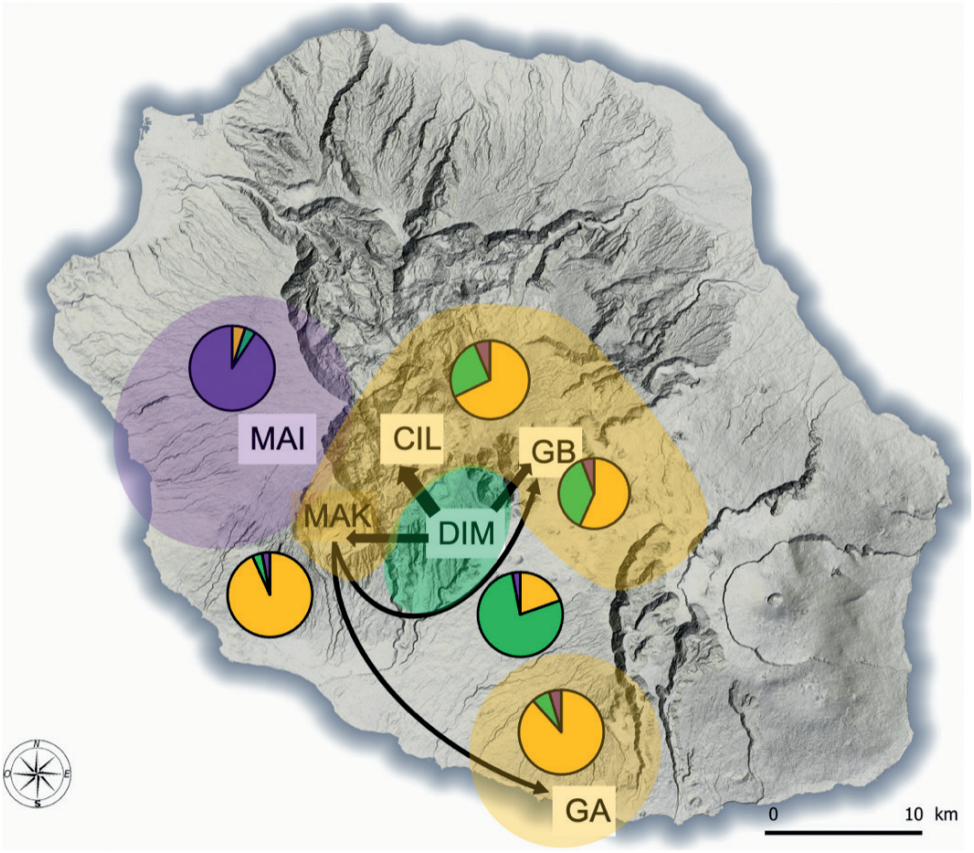


**Figure 2.** Distribution of microsatellite clusters based on Bayesian clustering analysis using STRUCTURE (pies) and Geneland (coloured areas) and map of the migratory pathways suggested by BIMr and BayesAss (black arrows, the thickness is proportional to the amount of gene flow) of the feral cats (N = 158 individuals) in Réunion Island, 2015-2016. Area codes: MAI for Maïdo, CIL for Cilaos, MAK for Makes, GB for Grand Bassin, DIM for Dimitile and GA for Grande Anse.

Pairwise $F_{ST}$ values ranged between 0.011 and 0.149 with a global $F_{ST}$ of 0.026 ($P < 0.001$). The highest values were for the comparison of Maïdo to the other areas. *Nei* distances showed the same pattern. For both indexes, 8 of the 10 loci showed p-values less than 0.001. Based on the two differentiation index values, three groups were distinguishable: (1) Maïdo, (2) Dimitile and (3) all other areas, suggesting an isolation of Maïdo particularly and Dimitile to a lesser extent, as previously suggested by the clustering analysis (Appendix 4).

Genetic distance amongst individuals showed no significant relationship with geographic distance either at the global scales (Mantel r-test, $P = 0.189$) or after excluding Grande Anse (Mantel r-test, $P = 0.385$).

Recent mean migration rates determined using BIMr ranged from nearly zero amongst most pairs of areas to nearly 0.05 between Grand Bassin and Dimitile

(Appendix 5). Based on non-overlapping 95% HPDIs, we only recorded significant asymmetric dispersal between Dimitile and Cilaos, with clearly highest migration from Dimitile to Cilaos (Appendix 5). Globally similar results were obtained using Bayes-Ass, suggesting asymmetric dispersal between these two areas. Moreover, all the mean values of recent migration rate were clearly higher compared to those from BIMr and six values had a confidence interval not including zero (Appendix 5), suggesting the migratory pathways presented in Fig. 2.

## Camera trapping and SECR results

During the camera-trapping survey, we collected 41,905 pictures including 376 (0.9%) pictures of feral cats. All photographed feral cats were identified and included in the study. Ten individuals were identified. There was no picture capturing more than one feral cat simultaneously (see details in Table 2).

**Table 2.** Summary of the results obtained from both camera trapping sessions of feral cats, 2016, Réunion Island. For each session, the trapping effort corresponds to the product of the number of occasions per session and the number of active cameras. The capture efficiency is the number of detections divided by 100 trap-days.

| Location | Period (days) | Date | Number of cameras | Trapping effort (trap-day) | Number of pictures | Number of cat pictures (%) | Total number of cats | Total number of recaptures | Capture efficiency (detections/100 trap-days) |
|---|---|---|---|---|---|---|---|---|---|
| Trail | 35 | 17 Feb – 23 Mar | 20 | 550 | 21,524 | 114 (0.5%) | 6 | 34 | 7 |
| Vegetation | 33 | 23 Mar – 25 Apr | 20 | 532 | 20,381 | 262 (1.3%) | 8 | 12 | 4 |
| | **68** | **17 Feb – 25 Apr** | **20** | **1082** | **41,905** | **376 (0.9%)** | **10** | **50** | **5.5** |

In total, we obtained 60 feral cat detections between 17 February and 25 April 2016, corresponding to 10 captures and 50 recaptures for 10 individuals. For cameras on trails, we obtained six captures and 34 recaptures of five individuals at 14 of the 20 cameras. When camera traps were placed under vegetation, eight feral cats were first detected (including four feral cats previously identified on trails) and five of them were recaptured (12 detections) at eight of the 20 cameras (Table 2; Appendix 6). The global trapping effort was 1,082 trap-nights with a capture efficiency of 5.5 detections/100 trap-nights. The mean maximum distance moved (MMDM ± se) by feral cat was 1,926 ± 589 m. We tested the effect of camera types and the linear time trend over occasions on the detection probability g0. No effect was detected.

The model with the greatest support was the null model (Table 3). This model had a maximum detection probability at each camera trap (g0) of 0.06 (95% CI [0.03; 0.09]) and a spatial scale of movement ($\sigma$) of 971 m (95% CI [791; 1,193]; Appendix 7). The estimated population density of feral cats was 0.25 feral cats/km$^2$ (95%CI [0.12; 0.47]). The mean home range was estimated at 15.0 km$^2$ ($HR_{95}$) with a core area of 2.5 km$^2$ ($HR_{50}$).

**Table 3.** Model selection testing the spatial effect of camera trap location (on trail vs. under vegetation) on the detection parameters (g0 and σ) on Réunion Island, 2016. D is the density, g0 is for the probability of feral cat detection at the home range centre and σ is the scale parameter of the detection function. Models are ranked by their AICc values. The best model (ΔAICc < 2) is in bold.

| | D | g0 | σ | Npar | AICc | ΔAICc | W (%) |
|---|---|---|---|---|---|---|---|
| **1.** | **~ 1** | **~ 1** | **~ 1** | **3** | **639.25** | **0.00** | **76** |
| 2. | ~ 1 | ~ location | ~ 1 | 5 | 641.60 | 2.35 | 24 |
| 3. | ~ 1 | ~ 1 | ~ location | 5 | 649.70 | 10.45 | 0 |
| 4. | ~ 1 | ~ location | ~ location | 7 | 680.84 | 41.59 | 0 |

Npar: number of estimated parameters, AICc: Akaike Information Criterion modified for small sample size, ΔAICc: difference in AICc values in relation to the most parsimonious model, W: model AICc weight.

## Discussion and conclusion

### Genetic diversity, structure and gene flow in feral cat populations

The genetic diversity of feral cats on Réunion Island is similar to that observed on cat populations recently introduced on other islands (Kerguelen, Pontier et al. 2005; Hawai'i, Hansen et al. 2007; Christmas & Cocos Island, Spencer et al. 2016). It is also similar to the diversity observed in non-insular contexts, in isolated populations with low dispersal rates (France, Say et al. 2003; Australia, Cowen et al. 2019). It is assumed that most feral cats of Réunion Island are descendants of cats introduced from France (Dreux 1990). Interestingly the genetic diversity of feral cats on Réunion Island is lower than that observed in Europe (Pierpaoli et al. 2003), which may be explained by a founding effect leading to a genetic drift as expected in such isolated contexts (Slatkin and Excoffier 2012; Bélouard et al. 2019).

Microsatellite analysis and Bayesian clustering analysis suggested significant structuring amongst studied populations. Genetic structure was strong compared to populations of Hawai'i (three sampled sites, $F_{ST} < 0.05$; Hansen et al. 2007) and the Kerguelen Archipelago (four sampled sites, $F_{ST} \leq 0.09$; Pontier et al. 2005). We found three genetic clusters amongst which, one was observed only at our highest sample site, Maïdo. This suggests very low gene flow between this site and other lower populations. The isolation of Maïdo was also supported by a lower allelic richness compared to other areas, as expected in isolated populations (Frankham 1996; Peter and Slatkin 2015). This pattern is probably due to the very rough topography of the island. Indeed, Maïdo is separated from other sites by a vertical cliff of more than 1 km, which probably represents a geographical obstacle for feral cat dispersal.

The second and third genetic clusters were detected in the five other areas (Dimitile, Cilaos, Grand Bassin, Makes, Grande Anse, Fig. 2). Although differentiation indexes indicated an isolation of Dimitile, feral cats sampled in this area were mostly assigned to a genetic group that was also detected in the four other areas. The low $F_{ST}$ and Nei distance estimates, coupled with a lack of isolation by distance, suggest that Grande Anse, Makes, Cilaos and Grand Bassin areas were weakly isolated from each other, despite large geographical distances between Grande Anse and the others (minimum of 20 km). This result suggests either or both natural and human-mediated

dispersal of feral cats amongst these areas. The human-mediated dispersal hypothesis is reinforced by the lack of genetic isolation through geographical distances, which would be expected if a progressive colonisation process occurred amongst neighbouring sites (Kimura and Weiss 1964; Slatkin 1993).

## Density and home range of feral cats

Comparing home range of feral cats from the literature is challenging because of the large diversity of the methods used, ranging from GPS tracking to SECR modelling (Jones and Coman 1982; Nogales et al. 2004; Bengsen et al. 2012; McGregor et al. 2015). However, our results suggest that feral cats at Maïdo are present at low density (0.25 feral cat/km$^2$) with large home ranges (15 km$^2$). To our knowledge, the only cases where feral cats live in such low densities in the tropics are also in mountainous habitats (Hawai'i, Smucker et al. 2000; Goltz et al. 2008). This suggests that some bottom-up limitation due to low densities of prey are occurring in these extreme habitats, resulting in a low carrying capacity for feral cats (see Liberg et al. 2000; see Bengsen et al. 2016). Furthermore, in the case of feral cats living at seasonal seabird breeding colonies (which is the case of both petrel species), the carrying capacity of their habitats fluctuate in relation to the phenology of the petrels.

This space use strongly contrasts to that observed in a low altitude area of Réunion Island transformed by human activities. A recent study has shown that, at sea level, cat density can reach 27 ± 2 cats/km$^2$ (Choeur 2021), with an average home range of 0.12 km$^2$ (Choeur et al. 2022). This peri-urban habitat is characterised by extremely abundant food resources for cats including anthropogenic food wastes, supplemental feeding and introduced prey such as mice, rats and lizards.

## Management implications and perspectives

The combined results of the genetic and behavioural studies of feral cats indicate that, in mountainous habitats of Réunion Island, such as Maïdo, harbouring Barau's Petrel colonies, cats are likely to be isolated and at low density. This is favourable for long term feral cat control. The genetic isolation implies there might be a low re-colonisation rate from surrounding cat populations (e.g. Lieury et al. 2015; Millon et al. 2019; Palmas et al. 2020).

In terms of feral cat control optimisation, the CMR study produced results that can be used to design the operations. First, in order to increase the number of feral cats exposed to control devices while minimising the number of devices, we propose to use the estimated sigma to optimise the spatial arrangement of the trapping grid (Goltz et al. 2008; Bengsen et al. 2012). In our case, with such an arrangement, each trap should be set every 950 to 1,000 m. This method minimises the number of cages to deploy (which minimises the human effort) while maximising the chance of a cat encountering at least one cage in its home range. Second, we suggest deploying traps near trails as the maximum detection probability has no variation between trail and vegetation cover for this site. This grid design presents the advantage of reducing the workload and

the time spent in the field by facilitating the access and maintenance of the traps. Of course, this recommendation is limited to habitats that have trails nearby. This design of device deployment can also be used after an intensive cat control to deploy camera traps for early detection of any re-invasive cats.

In addition to these recommendations, we propose trapping operations be conducted before the breeding season of Barau's Petrels (i.e. in austral winter, July and August, which correspond to the period when food availability for cats is the lowest, because of the absence of petrels). We also recommend the implementation of an early detection protocol, based on a network of camera traps to detect any re-invasion by cats and to respond with appropriate control actions.

For other sites located at lower altitudes, the absence of genetic isolation indicates strong connectivity between feral cat populations and, thus, a high risk of re-invasion after a feral cat control. Other strategies should be adopted to prevent or limit feral cat impact: 1) permanent feral cat control at colonies and in their vicinity and 2) predator-proof fences around bird colonies (Smith et al. 2020). However, the technical feasibility and financial costs of such operations may limit their implementation on the Island.

Feral cats are known to also prey upon other introduced mammals, such as rats or mice (Faulquier et al. 2009). Thus, in theory, a control of cats may result in the release of these prey, which in turn may impact birds, the so-called "mesopredator release effect" (Courchamp et al. 1999a). However, for such a release to occur, rat or mice populations must be controlled by feral cat predation (top-down control) rather than by their resources (bottom-up control; Courchamp et al. 1999a; Russell et al. 2009; Dumont et al. 2010). In the tropical context, it has been shown that rat and mice populations are controlled mostly by their resources through rain seasonality (Russell et al. 2011), which reduces the risk of a meso-predator release in case of feral cat control (Russell et al. 2011; Ringler et al. 2015). Furthermore, for long-lived animals, like seabirds, the population growth rate is more sensitive to change in adult survival than in breeding success (Le Corre 2008; Russell et al. 2009; Dumont et al. 2010). Feral cats prey upon adults and fledglings, whereas rats prey exclusively on eggs or chicks (Faulquier et al. 2009; authors' comm. pers.). Thus, even if rat population were released as a consequence of cat control, this would have less impact on the population growth of petrels than the impact of cats. Thus, we recommend to implement cat control at petrel colonies wherever possible.

Another more general recommendation would be to improve the public awareness and sensitisation at the scale of the entire island to stop human-mediated displacement of cats, to stop abandonment of domestic cats or kitten in the wild and to sterilise as many domestic cats as possible (Dias et al. 2017; Russell et al. 2018; Choeur et al. 2022).

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# Appendix 1

**Table A1.** Raw microsatellite genotypes for 158 individuals of feral cats from six geographical areas, in Réunion Island, 2015–2016. Area codes are described in Fig; 1.

| Samples | Area | 531 | 531 | 173 | 173 | 43 | 43 | 1027 | 1027 | 76 | 76 | 441 | 441 | 96 | 96 | 69 | 69 | 275 | 275 | 31 | 31 | X | Y |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LP0004 | CIL | 137 | 143 | 137 | 145 | 148 | 148 | 258 | 258 | 152 | 152 | 174 | 182 | 226 | 230 | 110 | 124 | 156 | 156 | 251 | 253 | 339151 | 7664054 |
| LP0005 | CIL | 143 | 145 | 145 | 147 | 148 | 150 | 258 | 258 | 152 | 152 | 174 | 182 | 236 | 244 | 110 | 110 | 156 | 158 | 235 | 255 | 338905 | 7664691 |
| LP0008 | CIL | 137 | 141 | 137 | 137 | 136 | 148 | 258 | 262 | 150 | 152 | 174 | 182 | 226 | 226 | 110 | 126 | 0 | 0 | 235 | 251 | 338905 | 7664691 |
| LP0009 | CIL | 133 | 133 | 137 | 137 | 148 | 150 | 262 | 262 | 154 | 154 | 170 | 182 | 238 | 244 | 110 | 128 | 156 | 160 | 235 | 235 | 342891 | 7664514 |
| LP0012 | CIL | 133 | 133 | 137 | 145 | 148 | 148 | 256 | 258 | 152 | 152 | 174 | 178 | 242 | 244 | 110 | 126 | 156 | 156 | 235 | 251 | 342962 | 7664490 |
| LP0013 | CIL | 133 | 137 | 141 | 149 | 148 | 148 | 258 | 258 | 150 | 154 | 174 | 182 | 230 | 244 | 110 | 126 | 156 | 162 | 235 | 239 | 342912 | 7664572 |
| LP0014 | CIL | 137 | 139 | 137 | 139 | 148 | 148 | 258 | 258 | 150 | 152 | 174 | 182 | 226 | 226 | 110 | 126 | 156 | 156 | 251 | 251 | 339151 | 7664054 |
| LP0016 | CIL | 145 | 145 | 125 | 131 | 140 | 150 | 250 | 258 | 152 | 154 | 174 | 186 | 226 | 230 | 110 | 126 | 156 | 156 | 255 | 255 | 341653 | 7663152 |
| LP0019 | CIL | 133 | 133 | 137 | 149 | 148 | 150 | 258 | 262 | 154 | 154 | 174 | 182 | 242 | 244 | 110 | 126 | 156 | 156 | 235 | 239 | 342891 | 7664514 |
| LP0020 | CIL | 143 | 147 | 137 | 145 | 148 | 148 | 258 | 268 | 138 | 152 | 174 | 182 | 226 | 226 | 110 | 110 | 154 | 156 | 235 | 235 | 338669 | 7662701 |
| LP0040 | CIL | 133 | 137 | 139 | 149 | 148 | 148 | 258 | 262 | 150 | 154 | 182 | 182 | 0 | 0 | 110 | 110 | 156 | 156 | 235 | 239 | 342891 | 7664514 |
| LP0041 | CIL | 143 | 143 | 141 | 141 | 148 | 148 | 258 | 258 | 152 | 154 | 178 | 182 | 236 | 244 | 110 | 118 | 156 | 158 | 235 | 235 | 343834 | 7664954 |
| LP0089 | CIL | 133 | 143 | 125 | 125 | 138 | 148 | 258 | 268 | 148 | 152 | 174 | 174 | 226 | 238 | 110 | 128 | 156 | 164 | 243 | 255 | 343614 | 7660602 |
| LP0092 | CIL | 133 | 147 | 137 | 145 | 148 | 150 | 258 | 262 | 154 | 154 | 178 | 182 | 226 | 226 | 124 | 124 | 0 | 0 | 235 | 243 | 341651,884 | 7663464,38 |
| LP0094 | CIL | 133 | 147 | 137 | 143 | 150 | 150 | 258 | 262 | 138 | 154 | 174 | 174 | 226 | 238 | 118 | 124 | 156 | 164 | 249 | 255 | 341651,884 | 7663464,38 |
| LP0120 | CIL | 133 | 143 | 143 | 145 | 138 | 148 | 258 | 258 | 150 | 152 | 174 | 182 | 232 | 244 | 110 | 128 | 0 | 0 | 251 | 253 | 343614 | 7660602 |
| LP0125 | CIL | 137 | 143 | 137 | 147 | 148 | 150 | 258 | 258 | 144 | 154 | 178 | 182 | 234 | 244 | 118 | 126 | 156 | 156 | 235 | 235 | 343614 | 7660602 |
| LP0146 | CIL | 133 | 133 | 143 | 147 | 148 | 148 | 262 | 268 | 148 | 152 | 182 | 194 | 226 | 232 | 124 | 126 | 156 | 158 | 235 | 255 | 341826 | 7660378 |
| LP0150 | CIL | 143 | 143 | 137 | 145 | 148 | 150 | 256 | 256 | 148 | 152 | 174 | 186 | 232 | 244 | 126 | 126 | 162 | 162 | 235 | 253 | 342568 | 7660656 |
| LP0153 | CIL | 143 | 143 | 145 | 145 | 142 | 150 | 258 | 258 | 124 | 152 | 174 | 178 | 226 | 244 | 110 | 110 | 156 | 158 | 235 | 253 | 341874 | 7660450 |
| LP0181 | CIL | 133 | 143 | 143 | 145 | 140 | 148 | 258 | 262 | 144 | 152 | 182 | 182 | 226 | 230 | 110 | 126 | 152 | 156 | 235 | 255 | 342917 | 7663413 |
| LP0182 | CIL | 143 | 143 | 125 | 141 | 148 | 154 | 258 | 258 | 152 | 152 | 178 | 186 | 226 | 226 | 110 | 126 | 156 | 170 | 243 | 249 | 340204,556 | 7658931,34 |
| LP0006 | DIM | 133 | 133 | 141 | 145 | 148 | 148 | 256 | 262 | 152 | 154 | 186 | 186 | 226 | 226 | 110 | 126 | 156 | 158 | 235 | 235 | 343194 | 7656559 |
| LP0007 | DIM | 133 | 143 | 125 | 145 | 148 | 148 | 258 | 258 | 152 | 154 | 174 | 174 | 226 | 244 | 110 | 122 | 156 | 162 | 235 | 255 | 344251 | 7656100 |
| LP0010 | DIM | 137 | 145 | 145 | 145 | 148 | 150 | 262 | 262 | 152 | 152 | 174 | 182 | 226 | 226 | 110 | 126 | 162 | 162 | 251 | 255 | 342242 | 7656286 |
| LP0011 | DIM | 137 | 143 | 145 | 145 | 148 | 150 | 258 | 258 | 152 | 152 | 186 | 186 | 232 | 238 | 124 | 126 | 156 | 158 | 235 | 251 | 345322,17 | 7657642,09 |
| LP0028 | DIM | 133 | 143 | 143 | 145 | 148 | 150 | 256 | 258 | 152 | 152 | 174 | 174 | 226 | 236 | 110 | 126 | 156 | 158 | 235 | 253 | 344056,112 | 7658441,64 |
| LP0029 | DIM | 137 | 147 | 145 | 145 | 148 | 152 | 258 | 260 | 152 | 152 | 174 | 174 | 226 | 226 | 110 | 126 | 156 | 158 | 235 | 255 | 344251 | 7656100 |
| LP0032 | DIM | 143 | 143 | 141 | 141 | 150 | 152 | 258 | 258 | 152 | 152 | 178 | 182 | 226 | 232 | 110 | 126 | 156 | 156 | 235 | 249 | 343844 | 7655518 |

| Samples | Area | 531 | 531 | 173 | 173 | 43 | 43 | 1027 | 1027 | 76 | 76 | 441 | 441 | 96 | 96 | 69 | 69 | 275 | 275 | 31 | 31 | X | Y |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LP0034 | DIM | 137 | 143 | 145 | 147 | 138 | 148 | 256 | 258 | 152 | 152 | 174 | 178 | 226 | 236 | 126 | 126 | 156 | 156 | 235 | 255 | 343974 | 7655595 |
| LP0036 | DIM | 137 | 143 | 137 | 145 | 148 | 148 | 258 | 258 | 152 | 152 | 182 | 186 | 232 | 236 | 124 | 126 | 156 | 158 | 235 | 253 | 345322,17 | 7657642,09 |
| LP0046 | DIM | 133 | 137 | 125 | 145 | 148 | 148 | 258 | 258 | 152 | 154 | 174 | 186 | 232 | 236 | 110 | 128 | 156 | 162 | 235 | 253 | 345311,993 | 7660541,02 |
| LP0052 | DIM | 143 | 143 | 125 | 145 | 148 | 150 | 258 | 258 | 152 | 154 | 174 | 182 | 226 | 244 | 110 | 122 | 156 | 158 | 235 | 235 | 344025 | 7656863 |
| LP0053 | DIM | 133 | 143 | 125 | 145 | 148 | 148 | 256 | 258 | 152 | 154 | 174 | 182 | 226 | 230 | 122 | 124 | 162 | 170 | 235 | 237 | 344251 | 7656100 |
| LP0056 | DIM | 139 | 145 | 137 | 137 | 140 | 150 | 258 | 262 | 152 | 152 | 178 | 178 | 232 | 232 | 110 | 124 | 156 | 160 | 235 | 235 | 343953 | 7655725 |
| LP0057 | DIM | 143 | 145 | 143 | 145 | 148 | 148 | 258 | 258 | 152 | 154 | 174 | 178 | 226 | 236 | 124 | 128 | 156 | 158 | 235 | 253 | 343660 | 7656552 |
| LP0058 | DIM | 147 | 147 | 145 | 145 | 148 | 150 | 256 | 258 | 0 | 0 | 174 | 178 | 236 | 238 | 114 | 118 | 156 | 158 | 243 | 251 | 344001 | 7655866 |
| LP0060 | DIM | 133 | 143 | 145 | 145 | 148 | 150 | 256 | 258 | 152 | 152 | 174 | 174 | 0 | 0 | 124 | 126 | 158 | 170 | 235 | 253 | 344109 | 7656689 |
| LP0072 | DIM | 145 | 145 | 145 | 145 | 148 | 150 | 258 | 262 | 152 | 154 | 182 | 182 | 236 | 244 | 110 | 110 | 0 | 0 | 235 | 255 | 344551,001 | 7657867 |
| LP0074 | DIM | 143 | 145 | 145 | 145 | 148 | 148 | 256 | 258 | 152 | 152 | 174 | 174 | 238 | 240 | 110 | 124 | 156 | 158 | 251 | 251 | 345312,053 | 7660387,19 |
| LP0075 | DIM | 137 | 145 | 145 | 145 | 148 | 150 | 256 | 258 | 152 | 154 | 182 | 186 | 232 | 236 | 124 | 128 | 156 | 158 | 235 | 253 | 344064,189 | 7658444,27 |
| LP0076 | DIM | 137 | 143 | 145 | 145 | 148 | 150 | 258 | 260 | 152 | 154 | 178 | 186 | 226 | 236 | 114 | 126 | 156 | 158 | 235 | 251 | 344553,72 | 7657861,89 |
| LP0085 | DIM | 133 | 143 | 139 | 145 | 148 | 150 | 258 | 262 | 138 | 152 | 174 | 178 | 242 | 244 | 126 | 130 | 156 | 160 | 235 | 255 | 345452,189 | 7660727,29 |
| LP0086 | DIM | 143 | 143 | 137 | 137 | 140 | 148 | 258 | 260 | 152 | 152 | 174 | 178 | 226 | 236 | 110 | 110 | 156 | 162 | 235 | 235 | 344056 | 7653594 |
| LP0087 | DIM | 137 | 145 | 125 | 147 | 148 | 148 | 260 | 262 | 152 | 152 | 174 | 174 | 226 | 226 | 120 | 122 | 156 | 162 | 255 | 255 | 344056 | 7653594 |
| LP0088 | DIM | 137 | 143 | 141 | 147 | 148 | 152 | 258 | 258 | 0 | 0 | 174 | 178 | 226 | 232 | 124 | 126 | 158 | 158 | 235 | 235 | 344086 | 7653441 |
| LP0096 | DIM | 133 | 143 | 139 | 145 | 148 | 148 | 258 | 262 | 152 | 152 | 170 | 186 | 226 | 236 | 118 | 120 | 156 | 158 | 235 | 235 | 344064,189 | 7658444,27 |
| LP0103 | DIM | 133 | 145 | 125 | 145 | 148 | 148 | 256 | 258 | 152 | 152 | 178 | 186 | 234 | 236 | 110 | 124 | 158 | 162 | 235 | 235 | 343762 | 7654114 |
| LP0109 | DIM | 143 | 145 | 145 | 145 | 148 | 148 | 258 | 258 | 152 | 152 | 174 | 182 | 242 | 244 | 110 | 110 | 158 | 170 | 235 | 251 | 343711 | 7652783 |
| LP0110 | DIM | 137 | 137 | 141 | 143 | 148 | 152 | 258 | 260 | 152 | 152 | 174 | 174 | 226 | 226 | 124 | 126 | 162 | 162 | 235 | 255 | 344401 | 7653628 |
| LP0114 | DIM | 137 | 147 | 145 | 145 | 138 | 148 | 260 | 262 | 152 | 152 | 174 | 174 | 226 | 226 | 110 | 126 | 158 | 162 | 235 | 255 | 343662 | 7653716 |
| LP0115 | DIM | 133 | 137 | 143 | 147 | 138 | 148 | 258 | 268 | 138 | 152 | 174 | 182 | 226 | 236 | 124 | 128 | 156 | 162 | 251 | 255 | 344169 | 7653841 |
| LP0117 | DIM | 143 | 143 | 139 | 147 | 148 | 150 | 258 | 262 | 152 | 152 | 178 | 182 | 232 | 236 | 110 | 110 | 156 | 158 | 249 | 257 | 344400 | 7653782 |
| LP0119 | DIM | 143 | 145 | 145 | 147 | 148 | 150 | 258 | 258 | 152 | 154 | 186 | 186 | 226 | 238 | 124 | 126 | 156 | 158 | 235 | 253 | 343946 | 7654171 |
| LP0122 | DIM | 143 | 143 | 145 | 145 | 148 | 150 | 258 | 260 | 152 | 152 | 174 | 182 | 226 | 244 | 124 | 128 | 156 | 158 | 235 | 253 | 343488 | 7654539 |
| LP0123 | DIM | 143 | 143 | 141 | 141 | 148 | 152 | 258 | 258 | 152 | 152 | 178 | 178 | 226 | 236 | 110 | 126 | 158 | 162 | 235 | 249 | 343745 | 7654812 |
| LP0124 | DIM | 137 | 145 | 145 | 147 | 148 | 150 | 258 | 260 | 152 | 152 | 174 | 178 | 232 | 236 | 110 | 124 | 156 | 158 | 235 | 235 | 344169 | 7653841 |
| LP0133 | DIM | 133 | 143 | 145 | 145 | 148 | 152 | 258 | 262 | 150 | 152 | 178 | 182 | 226 | 244 | 110 | 124 | 162 | 170 | 235 | 235 | 343745 | 7654812 |
| LP0138 | DIM | 137 | 145 | 145 | 145 | 148 | 148 | 256 | 258 | 152 | 152 | 174 | 174 | 232 | 238 | 110 | 110 | 154 | 156 | 235 | 235 | 344400 | 7653782 |
| LP0144 | DIM | 143 | 143 | 125 | 125 | 148 | 150 | 256 | 258 | 0 | 0 | 174 | 174 | 226 | 244 | 110 | 122 | 156 | 162 | 235 | 235 | 343776 | 7654707 |
| LP0147 | DIM | 143 | 143 | 145 | 145 | 148 | 148 | 258 | 258 | 152 | 152 | 174 | 182 | 226 | 244 | 110 | 118 | 162 | 170 | 235 | 253 | 343711 | 7652783 |
| LP0151 | DIM | 143 | 143 | 141 | 145 | 148 | 148 | 246 | 262 | 152 | 154 | 174 | 182 | 226 | 226 | 110 | 110 | 156 | 158 | 251 | 253 | 343660 | 7655007 |
| LP0158 | DIM | 137 | 145 | 145 | 145 | 148 | 150 | 258 | 258 | 0 | 0 | 174 | 186 | 232 | 238 | 124 | 126 | 156 | 158 | 235 | 251 | 344901,611 | 7657760,99 |

| Samples | Area | 531 | 531 | 173 | 173 | 43 | 43 | 1027 | 1027 | 76 | 76 | 441 | 441 | 96 | 96 | 69 | 69 | 275 | 275 | 31 | 31 | X | Y |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LP0164 | DIM | 143 | 145 | 145 | 145 | 150 | 154 | 262 | 268 | 130 | 150 | 182 | 182 | 226 | 234 | 124 | 126 | 156 | 158 | 255 | 255 | 343899 | 7654209 |
| LP0165 | DIM | 143 | 145 | 141 | 147 | 148 | 150 | 246 | 258 | 138 | 152 | 174 | 182 | 202 | 232 | 124 | 126 | 156 | 156 | 235 | 243 | 344056 | 7653594 |
| LP0175 | DIM | 137 | 143 | 145 | 147 | 148 | 148 | 256 | 260 | 150 | 152 | 178 | 182 | 236 | 238 | 110 | 124 | 156 | 158 | 0 | 0 | 343776 | 7654707 |
| LP0177 | DIM | 143 | 145 | 141 | 147 | 148 | 150 | 258 | 258 | 150 | 152 | 174 | 182 | 226 | 236 | 110 | 114 | 156 | 192 | 243 | 249 | 344905,252 | 7657584 |
| LP0179 | DIM | 143 | 147 | 141 | 145 | 148 | 148 | 258 | 258 | 150 | 154 | 174 | 174 | 226 | 238 | 114 | 124 | 156 | 158 | 243 | 251 | 343427 | 7655879 |
| LP0042 | GA | 143 | 143 | 145 | 147 | 148 | 148 | 256 | 258 | 148 | 152 | 178 | 182 | 226 | 232 | 124 | 124 | 156 | 156 | 235 | 243 | 350858,198 | 7636083,08 |
| LP0043 | GA | 133 | 139 | 145 | 147 | 148 | 148 | 246 | 262 | 150 | 154 | 174 | 174 | 226 | 226 | 122 | 124 | 158 | 158 | 235 | 255 | 350858,198 | 7636083,08 |
| LP0045 | GA | 133 | 133 | 141 | 145 | 148 | 148 | 258 | 262 | 138 | 152 | 174 | 178 | 230 | 244 | 124 | 126 | 162 | 166 | 235 | 255 | 350858,198 | 7636083,08 |
| LP0047 | GA | 143 | 145 | 145 | 145 | 148 | 150 | 258 | 262 | 146 | 150 | 178 | 178 | 226 | 230 | 120 | 128 | 156 | 156 | 235 | 255 | 350858,198 | 7636083,08 |
| LP0048 | GA | 137 | 143 | 141 | 143 | 138 | 150 | 258 | 258 | 152 | 152 | 182 | 186 | 230 | 238 | 110 | 126 | 156 | 162 | 235 | 251 | 350858,198 | 7636083,08 |
| LP0049 | GA | 139 | 143 | 143 | 143 | 140 | 142 | 254 | 258 | 132 | 150 | 174 | 178 | 226 | 240 | 124 | 126 | 154 | 158 | 235 | 243 | 350858,198 | 7636083,08 |
| LP0050 | GA | 143 | 143 | 145 | 147 | 148 | 150 | 258 | 258 | 152 | 154 | 182 | 182 | 0 | 0 | 126 | 126 | 156 | 158 | 235 | 235 | 350858,198 | 7636083,08 |
| LP0064 | GA | 143 | 145 | 143 | 147 | 140 | 154 | 258 | 258 | 146 | 152 | 174 | 178 | 226 | 226 | 120 | 124 | 156 | 156 | 249 | 251 | 350858,198 | 7636083,08 |
| LP0065 | GA | 143 | 143 | 125 | 145 | 142 | 148 | 256 | 258 | 152 | 152 | 178 | 178 | 232 | 236 | 124 | 126 | 156 | 156 | 243 | 249 | 350858,198 | 7636083,08 |
| LP0067 | GA | 143 | 147 | 143 | 145 | 138 | 148 | 254 | 268 | 152 | 152 | 162 | 178 | 228 | 230 | 110 | 110 | 156 | 158 | 235 | 245 | 350858,198 | 7636083,08 |
| LP0070 | GA | 133 | 143 | 125 | 141 | 138 | 150 | 258 | 258 | 152 | 154 | 0 | 0 | 226 | 236 | 118 | 120 | 156 | 162 | 235 | 245 | 350858,198 | 7636083,08 |
| LP0071 | GA | 133 | 143 | 141 | 145 | 148 | 148 | 258 | 262 | 152 | 152 | 174 | 182 | 236 | 244 | 110 | 126 | 156 | 166 | 235 | 245 | 350858,198 | 7636083,08 |
| LP0073 | GA | 133 | 143 | 125 | 145 | 148 | 154 | 258 | 262 | 152 | 152 | 178 | 178 | 226 | 236 | 110 | 124 | 156 | 162 | 239 | 243 | 350858,198 | 7636083,08 |
| LP0078 | GA | 133 | 143 | 125 | 137 | 138 | 146 | 246 | 262 | 152 | 152 | 162 | 178 | 226 | 226 | 124 | 126 | 134 | 156 | 235 | 255 | 350858,198 | 7636083,08 |
| LP0079 | GA | 133 | 143 | 125 | 145 | 142 | 148 | 258 | 268 | 152 | 152 | 178 | 182 | 226 | 226 | 110 | 124 | 156 | 156 | 239 | 243 | 350858,198 | 7636083,08 |
| LP0080 | GA | 133 | 143 | 125 | 137 | 146 | 150 | 258 | 262 | 152 | 152 | 162 | 182 | 226 | 236 | 118 | 126 | 156 | 156 | 235 | 235 | 350858,198 | 7636083,08 |
| LP0081 | GA | 143 | 143 | 143 | 147 | 138 | 150 | 254 | 258 | 148 | 152 | 178 | 178 | 230 | 232 | 120 | 128 | 156 | 158 | 235 | 255 | 350858,198 | 7636083,08 |
| LP0082 | GA | 143 | 147 | 125 | 147 | 140 | 146 | 258 | 258 | 152 | 154 | 174 | 178 | 226 | 232 | 124 | 126 | 156 | 166 | 235 | 243 | 350858,198 | 7636083,08 |
| LP0083 | GA | 143 | 145 | 137 | 145 | 148 | 148 | 258 | 258 | 146 | 152 | 178 | 182 | 236 | 236 | 110 | 126 | 156 | 162 | 235 | 255 | 350858,198 | 7636083,08 |
| LP0084 | GA | 143 | 143 | 137 | 141 | 138 | 150 | 246 | 258 | 152 | 154 | 178 | 182 | 226 | 226 | 120 | 126 | 134 | 156 | 235 | 235 | 350858,198 | 7636083,08 |
| LP0015 | GB | 143 | 147 | 137 | 145 | 148 | 150 | 256 | 258 | 152 | 154 | 178 | 182 | 226 | 244 | 110 | 124 | 164 | 168 | 235 | 235 | 348970 | 7658171 |
| LP0022 | GB | 143 | 147 | 137 | 147 | 148 | 148 | 258 | 266 | 152 | 152 | 174 | 182 | 236 | 236 | 110 | 116 | 152 | 156 | 235 | 253 | 349484 | 7658622 |
| LP0030 | GB | 143 | 143 | 137 | 145 | 154 | 154 | 258 | 258 | 152 | 152 | 174 | 174 | 226 | 244 | 110 | 110 | 156 | 156 | 235 | 251 | 347824 | 7653382 |
| LP0051 | GB | 141 | 143 | 125 | 145 | 142 | 142 | 256 | 262 | 150 | 152 | 174 | 182 | 226 | 236 | 124 | 128 | 156 | 156 | 253 | 255 | 349880 | 7659100 |
| LP0059 | GB | 143 | 147 | 147 | 147 | 148 | 148 | 256 | 258 | 152 | 152 | 162 | 178 | 236 | 236 | 110 | 110 | 156 | 158 | 235 | 253 | 349720 | 7658792 |
| LP0062 | GB | 145 | 147 | 141 | 145 | 148 | 150 | 258 | 258 | 152 | 152 | 174 | 174 | 242 | 244 | 110 | 120 | 156 | 158 | 251 | 251 | 349608 | 7658645 |
| LP0063 | GB | 143 | 145 | 125 | 147 | 148 | 148 | 256 | 258 | 152 | 152 | 182 | 182 | 236 | 244 | 110 | 126 | 158 | 162 | 235 | 245 | 349483 | 7658437 |
| LP0111 | GB | 143 | 143 | 137 | 137 | 140 | 148 | 258 | 258 | 152 | 152 | 174 | 186 | 226 | 236 | 114 | 126 | 156 | 156 | 253 | 255 | 347935,24 | 7658920,11 |
| LP0112 | GB | 145 | 145 | 131 | 137 | 148 | 150 | 258 | 258 | 152 | 152 | 174 | 182 | 236 | 244 | 110 | 126 | 156 | 156 | 235 | 255 | 349481 | 7658623 |

| Samples | Area | 531 | 531 | 173 | 173 | 43 | 43 | 1027 | 1027 | 76 | 76 | 441 | 441 | 96 | 96 | 69 | 69 | 275 | 275 | 31 | 31 | X | Y |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LP0142 | GB | 143 | 143 | 145 | 147 | 148 | 148 | 258 | 266 | 152 | 152 | 174 | 178 | 236 | 236 | 110 | 114 | 0 | 0 | 253 | 255 | 347932 | 7661950 |
| LP0145 | GB | 143 | 145 | 137 | 147 | 148 | 148 | 258 | 258 | 152 | 152 | 174 | 174 | 226 | 244 | 110 | 110 | 156 | 156 | 235 | 255 | 349883 | 7659101 |
| LP0148 | GB | 143 | 143 | 137 | 145 | 150 | 150 | 258 | 258 | 146 | 152 | 174 | 182 | 226 | 226 | 110 | 110 | 156 | 156 | 235 | 255 | 348429 | 7661450 |
| LP0167 | GB | 133 | 143 | 145 | 145 | 0 | 0 | 0 | 0 | 150 | 150 | 178 | 182 | 232 | 234 | 114 | 126 | 156 | 156 | 235 | 255 | 348116 | 7655939 |
| LP0168 | GB | 133 | 143 | 125 | 145 | 144 | 148 | 258 | 258 | 150 | 152 | 174 | 174 | 226 | 230 | 110 | 110 | 158 | 162 | 235 | 243 | 350872 | 7658655 |
| LP0169 | GB | 133 | 145 | 131 | 141 | 148 | 150 | 258 | 258 | 152 | 152 | 174 | 174 | 226 | 246 | 110 | 110 | 156 | 158 | 235 | 255 | 349045 | 7660365 |
| LP0183 | GB | 139 | 143 | 145 | 145 | 138 | 150 | 256 | 262 | 150 | 150 | 178 | 178 | 202 | 234 | 124 | 124 | 156 | 156 | 235 | 255 | 349824,913 | 7658843,05 |
| LP0027 | MAI | 133 | 143 | 145 | 145 | 144 | 148 | 258 | 258 | 152 | 154 | 174 | 182 | 230 | 236 | 110 | 120 | 156 | 162 | 235 | 255 | 333677,367 | 7665497,52 |
| LP0037 | MAI | 133 | 143 | 137 | 145 | 148 | 148 | 258 | 258 | 152 | 152 | 178 | 178 | 226 | 244 | 120 | 124 | 134 | 156 | 235 | 255 | 332819,755 | 7667694,89 |
| LP0044 | MAI | 143 | 143 | 145 | 145 | 148 | 148 | 258 | 262 | 152 | 152 | 182 | 182 | 236 | 236 | 118 | 124 | 156 | 158 | 235 | 255 | 336242,024 | 7664056 |
| LP0054 | MAI | 133 | 133 | 137 | 145 | 142 | 148 | 250 | 260 | 148 | 152 | 178 | 178 | 226 | 230 | 112 | 118 | 156 | 158 | 235 | 255 | 334019,61 | 7658585,33 |
| LP0068 | MAI | 133 | 133 | 145 | 145 | 140 | 148 | 250 | 258 | 152 | 154 | 182 | 182 | 230 | 236 | 118 | 124 | 158 | 158 | 235 | 235 | 336340,044 | 7663521,99 |
| LP0069 | MAI | 133 | 143 | 145 | 145 | 148 | 148 | 258 | 258 | 152 | 152 | 174 | 178 | 230 | 236 | 110 | 118 | 156 | 156 | 235 | 235 | 336330,619 | 7663582,43 |
| LP0093 | MAI | 133 | 143 | 137 | 145 | 148 | 148 | 250 | 258 | 152 | 154 | 178 | 182 | 226 | 230 | 114 | 128 | 156 | 156 | 235 | 235 | 330867,51 | 7669268,36 |
| LP0104 | MAI | 143 | 143 | 145 | 147 | 140 | 150 | 250 | 258 | 138 | 152 | 174 | 182 | 226 | 236 | 110 | 128 | 156 | 158 | 243 | 255 | 330737,89 | 7668030,97 |
| LP0105 | MAI | 133 | 133 | 137 | 147 | 148 | 148 | 258 | 258 | 152 | 154 | 174 | 178 | 226 | 244 | 110 | 110 | 156 | 162 | 235 | 235 | 330404,71 | 7669412,77 |
| LP0106 | MAI | 133 | 133 | 137 | 137 | 148 | 152 | 250 | 260 | 152 | 152 | 178 | 178 | 228 | 230 | 110 | 124 | 156 | 156 | 235 | 235 | 333532 | 7662357 |
| LP0107 | MAI | 143 | 143 | 145 | 145 | 148 | 148 | 258 | 258 | 152 | 152 | 178 | 178 | 226 | 244 | 120 | 128 | 134 | 156 | 235 | 235 | 331878 | 7668510 |
| LP0113 | MAI | 133 | 143 | 137 | 145 | 148 | 148 | 258 | 258 | 152 | 152 | 178 | 182 | 230 | 244 | 110 | 124 | 156 | 162 | 235 | 235 | 333532 | 7662357 |
| LP0126 | MAI | 133 | 133 | 137 | 137 | 148 | 148 | 258 | 258 | 152 | 152 | 182 | 182 | 226 | 230 | 110 | 124 | 156 | 156 | 235 | 235 | 333532 | 7662357 |
| LP0131 | MAI | 133 | 143 | 137 | 145 | 144 | 148 | 258 | 262 | 150 | 154 | 178 | 182 | 230 | 244 | 120 | 124 | 162 | 162 | 235 | 235 | 330867,51 | 7669268,36 |
| LP0132 | MAI | 133 | 143 | 135 | 145 | 144 | 148 | 256 | 258 | 0 | 0 | 178 | 182 | 226 | 226 | 110 | 120 | 156 | 162 | 235 | 235 | 330537,86 | 7670690,27 |
| LP0134 | MAI | 143 | 143 | 135 | 145 | 148 | 148 | 258 | 262 | 0 | 0 | 182 | 182 | 226 | 244 | 110 | 124 | 156 | 158 | 235 | 255 | 333532 | 7662357 |
| LP0136 | MAI | 133 | 143 | 145 | 147 | 148 | 148 | 258 | 260 | 152 | 152 | 174 | 174 | 226 | 236 | 110 | 110 | 156 | 156 | 235 | 253 | 329934,94 | 7667151,6 |
| LP0137 | MAI | 133 | 143 | 145 | 145 | 152 | 152 | 258 | 258 | 152 | 154 | 174 | 178 | 226 | 236 | 110 | 120 | 156 | 158 | 235 | 255 | 330668,26 | 7667098,23 |
| LP0143 | MAI | 133 | 143 | 137 | 145 | 150 | 152 | 250 | 258 | 152 | 152 | 178 | 182 | 226 | 236 | 110 | 110 | 156 | 156 | 235 | 235 | 333609 | 7665097 |
| LP0152 | MAI | 133 | 133 | 137 | 145 | 144 | 148 | 258 | 262 | 130 | 154 | 182 | 182 | 226 | 230 | 110 | 110 | 156 | 162 | 235 | 235 | 333532 | 7662357 |
| LP0154 | MAI | 133 | 143 | 145 | 145 | 140 | 148 | 246 | 262 | 152 | 152 | 182 | 182 | 234 | 236 | 110 | 126 | 156 | 156 | 235 | 235 | 336242 | 7664056 |
| LP0173 | MAI | 137 | 143 | 135 | 145 | 148 | 148 | 256 | 258 | 152 | 152 | 178 | 182 | 228 | 230 | 110 | 110 | 156 | 160 | 235 | 235 | 329454,482 | 7666797 |
| LP0185 | MAI | 133 | 143 | 135 | 145 | 148 | 148 | 260 | 260 | 144 | 152 | 178 | 182 | 228 | 234 | 128 | 128 | 156 | 158 | 235 | 235 | 330668,259 | 7667098,23 |
| LP0017 | MAK | 143 | 143 | 127 | 139 | 148 | 150 | 258 | 258 | 152 | 152 | 174 | 182 | 226 | 236 | 110 | 110 | 156 | 158 | 235 | 255 | 336631 | 7657360 |
| LP0018 | MAK | 133 | 133 | 127 | 147 | 146 | 148 | 258 | 258 | 152 | 154 | 178 | 182 | 230 | 232 | 112 | 128 | 156 | 158 | 235 | 243 | 337083 | 7656762 |
| LP0021 | MAK | 143 | 143 | 145 | 145 | 148 | 148 | 258 | 258 | 150 | 152 | 174 | 182 | 230 | 230 | 124 | 126 | 156 | 158 | 255 | 255 | 337476 | 7654771 |
| LP0023 | MAK | 143 | 147 | 145 | 145 | 148 | 150 | 256 | 258 | 132 | 132 | 162 | 170 | 236 | 236 | 110 | 110 | 156 | 158 | 235 | 251 | 336649 | 7657420 |

| Samples | Area | 531 | 531 | 173 | 173 | 43 | 43 | 1027 | 1027 | 76 | 76 | 441 | 441 | 96 | 96 | 69 | 69 | 275 | 275 | 31 | 31 | X | Y |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LP0031 | MAK | 143 | 143 | 137 | 147 | 148 | 150 | 258 | 258 | 150 | 150 | 178 | 186 | 226 | 238 | 110 | 126 | 156 | 168 | 239 | 255 | 335467 | 7658028 |
| LP0033 | MAK | 133 | 147 | 145 | 145 | 140 | 148 | 258 | 268 | 152 | 152 | 182 | 182 | 226 | 232 | 110 | 110 | 156 | 156 | 235 | 235 | 336325 | 7657861 |
| LP0035 | MAK | 133 | 145 | 125 | 131 | 138 | 148 | 256 | 262 | 150 | 152 | 182 | 182 | 226 | 226 | 110 | 124 | 154 | 164 | 243 | 249 | 336325 | 7657861 |
| LP0038 | MAK | 133 | 143 | 139 | 145 | 148 | 148 | 258 | 262 | 150 | 154 | 178 | 182 | 226 | 232 | 110 | 124 | 156 | 168 | 235 | 243 | 337056 | 7656624 |
| LP0039 | MAK | 143 | 143 | 137 | 145 | 138 | 142 | 256 | 262 | 138 | 154 | 174 | 182 | 0 | 0 | 110 | 128 | 156 | 156 | 235 | 235 | 335342 | 7658063 |
| LP0055 | MAK | 143 | 143 | 145 | 147 | 148 | 150 | 258 | 268 | 152 | 152 | 174 | 182 | 226 | 236 | 110 | 124 | 0 | 0 | 235 | 243 | 334516,79 | 7657580,56 |
| LP0061 | MAK | 143 | 143 | 137 | 147 | 148 | 148 | 258 | 258 | 148 | 152 | 172 | 186 | 226 | 226 | 110 | 110 | 156 | 162 | 243 | 255 | 336881,726 | 7655700,6 |
| LP0066 | MAK | 143 | 143 | 137 | 147 | 138 | 148 | 258 | 258 | 150 | 152 | 172 | 178 | 226 | 226 | 110 | 126 | 156 | 162 | 243 | 251 | 337776,677 | 7656129,04 |
| LP0091 | MAK | 143 | 143 | 137 | 145 | 138 | 150 | 258 | 258 | 150 | 152 | 178 | 182 | 226 | 238 | 126 | 126 | 156 | 168 | 239 | 251 | 334840,713 | 7657807,07 |
| LP0095 | MAK | 133 | 143 | 131 | 141 | 148 | 148 | 262 | 262 | 132 | 154 | 174 | 182 | 234 | 236 | 118 | 124 | 134 | 156 | 235 | 251 | 336879,698 | 7656881,21 |
| LP0097 | MAK | 143 | 143 | 145 | 145 | 142 | 142 | 246 | 262 | 152 | 152 | 182 | 182 | 232 | 232 | 124 | 126 | 156 | 156 | 235 | 255 | 335364 | 7658061 |
| LP0098 | MAK | 133 | 143 | 131 | 145 | 148 | 148 | 258 | 262 | 150 | 154 | 174 | 174 | 226 | 236 | 110 | 124 | 156 | 156 | 235 | 255 | 336879,698 | 7656881,21 |
| LP0099 | MAK | 133 | 133 | 127 | 137 | 148 | 148 | 246 | 256 | 150 | 154 | 178 | 182 | 226 | 232 | 114 | 128 | 156 | 162 | 235 | 235 | 337446 | 7656509 |
| LP0100 | MAK | 133 | 133 | 137 | 141 | 138 | 148 | 256 | 262 | 130 | 152 | 174 | 182 | 224 | 236 | 110 | 110 | 134 | 134 | 235 | 235 | 337758 | 7656424 |
| LP0101 | MAK | 139 | 147 | 127 | 141 | 148 | 150 | 256 | 262 | 152 | 154 | 174 | 178 | 0 | 0 | 110 | 110 | 0 | 0 | 235 | 251 | 336694 | 7655803 |
| LP0102 | MAK | 133 | 147 | 137 | 141 | 148 | 148 | 262 | 262 | 152 | 154 | 178 | 182 | 230 | 232 | 110 | 124 | 156 | 158 | 235 | 251 | 335104 | 7658028 |
| LP0108 | MAK | 133 | 133 | 137 | 137 | 138 | 148 | 258 | 258 | 152 | 152 | 162 | 178 | 236 | 236 | 110 | 126 | 134 | 168 | 235 | 235 | 336970,406 | 7657053,94 |
| LP0116 | MAK | 143 | 145 | 141 | 149 | 148 | 150 | 258 | 262 | 152 | 152 | 172 | 174 | 202 | 238 | 124 | 124 | 158 | 164 | 247 | 255 | 337784 | 7656044 |
| LP0118 | MAK | 143 | 143 | 137 | 139 | 150 | 150 | 258 | 262 | 152 | 152 | 174 | 178 | 226 | 226 | 110 | 114 | 156 | 156 | 235 | 255 | 337890 | 7655551 |
| LP0121 | MAK | 133 | 139 | 137 | 147 | 146 | 148 | 256 | 258 | 152 | 152 | 178 | 182 | 232 | 232 | 110 | 110 | 0 | 0 | 235 | 243 | 337402 | 7655051 |
| LP0128 | MAK | 133 | 143 | 127 | 145 | 148 | 150 | 258 | 258 | 152 | 154 | 178 | 182 | 226 | 230 | 110 | 120 | 156 | 156 | 235 | 243 | 334840,713 | 7657807,07 |
| LP0130 | MAK | 139 | 143 | 143 | 145 | 148 | 154 | 258 | 258 | 150 | 152 | 174 | 186 | 226 | 236 | 110 | 110 | 164 | 164 | 235 | 255 | 337758 | 7656424 |
| LP0139 | MAK | 133 | 143 | 145 | 147 | 142 | 142 | 258 | 258 | 150 | 152 | 162 | 182 | 226 | 226 | 126 | 128 | 156 | 156 | 235 | 255 | 337568 | 7656070 |
| LP0140 | MAK | 143 | 145 | 125 | 125 | 148 | 150 | 258 | 262 | 0 | 0 | 174 | 174 | 226 | 226 | 110 | 110 | 158 | 158 | 243 | 251 | 337758 | 7656424 |
| LP0141 | MAK | 133 | 143 | 131 | 141 | 148 | 148 | 262 | 262 | 0 | 0 | 174 | 182 | 226 | 226 | 110 | 124 | 156 | 156 | 251 | 255 | 336970,406 | 7657053,94 |
| LP0149 | MAK | 143 | 143 | 137 | 145 | 148 | 150 | 246 | 258 | 132 | 152 | 174 | 182 | 226 | 226 | 110 | 118 | 156 | 156 | 251 | 255 | 336970,406 | 7657053,94 |
| LP0166 | MAK | 143 | 145 | 125 | 137 | 148 | 148 | 246 | 268 | 150 | 152 | 182 | 186 | 226 | 234 | 110 | 110 | 156 | 156 | 235 | 253 | 337069 | 7656738 |

## Appendix 2

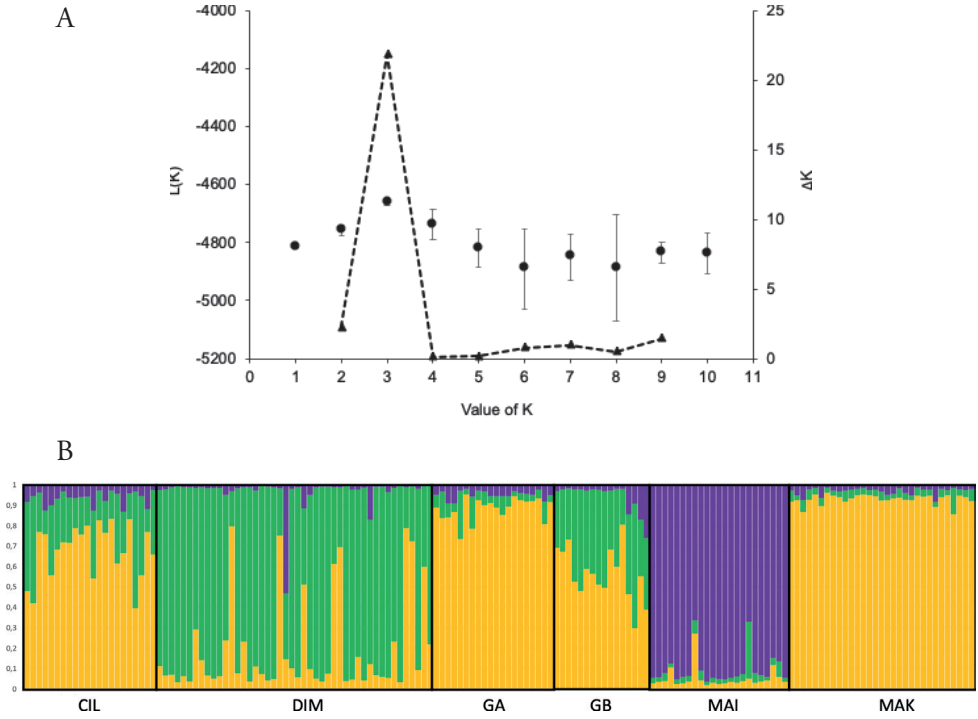


**Figure A1.** Cluster analysis of 158 feral cats from six geographical areas, on Réunion Island, 2015–2016 **A** detection of the number of genetic clusters K using the log-likelihood mean values L(K) (black circles; ± standard deviation) and ΔK statistic (black triangles; Evanno et al. 2005) as derived from STRUCTURE with K ranging from 1 to 10 with each value obtained by averaging the posterior probabilities over 10 independent runs **B** proportional membership probability to a given genetic cluster. Colours correspond to genetic clusters. Area codes are detailed in Fig. 1.

## Appendix 3

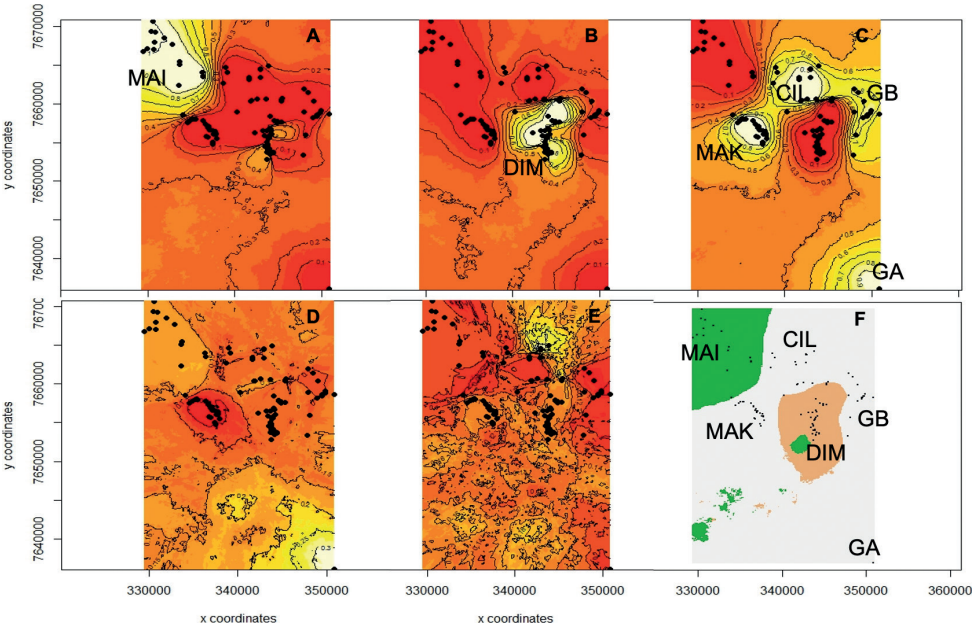


**Figure A2.** Spatial distribution of each group defined by Geneland for sampled feral cats (n = 158), on Réunion Island, 2015–2016. Black dots represent sample locations **A, B, C, D** and **E** are maps of posterior probability to belong to each group. Clusters are indicated by areas with different intensities of colour. Probability of population membership increases as shading intensity decreases (values of probability are indicated on each contour line) **F** shows the mode map of the posterior probability to belong to each group (see Table 1 for area codes). Unit of axis is metre.

## Appendix 4

**Table A2.** Pairwise $F_{ST}$ (above diagonal) and Nei distance estimates (below diagonal) for 6 areas where feral cats were sampled (n = 158) on Réunion Island, 2015–2016. Area codes are described in Fig. 1.

| Area/Area | MAI | MAK | CIL | DIM | GB | GA |
|---|---|---|---|---|---|---|
| MAI | – | 0.120*** | 0.132*** | 0.145*** | 0.149*** | 0.136*** |
| MAK | 0.043*** | – | 0.011 NS | 0.096*** | 0.036 NS | 0.049 NS |
| CIL | 0.047*** | 0.003 NS | – | 0.100*** | 0.055 NS | 0.093 NS |
| DIM | 0.055*** | 0.030*** | 0.032*** | – | 0.056 NS | 0.096*** |
| GB | 0.057*** | 0.012 NS | 0.018 NS | 0.019 NS | – | 0.097 NS |
| GA | 0.048*** | 0.015 NS | 0.028* | 0.030*** | 0.031 NS | – |

## Appendix 5

**Table A3.** Posterior mean and mode migration rates over the last generations amongst the six geographical groups of sampled feral cats on Réunion Island, 2015–2016. 95% high density predictive interval (HDPI) estimated by software BIMR and means of the posterior distributions of the migration rate (with 95% confidence intervals) using BayesAss are indicated. Asymmetric immigration is shown in bold text. Means values using BayesAss with a confident interval not including zero are in italic. Area codes are described in Fig. 1.

| From-Into | BIMr | | BayesAss | |
|---|---|---|---|---|
| | mean, mode | HDPI 95CI | mean | 95CI |
| CIL-CIL | 1, 1 | [1;1] | 0.681 | [0.653;0.709] |
| CIL-DIM | **6.90e-09, 1.05e-08** | **[2.41e-13;1.79e-08]** | **0.016** | **[-0.006;0.037]** |
| CIL-GA | 1.27e-08, 1.32e-08 | [4.27e-13;3.35e-08] | 0.014 | [-0.012;0.040] |
| CIL-GB | 2.70e-08, 2.94e-08 | [4.56e-13;8.12e-08] | 0.015 | [-0.013;0.044] |
| CIL-MAI | 4.60e-09, 1.13e-08 | [3.37e-13;1.23e-08] | 0.012 | [-0.011;0.035] |
| CIL-MAK | 3.19e-09, 5.80e-09 | [1.09e-13;8.41e-09] | 0.012 | [-0.011;0.036] |
| DIM-CIL | **0.020, 0.101** | **[2.08e-12;0.126]** | ***0.186*** | ***[0.098;0.275]*** |
| DIM-DIM | 0.903, 1 | [0.50;1] | 0.913 | [0.859;0.967] |
| DIM-GA | 1.29e-08, 1.21e-08 | [1.06e-12;3.40e-08] | 0.088 | [-0.005;0.179] |
| DIM-GB | 2.67e-08, 2.91e-08 | [1.56e-13;8.02e-08] | ***0.203*** | ***[0.130;0.276]*** |
| DIM-MAI | 4.66e-09, 9.99e-10 | [1.98e-13;1.23e-08] | 0.048 | [-0.017;0.113] |
| DIM-MAK | 3.17e-09, 3.73e-09 | [3.47e-13;8.36e-09] | *0.133* | *[0.015;0.250]* |
| GA-CIL | 6.80e-09, 2.28e-09 | [3.96e-13;1.79e-08] | 0.012 | [-0.011;0.035] |
| GA-DIM | 0.009, 1.16e-08 | [1.23e-12;0.065] | 0.007 | [-0.007;0.022] |
| GA-GA | 1, 1 | [1;1] | 0.680 | [0.655;0.705] |
| GA-GB | 2.67e-08, 1.42e-08 | [5.34e-13;8.00e-08] | 0.015 | [-0.013;0.044] |
| GA-MAI | 4.58e-09, 1.11e-08 | [9.69e-14;1.21e-08] | 0.012 | [-0.011;0.035] |
| GA-MAK | 3.20e-09, 1.24e-09 | [1.09e-13;8.36e-09] | **0.011** | **[-0.011;0.032]** |
| GB-CIL | 6.73e-09, 1.31e-08 | [9.26e-13;1.79e-08] | 0.012 | [-0.011;0.034] |
| GB-DIM | 0.047, 0.176 | [1.18e-12;0.291] | **0.007** | **[-0.006;0.020]** |
| GB-GA | 1.28e-08, 1.03e-08 | [2.21e-13;3.38e-08] | 0.013 | [-0.012;0.038] |
| GB-GB | 1, 1 | [1;1] | 0.682 | [0.653;0.711] |
| GB-MAI | 4.59e-09, 2.31e-09 | [3.24e-13;1.22e-08] | 0.012 | [-0.010;0.033] |
| GB-MAK | 3.20e-09, 1.58e-09 | [1.52e-13;8.43e-09] | 0.010 | [-0.008;0.027] |
| MAI-CIL | 6.78e-09, 2.40e-09 | [4.07e-14;1.77e-08] | 0.075 | [-0.007;0.157] |
| MAI-DIM | 0.008, 1.83e-08 | [3.09e-12;0.051] | 0.024 | [-0.012;0.059] |
| MAI-GA | 1.27e-08, 1.01e-08 | [9.34e-13;3.36e-08] | 0.054 | [-0.023;0.130] |
| MAI-GB | 2.64e-08, 1.23e-08 | [2.92e-12;8.00e-08] | 0.019 | [-0.016;0.055] |
| MAI-MAI | 1, 1 | [1;1] | 0.894 | [0.818;0.970] |
| MAI-MAK | 3.17e-09, 1.87e-09 | [1.81e-13;8.33e-09] | *0.089* | *[0.011;0.166]* |
| MAK-CIL | 6.72e-09, 2.31e-09 | [6.59e-14;1.77e-08] | 0.036 | [-0.024;0.091] |
| MAK-DIM | 0.013, 1.23e-08 | [3.97e-13;0.085] | 0.034 | [-0.006;0.073] |
| MAK-GA | 1.29e-08, 1.22e-08 | [6.76e-13;3.423e-08] | ***0.152*** | ***[0.047;0.257]*** |
| MAK-GB | 2.63e-08, 1.34e-08 | [2.83e-12;7.89e-08] | *0.065* | *[0.004;7.0.126]* |
| MAK-MAI | 4.63e-09, 3.98e-09 | [9.623e-14;1.23e-08] | 0.022 | [-0.016;0.060] |
| MAK-MAK | [9.623e-14;1.23e-08] | [9.623e-14;1.23e-08] | 0.747 | [0.619;0.875] |

## Appendix 6

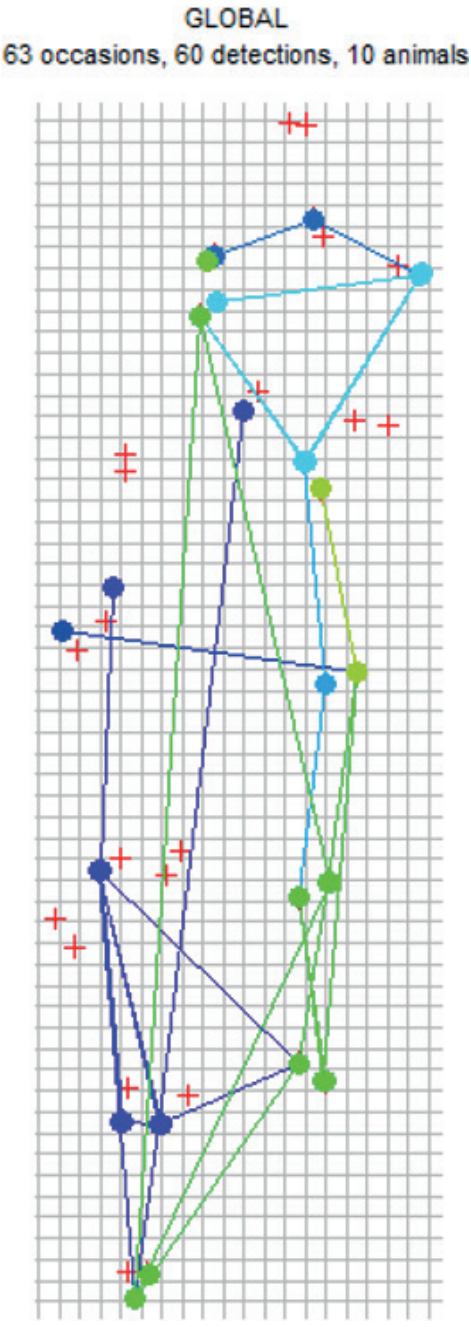


**Figure A3.** Plot of detection histories of feral cat over the detector maps during the study period, in Réunion Island, 2015-2016. Red crosses are for camera-trap locations and each dot represents a capture event (one colour per individual).

## Appendix 7

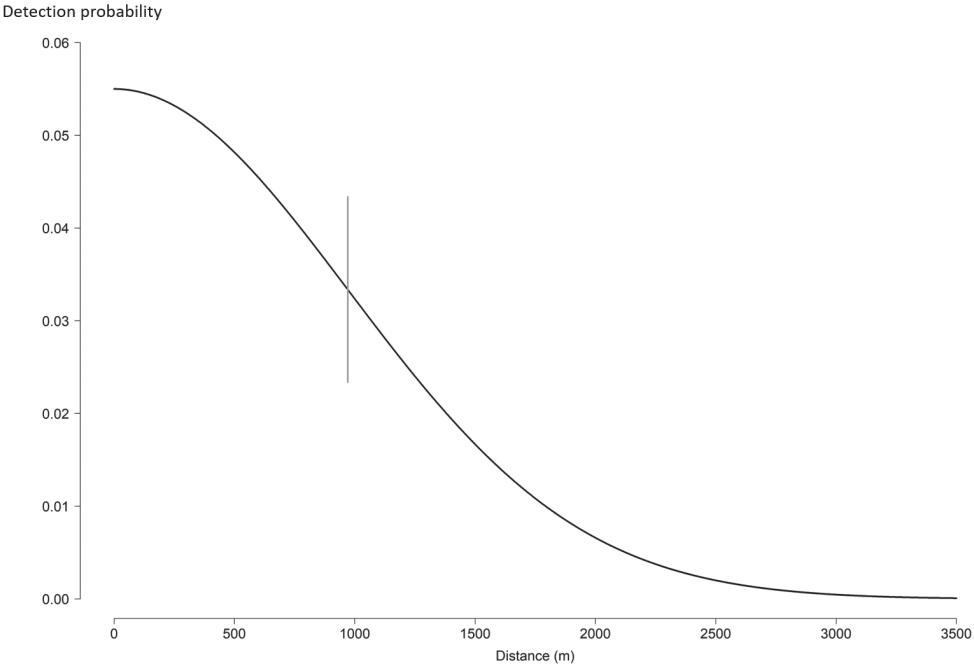


**Figure A4.** Variation of the detection probability with the distance of the home range centre. The dark grey mark is for the value of the spatial scale of the movement parameter for a half-normal detection function.

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# Spreading of the cup plant (*Silphium perfoliatum*) in northern Bavaria (Germany) from bioenergy crops

L. Marie Ende[1], Marianne Lauerer[1]

**1** *Ecological Botanical Gardens, University of Bayreuth, Bayreuth Center for Ecology and Environmental Research (BayCEER), Universitätsstraße 30, 95447 Bayreuth, Germany*

Corresponding author: L. Marie Ende (marie.ende@uni-bayreuth.de)

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## Abstract

Invasive species can be the cause of severe problems for biodiversity, economy and human health. The cup plant (*Silphium perfoliatum*) is native to eastern North America and is increasingly cultivated in Germany as a new bioenergy crop. Its growth characteristics and autecology do not exclude a possible invasive potential. However, there are hardly any studies on this to date. In this study, habitat requirements for spontaneous colonization and establishment of the cup plant were investigated. Therefore, a 15 m radius around eleven cup plant fields in northern Bavaria (Germany) was examined. Data on cup plant colonization, habitat type, vegetation structure, ground cover, and further site conditions using the Ellenberg indicator values were collected and analyzed by logistic regression models. Spontaneously colonized cup plants were found in a wide range of habitats. Open habitats and human settlement areas were particularly suitable, especially field margins and agricultural paths. A portion of open soil of about 25% was preferentially colonized. Cup plants occurred predominantly within the first few meters of the field margin and increasingly around cup plant fields that have existed for a longer period. Favorable for the development of stems and thus for flowering, fruiting and establishing are warmer sites with a high herb layer. Individual plants that had developed a stem persist for several years and increased their stem number over time. The number of stem-developing individuals also increased over time. Thus, there exist an enormous potential for spread in the future. However, an invasive potential could not be confirmed based on the present study, because a threat of biodiversity was not proven.

## Keywords

alien, bioenergy crop, casual occurrences, dispersal distance, distribution, establishment, habitat requirements, invasive potential

## Introduction

An increasing number of plant species are being introduced by humans into regions where they do not occur naturally (Seebens et al. 2017). Some of these plants can settle permanently within these new areas, often at ruderal sites but also in natural and semi-natural habitats (Kowarik and Rabitsch 2010). A few become invasive and cause severe problems for biodiversity, economy and human health (Vitousek et al. 1996; Andersen et al. 2004; Kowarik and Rabitsch 2010). Some of these species were introduced unintentionally, and others intentionally e.g. as crops. The example of the Jerusalem artichoke (*Helianthus tuberosum*) shows that large-scale cultivation of plants without previous investigation of potential invasiveness can be fatal. This species is firmly established in many European countries and is partly responsible for soil erosion and suppression of native vegetation in the floodplains of rivers (Kowarik and Rabitsch 2010). Another introduced crop that has increasingly been cultivated in Germany in recent years is the cup plant (*Silphium perfoliatum*). So far, there are hardly any studies on the possible invasiveness of this species. The cup plant was introduced to Germany in 1762 as an ornamental plant (Brennenstuhl 2010). Since 2004 it has been cultivated as a bioenergy crop (Frölich et al. 2016). Good yields, low effort after establishment and many ecological advantages compared to the predominantly cultivated biogas crop maize (*Zea mays*) are reasons for increasing cultivated cup plant fields (Frölich et al. 2016). In 2021, the cup plant was cultivated on around 10,000 ha of arable land in Germany, nearly tripling the area in one year (FNR 2022).

The cup plant is a perennial, yellow-flowering hemicryptophyte of the Asteraceae family and is native to the prairies of eastern North America (Stanford 1990; Jäger 2017). It grows 2–4 m high and can develop several hundred flower heads, each with 20–30 fruits (Stanford 1990; Dauber et al. 2016; Ende et al. 2021). The flowering period is from July on, fruits ripen from August onwards and then drop out (Jabłoński and Kołtowski 2005; Penskar and Crispin 2010; Wrobel et al. 2013; Jäger 2017). Seeds have a physiological dormancy, which can be broken by low or alternating temperatures, so that germination under Central European climate is possible (Gansberger et al. 2017). The high productivity and high reproductive potential of cup plant could lead to invasiveness, in case of spontaneous spread into sensitive habitats that are relevant for nature conservation. Following EU legislation, a species is being classified as invasive if its spread threatens biodiversity (Article 3 No. 2 EU-Regulation No. 1 143/2014). The Netherlands and Russia already classify the cup plant as potentially invasive (Matthews et al. 2015; Vinogradova et al. 2015). Detections of spontaneous occurrences of cup plants have been made in several European countries and for 15 of Germany's 16 federal states (Roskov et al. 2019; GBIF 2021). However, knowledge of dispersal distance and requirements on soil, vegetation or habitat for spontaneous colonization is low. Therefore, we investigated an area within a 15 meter radius around eleven cup plant fields in northern Bavaria (Germany) and collected data on cup plant occurrence in combination with habitat type, vegetation structure, ground cover, and other site conditions using the Ellenberg indicator values (Ellenberg and Leuschner 2010). We asked the following questions:

What are the crucial site conditions that enable a spontaneous colonization of cup plants and subsequently their establishment? How do establishing (stem-developing) individuals develop over three years? Spontaneous colonization and establishment in habitats that are relevant for nature conservation would be more problematic for biodiversity than, for example, in ruderal sites or roadsides (Nehring et al. 2015). Our study is limited to northern Bavaria over three years. Nevertheless, the results provide important insights into the habitat requirements and development of cup plant that are valid beyond this small region and are essential for assessing the possible invasiveness of cup plant.

## Materials and methods

### Data collection

Data were collected between 19 May and 3 Aug. 2020 in 15 m-radius around eleven cup plant fields in Upper Franconia and Upper Palatinate in Bavaria, Germany (Suppl. material 1). We recorded several parameters in invaded and uninvaded plots (Table 1). Plots had a size of 1 m × 1 m and a distance of ≥ 1 m from the field margin. Invaded plots were chosen by carefully searching the area for spontaneously colonized cup plants. Around detected individuals, we investigated a plot (= invaded plot). As control, we additionally investigated plots without cup plant (= uninvaded plots) randomly selected in the same sites using the tool "Create Random Points" in GIS (ArcGIS Version 10.2.2). The preliminary mapping of habitat types by Ende and Lauerer (2020) served as the basis for selecting uninvaded plots. They mapped habitat types up to the second subgroup according to LfU (2014). For each cup plant field, plots were selected for each presented habitat type. The number of these plots was calculated as the area of the respective habitat type around the respective field divided by 100. The minimum distance between plots (invaded and uninvaded plots alike) was 2 m. On site, we located the selected uninvaded plots using an aerial photograph (LDBV 2020) and a GPS device (eTrex Legend HCx by Garmin). We excluded plots with 100% sealed area or 100% open water area, plots in private, fenced land as well as agricultural fields. In sum, we collected data in 549 plots (224 invaded and 325 uninvaded plots).

### Monitoring of establishing individuals from 2017 to 2020

In 2017, Ende and Lauerer (2020) recorded the cup plants that had developed stems and that are considered as establishing cup plants in the present study in a 20 m radius of 15 cup plant fields in northern Bavaria with a GPS device (eTrex30 by Garmin). In 2020, a 15 m radius of eleven fields was investigated. For the comparison of the numbers of establishing individuals, we extracted the data of the 15 m-radius of the eleven fields that we mapped in 2020 of the whole data of 2017. In 2017, 20 of the establishing individuals were monitored annually from 2017 to 2020 between late July and mid-October. Survival was documented, and stems were counted.

**Table 1.** Parameters recorded in invaded and uninvaded plots and description of the underlying methodology. Abbreviation: EIV = Ellenberg indicator value.

| Parameter | Method |
|---|---|
| Habitat type | According to LfU (2014) until third subgroup |
| Number of spontaneously colonized cup plants | Counted, independent of developmental stage |
| Number of establishing cup plants | Counted, considered were plants that had developed at least one stem |
| Distance to the field | Distance between plot center and field margin, for invaded plots measured on site with a measuring tape, and for uninvaded plots calculated using GIS, accurate to 1 m both |
| Height of the herb layer | Mean maximum plant height (without cup plant), measured with a folding rule, accurate to 5 cm |
| Cover of herb layer (height < 1.5 m) | Visually estimated (without cup plant), accurate to 1% in the sections from 0 to 10% and from 90 to 100%, accurate to 5% in the section from 10 to 90% |
| Cover of shrub layer (height between 1.5 and 5 m) | Visually estimated, accurate to 1% in the sections from 0 to 10% and from 90 to 100%, accurate to 5% in the section from 10 to 90% |
| Cover of tree layer (height > 5 m) | |
| Cover of litter | |
| Portion of open soil | |
| Cover of paved ground | Visually estimated, accurate to 1% in the sections from 0 to 10% and from 90 to 100%, accurate to 5% in the section from 10 to 90%, considered were gravel, pavement, cement and tarmac |
| Dominant species of herb layer | Cover per species was visually estimated, accurate to 1% in the sections from 0 to 10% and from 90 to 100%, accurate to 5% in the section from 10 to 90%, considered were those species which together accounted for 75% of the total cover of herb layer |
| EIV light availability | Mean weighted Ellenberg indicator values, according to the cover of dominant species. Values were taken from Jäger (2017). |
| EIV soil nutrients | |
| EIV soil reaction | |
| EIV soil moisture | |
| EIV temperature | |
| Age of the nearest cup plant field | Survey of farmers |

## Data analysis

Statistical analysis and data visualization were performed with R (R Core Team 2019). We used logistic regression models (generalized linear models with binomial distributed residuals) to analyze the binary data presence and absence of cup plants respectively presence and absence of establishing (stem-developing) cup plants as dependent variables. For the analysis of cup plant establishing, we used only the plots with spontaneously colonized cup plants (invaded plots). First, we individually tested the parameters (explanatory variables) using univariate models. Depending on data structure, we occasionally exerted log or quadratic data transformation. Log transformation was natural logarithm with + 0.1 in case of zero values in data. Models were checked for influential outliers, which were defined as samples with a cook's distances > 4/(sample size) and residuals > 3. No such influential outliers occurred. Furthermore, we tested the correlations between explanatory variables. None of them had a Spearman's rho > 0.7. With all of the explanatory variables that were significant in univariate models, we built global multivariate models. We reduced the global models stepwise by using the Akaike information criterion (AIC) with the "step" function. Plots without herb layer or those where not all EIVs (Ellenberg indicator values) were available would be excluded from the global model (n = 100).

To avoid this, missing EIVs were filled using the mean value of the respective EIV calculated of all plots of the same habitat type (third subgroup) and the same site. If there were no plots of the same habitat type and site with complete EIVs, the respective plots were excluded of analysis (n = 3). We checked collinearity in global models with the variance inflation factor (function "vif" of "car" package by Fox and Weisberg (2019)). Values > 10 were achieved in the global model of cup plant colonization for the parameter habitat type in the subgroup levels. Therefore, we inserted the main groups of habitat types in the global model. Results of subgroups were analyzed descriptively. We calculated *p*-values of the parameters in multivariate models with the Wald-test of "Anova" function of "car" package (Fox and Weisberg 2019). Significant differences between the habitat types in the final model of cup plant colonization were analyzed using the Tukey's post-hoc test ("glht" function of "multcomp" package by Hothorn et al. (2008)). For analyzing the stem number of establishing individuals over time, a linear regression model was aimed for. Because the assumptions, i.e. normality and homoscedasticity of residuals, visually checked, were not satisfied, we executed a correlation analysis.

Significance level was always $p < 0.05$. We used the function "ddply" of "plyr" package (Wickham 2011) for descriptive statistics and the function "visreg" of "visreg" package (Breheny and Burchett 2017) for visualization of logistic regression results. For evaluation of model results, distributions of all explanatory variables are shown in Suppl. material 2.

## Results

### Spontaneous occurrences of cup plant

Spontaneously colonized cup plants were found within 15 m radius of each of the eleven surveyed fields. 224 of the 549 plots (41%) had spontaneous occurrences with 1 to 60 individuals per plot and 2 in median. The probability of spontaneous occurrence of cup plants was significantly affected by habitat type specified as main group (Table 2). Open habitats and human settlement areas showed a significantly higher probability of cup plant occurrence than woody habitats (shrubs, trees and forests) or inland waters, including riparian areas (Fig. 1A). Looking at the subgroups of habitat types, most occurrences of cup plants were in fringes, ruderal areas and perennial herb communities with low or moderate species richness (habitat types K11 and K12, Table 3), which were mainly grass dominated field margins. 63 to 77% of these plots were invaded. The other subgroup of open habitats was grassland, which was less invaded than fringes, ruderal areas and perennial herb communities. Cup plant occurrences were found in intensively used grasslands in use (G11, 20% invaded plots) or lain fallow (G12, 14% invaded plots). Extensively used grasslands (G21) and lawns (G4) had no occurrences of cup plants at all. However, cup plants were also found frequently on unpaved and paved cycle paths, footpaths and agricultural paths (V32, V33). Both habitat types belong to the main

**Table 2.** Results of the final logistic regression model of spontaneous cup plant colonization depending on environmental variables. (Logistic regression, $p < .001$, $n = 546$). Significant parameters are shown in bold. Abbreviation: EIV = Ellenberg indicator value.

| Parameter | | Estimate | SE | *p*-value |
|---|---|---|---|---|
| **Habitat type main group compared to IW (Inland waters, including riparian areas)** | **OH (Open habitats)** | **15.32** | **580.2** | **.002** |
| | **HS (Human settlement areas)** | **15.19** | **580.2** | |
| | **WH (Woody habitats)** | **13.73** | **580.2** | |
| **log (Distance to the field)** | | **- 1.668** | **0.151** | **< .001** |
| EIV soil nutrients | | 1.482 | 0.927 | .110 |
| (EIV soil nutrients)^2 | | - 0.129 | 0.082 | .116 |
| EIV temperature | | 0.521 | 0.315 | .099 |
| **Age of nearest cup plant field** | | **0.329** | **0.076** | **< .001** |
| log (Height of herb layer + 0.1) | | 0.321 | 0.167 | .054 |
| **Portion of open soil** | | **0.069** | **0.028** | **.013** |
| **(Portion of open soil)^2** | | **- 0.001** | **< 0.001** | **.005** |
| Cover of herb layer | | - 0.012 | 0.007 | .067 |

group of human settlement areas and had 49 to 60% invaded plots. Other traffic areas such as paved roads (V12) and green spaces along traffic routes (V51) were not colonized by cup plant. From the main group of woody habitats (shrubs, trees and forests), most habitat types were invaded, however partly in few plots (2 to 23%). Cup plants occurred in tree rows and tree groups (B31, 23% invaded plots), deciduous and coniferous plantations (L71, N71, N72, 2 to 13% invaded plots), pine forests (N11, 20% invaded plots) and woodland mantles (W12, 14% invaded plots), however not in scrubs and hedges (B11) or copses (B21). There were also no occurrences of cup plants in riparian areas of naturally arisen running waters (F14) and ditches (F21).

Apart from habitat type, the spontaneous occurrence of cup plants was also significantly negatively dependent on distance to the field (Table 2). Within the first 3 m distance to the field, the probability of cup plant occurrence decreased nearly by half (Fig. 1B). In mean, spontaneously colonized cup plants had a distance of 2.1 m to the field. The maximum distance was 14 m. Besides, the probability of cup plant occurrence increased significantly with the age of the nearest cup plant field, which ranged from 2 to 11 years (Table 2, Fig. 1C). There was an increase in probability of cup plant occurrence of around 6% per year. The portion of open soil also significantly affected the probability of cup plant occurrence. The cup plant preferred an open soil portion of about 25% (Fig. 1D). More or less open soil resulted in lower probability of cup plant occurrence. More than 50% open soil was mainly found on unpaved, heavily compacted paths, occasionally also under dense scrubs or hedges. EIVs for soil nutrients and temperature as well as height of the herb layer added information to the model, but they were no significant parameters. Ellenberg indicator values (EIV) for soil reaction, light availability and soil moisture, covers of litter, shrub and tree layer, and cover of paved ground had no influence on the probability of cup plant occurrence.

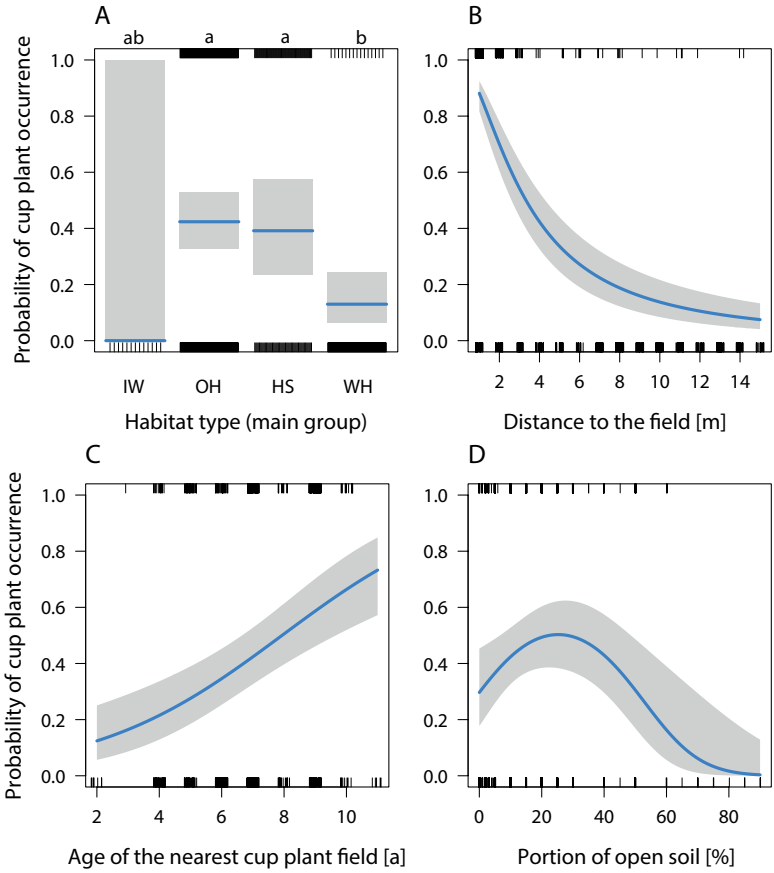


**Figure 1.** Probability of cup plant occurrence depending on the significant parameters of the final model (Table 2) **A** habitat type (main groups). Abbreviations: IW = Inland waters, including riparian areas, OH = Open habitats, HS = Human settlement areas, WH = Woody habitats (shrubs, trees and forests) **B** distance to the field **C** age of the nearest cup plant field **D** portion of open soil. Parameters were fitted by the final model (Logistic regression, Table 2) with all other parameters held constant on median. The fitted probabilities of cup plant occurrence (blue line) and the 95%-confidence interval (grey band) are given. In addition, invaded plots (dashes in the top) and uninvaded plots (dashes in the bottom) are shown with a slight offset in case of multiple plots of one value. ($n$ = 546).

## Establishing of cup plants

Establishing cup plants, by which we mean those that had developed a stem, were also found within 15 m radius of each of the eleven surveyed fields. In 132 of the 224 plots (59%) where cup plant occurred spontaneously, between 1 and 13 individuals were establishing. The median was 1. One of the essential parameters for establishing was EIV temperature (Table 4). The higher the value, i.e. the warmer the site, the higher the probability of establishing (Fig. 2A). It must be mentioned that the values only cover a small range from 5 to 8, while the entire range is from 1 to 9. Distance to the

**Table 3.** Mapped habitat types and their spontaneous colonization by the cup plant. Grouping, naming and abbreviations preceding the habitat types are based on LfU (2014). ($n$ = 549).

| Main group | Subgroup | | | Number of plots (invaded/ uninvaded) | Portion of plots invaded/ with establishing (stem-developing) cup plants [%] |
|---|---|---|---|---|---|
| | First | Second | Third | | |
| Inland waters including their riparian areas | F: Running waters | F1: Naturally arisen | F14: Moderately modified | 3 (0/3) | 0/0 |
| | | F2: Anthropogenic generated | F21: Ditches | 10 (0/10) | 0/0 |
| Open habitats | G: Grasslands | G1: Intensively used | G11: In use | 54 (11/43) | 20/20 |
| | | | G12: Lain fallow | 7 (1/6) | 14/14 |
| | | G2: Extensively used | G21: On moist to moderate dry sites | 17 (0/17) | 0/0 |
| | | G4: Trampled grass and park lawns | | 4 (0/4) | 0/0 |
| | K: Fringes, ruderal areas and perennial herb communities | K1: Of planar to high montane zone | K11: Species-poor | 59 (37/22) | 63/37 |
| | | | K12: Moderate species-rich | 138 (106/32) | 77/42 |
| Human settlement areas | V: Traffic area | V1: Roads | V12: Paved | 2 (0/2) | 0/0 |
| | | V3: Cycle paths, footpaths, agricultural paths | V32: Paved | 57 (34/23) | 60/42 |
| | | | V33: Unpaved | 41 (20/21) | 49/20 |
| | | V5: Green spaces along traffic routes | V51: Of young to medium age | 5 (0/5) | 0/0 |
| Woody habitats (shrubs, trees and forests) | B: Copses, thickets, scrubs, hedges and cultivated woody plants | B1: Scrubs and hedges | B11: Of predominantly native, site-appropriate species | 16 (0/16) | 0/0 |
| | | B2: Copses | B21: Of predominantly native, site-appropriate species | 1 (0/1) | 0/0 |
| | | B3: Tree rows and tree groups | B31: Of predominantly native, site-appropriate species | 26 (6/20) | 23/19 |
| | L: Deciduous (mixed) woodlands and forest plantations | L7: Deciduous (mixed) plantations, not site-appropriate | L71: Of predominantly native species | 8 (1/7) | 13/0 |
| | N: Coniferous (mixed) woodlands and forest plantations | N1: Pine forests | N11: On nutrient-poor, base-deficient sites | 5 (1/4) | 20/0 |
| | | N7: Coniferous plantations | N71: Structure-poor age-cohorts | 34 (4/30) | 12/3 |
| | | | N72: Structure-rich | 48 (1/47) | 2/2 |
| | W: Woodland mantles, pioneer stages of woodland, special forms of woodland use | W1: Woodland mantles | W12: On moist to moderate dry sites | 14 (2/12) | 14/7 |

**Table 4.** Results of the final logistic regression model of cup plant establishing depending on environmental variables. (Logistic regression, $p < .001$, $n$ = 223). Included in the analysis were only the plots with spontaneous cup plant occurrence. Significant parameters are shown in bold. Abbreviation: EIV = Ellenberg Indicator Value.

| Parameter | Estimate | SE | $p$-value |
|---|---|---|---|
| **EIV temperature** | **1.067** | **0.356** | **.003** |
| EIV soil moisture | - 0.430 | 0.270 | .111 |
| **Distance to the field** | **0.207** | **0.087** | **.017** |
| **Height of the herb layer** | **0.020** | **0.007** | **.003** |

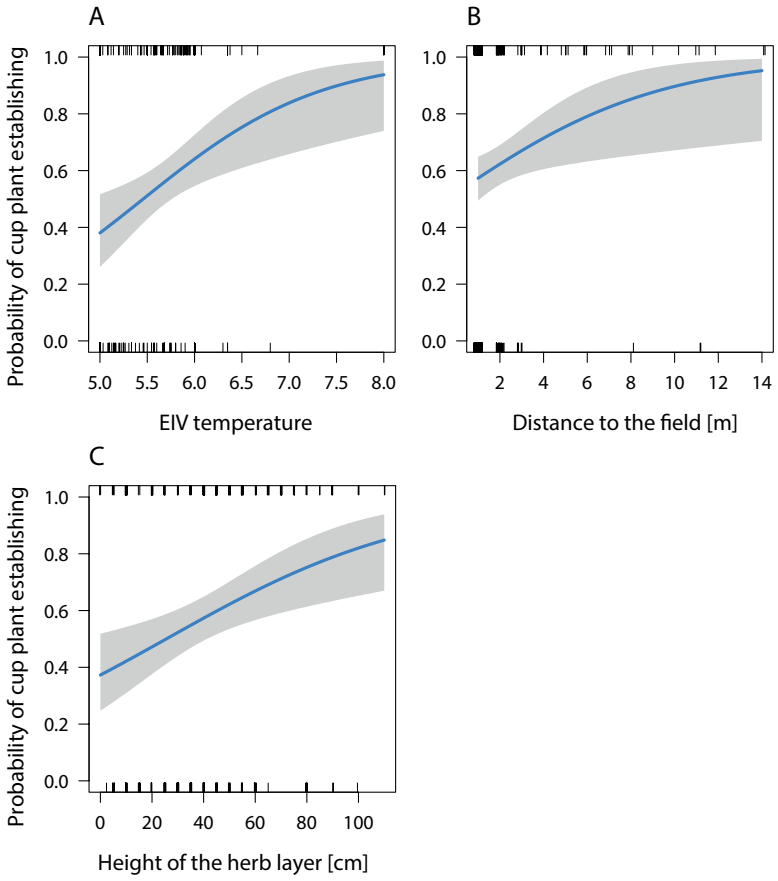


**Figure 2.** Probability of cup plant establishing depending on the significant parameters of the final model (Table 4) **A** EIV temperature. Abbreviation: EIV = Ellenberg indicator value **B** distance to the field **C** height of the herb layer. Parameters were fitted by the final model (Logistic regression, Table 4) with all other parameters held constant on median. The fitted probabilities of cup plant establishing (blue line) and the 95%-confidence interval (grey band) are given. In addition, plots with (dashes in the top) and without (dashes in the bottom) establishing cup plants are shown with a slight offset in case of multiple plots of one value. ($n$ = 223).

field was a significantly affecting parameter once again. However, the probability of establishing increased with increasing distance to the field (Table 4, Fig. 2B). Thus, it is the opposite way than regarding cup plant occurrence in general where the relationship between distance to the field and probability of cup plant occurrence was significantly negative. Establishing cup plants had a mean distance of 2.5 m to the field. Height of the herb layer also showed a significantly positive relationship with the probability of cup plant establishing (Table 4, Fig. 2C). At a very low vegetation height of a few centimeters, cup plants were establishing in about 40% of the invaded plots. At a vegetation height of 1 m, establishing cup plants were found in 80% of the plots. EIV soil moisture was also a parameter of the final model, but it was not significant. All other

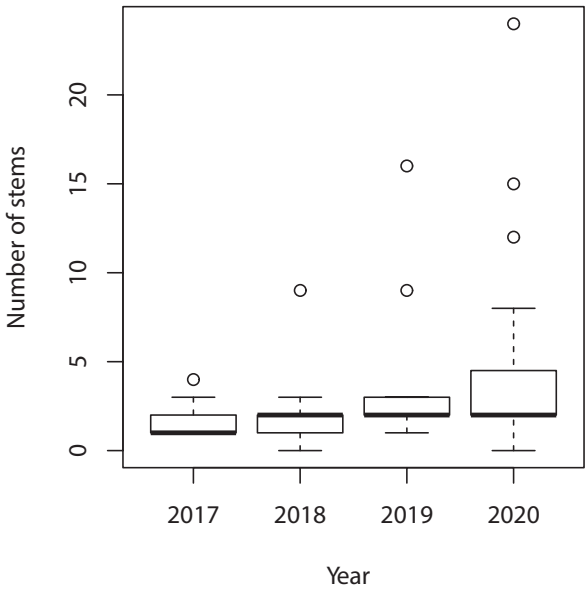


**Figure 3.** Number of stems of the establishing individuals monitored over the years. ($n$ = 20, except in 2018 $n$ = 19).

parameters (EIVs for soil reaction, light availability and soil nutrients, covers of litter, herb, shrub and tree layer, covers of paved ground and of open soil, age of the nearest cup plant field, and habitat type) did not affect the probability of cup plant establishing. Cup plants were establishing in all of the habitat types with cup plant occurrences except deciduous plantations and pine forests (habitat types L71 and N11, Table 3).

### Development of establishing cup plants over time

In 2017, 46 establishing (stem-developing) cup plants were mapped within the 15 m radius of the eleven surveyed fields, whereas in 2020 there were 295 establishing individuals. On average, this corresponds to almost a doubling per year. The 20 establishing individuals of 2017 that were monitored over the years were proven every year until 2020, with one exception: One individual plant was missing in 2018; however, it was found alive in the following years. The number of stems of these 20 individuals increased significantly over the years (Fig. 3, Spearman's rho = .38, $p < .001$).

## Discussion

The present study is the first one which comprehensively investigates habitat requirements of the cup plant for spontaneous colonization and establishment in Germany. Results showed that cup plants were able to invade a wide range of habitats and were found around all of the eleven investigated fields in northern Bavaria.

One of the most crucial parameters for spontaneous cup plant occurrence was the distance to the cup plant field. On average, cup plants were spread at distances of only 2.1 m, and there was a strongly negative relationship between cup plant occurrence and distance to the field (Fig. 1B). Fruits as well as root parts if they contain buds can serve as diaspores (Stanford 1990; Czarapata 2005). Dispersal of root parts would presuppose a cutting or damage of roots which could happen e.g. when ploughing. Because all of the investigated fields were not ploughed since cup plant is cultivated there, we assume that all of the detected spontaneously settled cup plants are developed from seeds. The vectors for the dispersal of cup plant fruits have not been well investigated so far. According to Jäger (2017), fruits are dispersed by shaking of fruiting stems which fits in with the low dispersal distances in the present study. However, single individuals were still found up to 14 m distant to the field, which nearly corresponds to the limit of investigated radius of 15 m. This dispersal distance cannot be achieved by shaking. Hence, there must be another dispersal vector. The fruits of cup plant are equipped with two tiny wings that could enable them to fly (Kowalski and Wierciński 2004) and thus be carried over longer distances. Nevertheless, wind dispersal seems not to play a major role, because most of the cup plants were detected in the immediate vicinity of the fields. Ende and Lauerer (2020) documented a spread of cup plants of up to 700 m along a street and presumed agricultural machines as vector. In fact, losing of crop and thus of fruits from the trailer is quite possible and must be kept in mind regarding the prevention of cup plant spread. A dispersal of the fruits and of root parts via water would also be conceivable and was suspected by Ende et al. (2021) and Vladimirov (2021). Altogether, we suspect a certain dispersal potential of cup plant – even over longer distances.

The probability of spontaneous cup plant occurrence increased almost linearly with increasing field age (Fig. 1C). This is not surprising, because the spontaneously colonized cup plants are perennial and can persist over many years (Stanford 1990). Every year, plants can be added and accumulate to an increasing number with increasing field age. Cup plant fields are usually used for 10 to 15 years, sometimes even longer (Dauber et al. 2016; Frölich et al. 2016; Hartmann and Lunenberg 2016; Bernas et al. 2021). The fields investigated in our study were between 2 and 11 years old. A further increase in spontaneous colonization can be expected in the coming years. Together with the continuously increasing area that is cultivated with the cup plant (FNR 2022), this results in an enormous spreading potential for the future emanating from the fields throughout Germany.

Another important parameter for the spontaneous colonization of cup plants seems to be the portion of open soil. In our study, cup plants occurred preferentially on about 25% open soil (Fig. 1D). It is a matter of speculation why just this value is optimal for colonization. A lower portion of open soil goes along with a higher cover of vegetation, of paved ground, or of litter. However, none of these parameters was significantly decisive for cup plant occurrence. Notwithstanding this, these three factors could still impede germination and/or seedling development. It is known that cup plant seedlings develop slowly and are therefore very sensitive to competing vegetation in the first year (Köhler and Biertümpfel 2016). In the present study, a high portion of open soil was mainly found on unpaved paths and occasionally under dense scrubs

or hedges. Colonization is more difficult on paths due to strong soil compaction and under woody formations due to lower light availability. Stanford (1990) confirms that cup plants need full sun for optimal growth.

All other parameters (besides habitat type), i.e. Ellenberg indicator values (EIV) for soil reaction, light availability and soil moisture, cover of litter, of shrub and of tree layer, as well as the cover of paved ground, did not influence the probability of cup plant occurrence.

In the first year of growth, cup plants develop only a rosette of leaves. From the second year on it can develop stems, flowers, and respectively fruits (Wrobel et al. 2013). These stem-developing individuals were considered as establishing in the present study. Such establishing individuals were also found around each of the eleven surveyed fields. More than half of the plots that were colonized by cup plants also had establishing individuals (59%). A flower development could not be assessed because the cup plant blooms between July and October in Central Europe (Jabłoński and Kołtowski 2005; Wrobel et al. 2013; Jäger 2017) and data were collected between May and early August. However, most of these individuals had only one stem, were not very tall and less vigorous than the cultivated cup plants on the field. We therefore assume that most of these plants could only develop a few flowerheads at most.

In the study area the following habitat types were represented: open habitats, human settlement areas, woody habitats, and inland waters including their riparian areas. However, cup plant did not invade these habitat types equally (Fig. 1A). The habitat type had a significant impact on the probability of cup plant occurrence. Cup plants preferred open habitats such as field margins and human settlement areas especially paths, but also occurred spontaneously in intensively used grasslands. All these habitat types are cut regularly, so that successful fruit development and further spread are unlikely. This is reflected in the significantly positive relationship between the probability of cup plant establishing and the height of the herb layer in the present study (Fig. 2C). Due to the nutrient-rich and species-poor characteristic of the mentioned habitat types their value for biodiversity is quite low. Hence, an invasion of cup plant is initially unproblematic here. Open habitats without management were hardly represented in our study. However, cup plant occurrences in ruderal and fallow areas are frequently detected in Germany (Buhr and Kummer 2009; Brennenstuhl 2010; Nezadal et al. 2011; Schönfelder 2012; Klug 2015; Parolly and Rohwer 2016; Jäger 2017; Kämpfe 2017). There a successful fruit development and further spread is conceivable.

In the present study, the cup plant also invaded woody habitats, although the probability of its occurrence was only about one third as high as in open habitats. The herb layer of woody habitats is usually not managed, which is why reproduction and further spread of cup plant is potentially possible. However, an extensive spread in forests or plantations is not expected, because cup plant needs full sun for optimal growth (Stanford 1990). Additionally, the probability of establishing grew with increasing EIV temperature in the present study (Fig. 2A). The cup plant therefore prefers warmer sites for stem development than it needs for rosette stage. Stanford (1990) confirms that cup plants develop best at around 20 °C. In fact, only three of the cup plants that invaded forests and plantations, have developed stems. In tree rows and tree groups stem development succeeded more often because the required light, warmth and absent management is available there. Less management

could also be the reason for the positive relationship between the probability of cup plant establishing and distance to the field (Fig. 2B). In contrast to the general occurrence of cup plant, its establishing increased with the distance to the field. Usually, the habitats in the immediate field vicinity are intensively mowed and managed, which prevents stem development. Habitats farther away from the fields may be more heterogeneously structured, which is why stem development may succeed more often. In literature, there is little evidence of spontaneous detections of cup plants in woody habitats. Only Reuther and Tillich (1996) detected it in a scrub fringe in Germany. In its native range in eastern North America, it colonizes amongst others in woods, thickets and roadside ditches (Penskar and Crispin 2010). However, cup plants preferentially colonize there near rivers: in open prairie clearings in moist sandy bottomlands, in lakesides, and in ravines (Stanford 1990; Penskar and Crispin 2010; Gansberger et al. 2015). In Germany, too, the cup plant was frequently found in the riparian areas of standing and running water (Oberdorfer 1994; Brandes 2003; Wölfel 2013; Parolly and Rohwer 2016; Jäger 2017; Schönfelder 2017). In the present study, inland waters including their riparian area were not invaded. However, this habitat type was hardly represented in our study. Furthermore, not even a significant relationship between EIV soil moisture and cup plant occurrence or establishing could be proven in the present study. This was possibly because all the investigated sites had an average EIV soil moisture. But the preference of cup plants for moist sites was also confirmed by experimental studies in Germany by Ende et al. (2021), where it showed higher biomass and reproductive potential under moist soil conditions. Therefore, special attention must be paid to moist habitats because they are often valuable for nature conservation and could be colonized by the cup plant (Ende et al. 2021). In our study, other valuable ecosystems were also hardly represented. Only two sites fall into this category: A pine forest on a nutrient-poor, base-deficient site where one single spontaneously colonized cup plant in rosette stage occurred and a species-rich, extensively used grassland where no cup plant occurred. According to literature, no spontaneously colonized cup plants have been detected in valuable ecosystems in Germany so far. However, future colonization cannot be excluded.

Once a spontaneously colonized cup plant has developed stems, the question arises whether and how they develop over time. Our results showed that all of the monitored establishing cup plants survived and new ones were added over the observational period of three years. The number of establishing individuals increased six-fold within these three years. The number of stems per plant increased over this time. Boe et al. (2019) also observed that the cup plant develops more stems with increasing age. All these individuals might develop flowers and eventually reproduce. Therefore, the reproductive potential increases over time both per plant and per population. The factor time is therefore of great importance for assessing the invasive potential of the cup plant.

## Conclusion

The present study demonstrated an enormous spreading potential of the cup plant. Regarding the future, spontaneous occurrences are likely to expand as the number of cup plant fields increases. The cup plant is able to colonize and establish in a wide range

of habitats, especially in less managed open habitats with disturbances. An invasive behavior has not yet been detected. However, there are still some unanswered questions regarding its possible invasiveness. Further studies, especially on dispersal vectors and competitive strength, as well as further documentation of spontaneous occurrences, are necessary to assess the risk of the continuing spread of cup plants and its impact on the native flora and fauna. Until further knowledge is available, we recommend cautious handling of the cup plant. Fields should be located at a safe distance to valuable ecosystems and watercourses to avoid possible dispersal of diaspores via water and an invasion of these ecosystems. Agricultural machines should be cleaned thoroughly after use and covered before leaving the field to prevent dispersal of fruits over long distances.

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## Supplementary material 1

**Characteristics of cup plant fields around which data were collected**
Authors: L. Marie Ende, Marianne Lauerer
Data type: table (PDF file)

Link: https://doi.org/10.3897/neobiota.79.94283.suppl1

## Supplementary material 2

**Distribution of parameters in all plots independent whether invaded or uninvaded**
Authors: L. Marie Ende, Marianne Lauerer
Data type: figure (PDF file)

Link: https://doi.org/10.3897/neobiota.79.94283.suppl2

## Supplementary material 3

**Dataset invaded and uninvaded plots**
Authors: L. Marie Ende, Marianne Lauerer
Data type: table (csv document)

Link: https://doi.org/10.3897/neobiota.79.94283.suppl3

## Supplementary material 4

**Dataset establishing cup plants**

Authors: L. Marie Ende, Marianne Lauerer

Data type: table (csv document)



Link: https://doi.org/10.3897/neobiota.79.94283.suppl4