Native and invasive populations of the ectomycorrhizal death cap *Amanita phalloides* are highly sexual but dispersal limited

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Abstract

1. The ectomycorrhizal death cap *Amanita phalloides* is native to Europe but invasive in North America. To understand whether the fungus spreads underground using hyphae, or above ground using sexual spores, we mapped and genotyped sporocarps from European and American populations. Larger genetic individuals (genets) would suggest spread mediated by vegetative growth, while many small genets would suggest dispersal mediated by spores. To test whether genets are ephemeral or persistent, we also sampled from the same invasive populations over time.

2. We mapped 13 European and American populations between 2004-2007 and characterized each using amplified fragment length polymorphisms (AFLP). In 2014 and 2015, we resampled populations in California and added three new European populations. These populations and a subset of the specimens originally collected in 2004 were characterized using whole genome sequencing.

3. In every population and across all time points, sporocarps resolve into small, apparently short-lived genets. Sporocarps nearer each other are more closely related, suggesting spores land and germinate near parent sporocarps.

4. *A. phalloides* uses spores to move across landscapes. Spores travel very short distances and individuals appear ephemeral. The death cap's life history suggests yearly sporocarp removal as a strategy for control of this deadly fungus.

Introduction

Invasions drive global change (Vitousek *et al.*, 1997; Dukes & Mooney 1999; Ravi *et al*., 2009) but invasion biology often focuses on plants and animals in isolation (Shea & Chesson 2002), ignoring the role mutualists may play in invasion dynamics. Mutualist ectomycorrhizal (ECM) fungi provide resources to plants in exchange for photosynthetically derived carbon (Smith & Read, 2008), and consequently introduced or invasive ECM fungi impact local ecosystems (Litchman 2010). But while the mechanisms enabling the spread of invasive plants, and the roles of ECM in facilitating plant invasions, are increasingly documented (Welk *et al*., 2002; Williams *et al*., 2005; Barker *et al*., 2017), the mechanisms enabling the spread of invasive ECM fungi in association with native plants are scarcely explored (Desprez-Loustau *et al*., 2007, but see Nuñez *et al.*, 2013).

Although invasions by plants are often mediated by symbiotic associations with introduced or invasive ECM fungi (and vice versa; Richardson *et al*., 2000; Hayward *et al*., 2015; Teste *et al.*, 2019), introduced ECM fungi can also invade independently of introduced or invasive plants (Díez *et al*., 2005; Pringle *et al*., 2009; Berch *et al*., 2016; Vargas *et al*., 2019). A fungus invading on its own is likely to have an invasion dynamic distinct from a fungus associating with an invasive plant (Dickie *et al*. 2017). However data tracking ECM invasions are limited, as are data describing potential impacts. Open questions include whether these fungi persist as mutualists, behave as parasites, or displace native ECM species (Chapela *et al*., 2001; Vellinga *et al*., 2009).

The death cap *Amanita phalloides* is an invasive ECM fungus spreading through the endemic coastal live oak woodlands of California (Pringle & Vellinga, 2006, Pringle *et al*., 2009, Wolfe *et al*., 2010, Wolfe & Pringle 2011). Native to Europe, it now grows across the Southern Hemisphere, but its invasion dynamic is best described in North America, and specifically in the United States. The fungus first appeared in California and the Northeast U.S. in the mid-20th century (Pringle *et al*., 2009), and it has since spread through California and north into Washington and British Columbia (Ammirati *et al*., 1977; Pringle *et al*., 2009; Berch *et al*., 2016). In contrast, populations on the East Coast appear confined within initial points of introduction, despite nearly 50 years of monitoring (Tanghe & Simons 1973; Tanghe, 1983; Pringle & Vellinga, 2006).

While the death cap is clearly invading California, how it spreads is unknown. Fungi reproduce and disperse using vegetative fragments or spores (sexual or asexual). At one time spores were assumed to mediate dispersal across continents and oceans (Peay *et al.*, 2010; Golan & Pringle 2017), but recent data suggest the sexual spores of many mushroom forming Basidiomycete fungi fall just next to parent sporocarps (Galante *et al*. 2011; Peay & Bruns 2014). Steady vegetative growth can result in enormous mycelia (e.g., *Armillaria* spp.), but whether fragmentation enables colonization of empty habitats is often unclear (Smith *et al*., 1992; Anderson *et al*., 2018).

Directly observing the dispersal of fungi in nature is challenging. Spores are difficult to track and often, vegetative mycelia are hidden in substrates. But dispersal can be inferred by extending the concept of a genetic individual, or *genet* (*c.f.* Harper, 1977; a term originally developed by plant demographers) to include fungi. In the context of *A. phalloides*, we define a genet as the body (or mycelium) generated by the fusion of two germinating, haploid (or monokaryotic) spores, and assume the diploid body (or the dikaryon) is the dominant phase of the life cycle (Rayner, 1991; Dahlberg & Stenlid, 1994; Anderson & Kohn, 1995; Booth 2014). Vegetative growth would result in large genets, while frequent colonization by sexual spores would result in many small genets (Dahlberg & Stenlid, 1994). Practically, genet size is measured by genotyping sporocarps. Many genetically identical sporocarps scattered across a habitat characterize large genets; distances among genetically identical sporocarps define the size of the genet. Genets of other ECM species range from centimeters to hundreds of meters (Gryta *et al*., 1997; Redecker *et al*., 2001; Sawyer *et al*., 2001; Bergemann *et al*., 2002; Dunham *et al*., 2003; Lian *et al*., 2006; Rubini *et al*., 2011), and like these fungi, *A. phalloides* may propagate using either fragments or spores, or both.

By physically mapping and genotyping sporocarps from multiple populations in European and American forests, and from the same Californian sites over time, we generated data on genet size to ask 1) does the death cap establish across landscapes using vegetative hyphae or sexual spores, 2) can the same individuals persist in a population over time, and 3) does genet size differ between native and invasive ranges?

Materials and Methods

Collections and Mapping

Sporocarps of *A. phalloides* were collected from populations throughout Europe and the United States (Fig. **1**, Table **1,** Supporting Information Table **S1a,b**). We define a population as a group of sporocarps occurring within an area no larger than 75m by 75m. Most populations are kilometers away from each other (Fig. **1**), but at Point Reyes National Seashore (PRNS) in California, individuals are continuously distributed along Limantour Road. Populations from PRNS were delineated arbitrarily by walking across the road or at least 75m away before collecting and naming a distinct population (Wolfe & Pringle, 2011). Global Position System (GPS) coordinates were recorded for each location within 5 m of accuracy (Table **1**). Due to small-scale GPS inacuracies, we are unable to overlay the spatial maps of sporocarps from different years (when we sampled over time at a single site).

The majority of sporocarps were collected from forests dominated by native species of Fagaceae and Pinaceae. Populations from the former Centre d'Ecologie des Système Aquatiques Continentaux (CESAC) surrounded a planted *Cedrus libani* on what is now the Marvig campus of the CNRS Institute and Toulouse III University, and the population from the Escola Superior Agrária de Coimbra was collected from a disturbed site on the Polytechnical Institute of Coimbra campus. In New Jersey, *A. phalloides* grows in planted forests of native *Pinus strobus* (Wolfe *et al*., 2010; Wolfe & Pringle, 2011), and in Rochester, NY, in a municipal park with *Pinus strobus*, *P. resinosa*, *Tsuga canadiensis*, and *Betula* spp. In California, *A. phalloides* were collected from relatively undisturbed coast live oak woodlands in association with *Quercus agrifolia* (Wolfe & Pringle, 2011).

Every sporocarp in a population consisting of more than two sporocarps was carefully mapped using one of three mapping methods depending on the tools available at each site. A full description of each mapping method can be found in Supporting Information Methods **S1**.

DNA extraction and sequencing

Amplified Fragment Length Polymorphism (AFLP) data were generated between 2005 and 2007 from a total of 221 sporocarps, and genome sequence data were generated between 2015 and 2016 from a total of 86 sporocarps (Table **1**). Approximately 50 mg of cap tissue from each sporocarp was placed in a 2.0 ml microcentrifuge tube with four to five 3 mm glass beads, and

macerated using a MiniBeadbeater-8 (BioSpec Products Inc., Bartlesville, OK) set at 75% speed for one minute. For AFLP samples, genomic DNA was extracted from tissue using a Qiagen DNeasy Tissue kit (Qiagen, Hilden, Germany) according to manufacturer's specifications. Any extractions of low quality (as measured on a NanodropTM Spectrophotometer [NanoDrop Technologies, Wilmington, DE]) were re-extracted. Extraction blanks were extracted along with samples and used as a control to test for potential contamination.

For genome samples, 700 µl of CTAB buffer (2% CTAB, 2% PVP, 100 mM Tris-HCL [pH 8.0], 20 mM EDTA [pH 8.0], and 1.4 mM NaCl) was added after maceration, and samples were left to incubate at 60ºC for one hour. Next, 700 µl of a 24:24:1, by volume, of a phenol:chloroform:isoamyl alcohol solution was added to each sample and samples were gently mixed at room temperature for ten minutes, followed by centrifugation at room temperature at 13,000 rpm for ten minutes. The aqueous phase (~650-700 µl) of each sample was then carefully transferred to a new 2.0 ml tube. 700 µl of the phenol:chloroform:isoamyl alcohol solution was again added to each sample, and samples were inverted and mixed at room temperature for ten minutes, followed by centrifugation at room temperature at 13,000 rpm for 10 minutes, at which point the aqueous phase was again transferred to a new 2.0 ml tube. Approximately 1,400 ml of 100% ethanol was added to each sample, and samples were incubated at -20ºC for 45 minutes, and then centrifuged at 4ºC at 13,000 rpm for ten minutes. The supernatant was discarded, and the pellet dried on a ThermoSci DNA SpeedVac at room temperature for ten minutes, or until dry, and finally resuspended in 400 µl of 10 mM Tris-HCL (pH 8.0) and transferred to a new 1.5 ml tube. To further purify genomic DNA, 12 µl of RNase A (Qiagen, Hilden, Germany) was added to each sample, and each sample incubated at 37ºC for one hour. 16 µl of 5 M NaCl and 860 µl of 100% ethanol were then added to each tube and the solution left to precipitate at -20ºC for one hour, after which each tube was centrifuged at 4ºC at 13,000 rpm for 15 minutes and the supernatant discarded. A final washing was performed with 500 µl of 75% ethanol; solutions were centrifuged at 4ºC at 13,000 rpm for ten minutes and the supernatant discarded. Finally, the resulting pellet was resuspended in 200 µl of 10mM Tris-HCl (pH 8.0). 5 ml of an Oxygen AxyPrep Mag PCR Clean-Up kit (Fisher Scientific, Pittsburg, PA, USA) was used per manufacturer instructions to remove any remaining impurities. DNA was stored at -80ºC until it was provided to the University of Wisconsin-Madison Biotechnology Center.

Amplification and visualization of AFLP markers

The AFLP protocol is a DNA fingerprinting technique that does not require prior DNA sequence information as it is based on a selective polymerase chain reaction (PCR) amplification of adaptor-ligated restriction fragments formed from digested genomic DNA (Vos *et al.*, 1995). Mutations at restrictions sites result in the presence or absence of fragments of different sizes and enable individuals to be distinguished from one another. AFLP data were generated for sporocarps collected from 2004-2007, before genome data of *A. phalloides* were available, and at the time AFLP was a genetic fingerprinting method of choice.

We adapted the AFLP protocol of the Applied Biosystems Microbial Fingerprinting Kit (Applied Biosystems [ABI], Foster City, CA, USA) for use with our samples. Typically, ligated fragments are amplified with increasingly selective primers in order to randomly subset the number of fragments to a quantifiable number. But to generate consistent results, we first amplified with no selective primers, and then proceeded to the +1 preselective (one additional base pair on the primer) and +2 (two additional base pairs) selective amplifications. Moreover, because *A. phalloides* has a smaller genome (~45 mb) than most organisms for which the ABI AFLP kit is typically used, we modified the protocol to use less selective primers: we designed +2 selective primers for the *Mse*I restriction site, instead of the +3 typically used on larger genomes. The primer combinations we used were: *Eco*RI-AC/*Mse*I-CT (where AC are the two additional base pairs on the *Eco*RI site and CT are the two base pairs on the *Mse*I site), *Eco*RI-TG/*Mse*I-CC, *Eco*RI-AC/*Mse*I-CT. Amplification products were denatured in formamide and visualized on a 3730 ABI capillary sequencer. Data were analyzed using GeneMapper version 4.0 (ABI). Potential markers were scored using GeneMapper, and then inspected by eye. Clear presence/absence patterns were recorded as 1 or 0, respectively. A total of 102 loci across 221 specimens were recovered and converted to a GenAlEx v6.5 format for downstream analyses (Peakall & Smouse, 2012).

Genome sequencing, read filtering and variant calling

In 2016, genomes were sequenced using an Illumina HiSeq2500 platform at the University of Wisconsin-Madison Biotechnology Center, typically with a 550 bp insert size and 251 bp paired-end reads (13 specimens were prepared with 350-bp inserts; often these specimens represent older collections [Supporting Information Table S**1b**]). Sequencing was performed using two

flow cells with 48 samples and two lanes each (ten samples either failed quality control or were irrelevant to this study). Mean sequencing depth of each sample ranged from 10.56 to 150.86 (Supporting Information Table S**1b**). Sequence data were filtered using Trim Galore! (v0.4.5) (https://github.com/FelixKrueger/TrimGalore). Adapter trimming was set to the highest stringency such that even a single nucleotide of overlap with the adapter sequence was trimmed from a given read. After trimming, reads reduced to 100 bp or shorter, and those with quality scores less than 30 were discarded.

A sporocarp from Dunas de Mira, São Jacinto, Portugal (specimen number 10511) was also sequenced on four SMRTcells on a PacBio RS II Sequel platform, also at the University of Wisconsin-Madison Biotechnology Center (Supporting Information Table **S1b**). This resulted in raw coverage of 47x with N50 read length of 6,310 bp.

After troubleshooting of genome assembly pipelines (summarized in Supporting Information Table **S2**), the final assembly was performed using an in-house hybrid approach. First, Illumina data were subjected to a second round of filtering using Trimmomatic v0.35 (Bolger *et al*., 2014) with the following parameters: ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10 CROP:245 LEADING:30 TRAILING:30 SLIDINGWINDOW:4:25 MINLEN:100. PacBio data were filtered to remove sequences shorter than 500 bp and error corrected with the Illumina data using FMLRC (Wang *et al*., 2018) with default settings. Error-corrected PacBio reads were then used to simulate 20x coverage 3 kb insert size libraries with wgsim (https://github.com/lh3/wgsim) and parameter setting as follows: -e 0.0 -d 3000 -s 500 -1 100 -2 100 -r 0.0 -R 0.0 -S 123 -N 5000000. Illumina data and simulated long-range libraries were then assembled using AllpathsLG v52400 (Gnerre *et al*., 2011), setting HAPLOIDIFY=True. The resulting assemblies were subjected to further scaffolding using error-corrected PacBio data with the software LINKS v.1.8.5 (Warren *et al*., 2015) with -d 2000,5000,10000,15000,20000 -t 20,20,5 and a kmer value of 29. Scaffolds were extended and gap-filled using PBJelly v.15.8.24 and finally polished using Pilon v.1.2 (Walker *et al*., 2014). Polished assemblies were evaluated with QUAST (Gurevich *et al*., 2013) and BUSCO v.2 with the Basidiomycota database v.9 (Simão *et al*., 2015).

After genomes were assembled, single nucleotide polymorphisms (SNPs) and indels were identified using the Genome Analysis Toolkit (GATK) software v3.8-0-ge9d806836 (McKenna *et al*., 2010), following GATK best practices (Broad Institute 2019). Illumina reads from each of the 86 Illumina genome libraries were first mapped to the hybrid assembly using bwa-0.7.9 (Li

2013) with the following parameters: mem -M -t 8 -v 2. Mapping rates for *A. phalloides* specimens ranged from 20.0% - 95.3%, with a median mapping percentage of 86.1% (only older specimens mapped at less than 50%). The mapping rate of 10511's Illumina reads to the 10511 hybrid assembly was 93.8%. Duplicate reads were marked, and the GATK program Haplotypecaller was used to call variants simultaneously on all samples, using MODE=DISCOVERY and type=GVCF. This procedure resulted in 212,119 indels and 1,580,133 SNPs.

To eliminate any variants that may have been called as a result of sequencing errors, the raw VCF file was filtered using VCFtools-0.1.14 (Danecek *et al*., 2011). We took a conservative approach: loci with quality scores greater than 30 (--minQ 30); with sequencing depths greater than the approximate mean depth across all loci and individuals (60X, or --min-meanDP 60); with a minor allele frequency (MAF) greater than 0.006 (1/2N, where N=86 diploid individuals); and with no more than 50% missing data (--max-missing 0.5), were kept in our dataset. To test how different filtering criteria affected the ability to distinguish genotypes, the raw VCF file was subjected to a variety of alternative filtering parameters (Supporting Information Table **S1b**, Fig. **S1**). To remove any remaining erroneously called variants, we removed loci deviating from Hardy-Weinberg equilibrium, calculated per population using an exact test as defined by Wigginton *et al*., (2005) (p-value cut-off 0.01; Supporting Information Table **S1b**). A total of ten VCF files were produced: one encompassing all 86 individuals, and the remaining nine containing a given population with loci deviating from Hardy-Weinberg equilibrium removed. Each VCF file was then converted to a .raw file format using PLINK v1.9 (Purcell *et al*., 2007) for subsequent analyses in R v3.4.4 (R Core Team 2013).

Intrapopulation and interpopulation patterns of polymorphism and divergence

Statistical analyses were performed using R v3.4.4 and all scripts are available at https://github.com/jacobgolan/genets. To calculate the genetic relationships among sporocarps of both the AFLP and SNP datasets, we quantified the number of AFLP or SNP differences for every pair of sporocarps within a given population, and any sporocarp pair with a difference of zero was considered as belonging to the same genet (Fig. **2**, Fig. **3**, Fig. **4**). Missing data are rare (no AFLP data are missing, and only 0.73% of SNP data are missing), but a SNP locus at which two samples differed by missing data was counted as "not different" so as to facilitate

comparisons among pairs of samples: each pairwise comparison using SNP data was scaled to $m(m-x)^{-1}$, where m is the total number of loci and x is the number of missing sites (Kamvar *et al.*, 2014). To understand how many markers are required to fully resolve genetic relationships among AFLP and SNP genotypes, we randomly sampled 100 loci 1,000 times without replacement, and calculated the number of unique genotypes for each random sample (Supporting Information Methods **S2**, Fig. **S1**). Curves of these data were constructed by adapting the *poppr* command from the package *poppr* v2.8.3 (Kamvar *et al.*, 2014).

To understand genetic relationships among sporocarps collected across space and time, we first took an ordination approach. Using *ape* v5.3 (Paradis & Schliep, 2018), we conducted a principle coordinate analysis (PCoA) on pairwise differences of sporocarp AFLP and/or SNP profiles. We plotted individual sporocarps using eigenvalues from the first two principal coordinates. Axes are centered around the mean of eigenvalues per axis and scaled by the standard deviation of eigenvalues per axis. The first two axes capture 27.42% and 16.27% of the variance in the AFLP data, and 16.95% and 9.75% of the variance in the SNP data. Ellipses were drawn to encompass a 0.95 confidence interval for each geographic region for AFLP data (Fig. **5**), and labelled to highlight specific genetic and spatial clusters for SNP data (Fig. **6**).

To test whether closely related sporocarps grow near each other, we performed Mantel tests to test for correlations between genetic and physical distances. Analyses were performed using *vegan* 2.5-5 on each population and then for geographic regions (e.g., California) and subpopulation clusters identified from our PCoA analyses (Supporting Information Table **S3**; Oksanen *et al.*, 2018). To estimate the physical area of each population we first drew a polygon around the perimeter of a population (as defined by sporocarps at the population's edges) and then estimated the smallest four-sided parallelogram that would completely enclose all sporocarps using in-house R scripts and *alphahull* v2.1 (Pateiro-López & Rodríguez-Casal, 2010).

Finally, to infer population differentiation between adjacent locations sampled in the same year, and between the same location sampled over time (e.g., relationships between Drake 2 and Drake 3, both sampled in 2004, 2014, and 2015), we calculated F_{ST} for every pair of populations and subpopulations with *hierfstat* v0.04-22 (Weir & Cockerham, 1984; Goudet, 2005).

Results

AFLP and SNP data

Sequencing resulted in 103 AFLP bands from 221 *A. phalloides* genomes and 1.2 raw Illumina reads (an average of 3.6 M per sporocarp) from 86 *A. phalloides* genomes. The assembled Illumina-PacBio hybrid genome of specimen 10511 (Mira, São Jacinto, Portugal) resulted in a 35.5 Mb assembly covering approximately 77% of the estimated 43 Mb genome. The assembly encompasses 605 scaffolds with an N50 of 320 kb, and gene space completeness is estimated at 94.4%. The 86 full genomes mapped to the 10511 assembly provided 297,133 high quality SNPs. Sporocarps with identical genotypes were found exclusively in the AFLP dataset (Supporting Information Fig **S2a,b**, Fig. **S3**). The two most closely related SNP genotypes were collected from Dunas de Mira, São Jacinto, Portugal (specimens 10511 and 10512), and they differed by approximately 24,250 SNPs (scaling for missing data; Fig. **2**, Supporting Information Table **S1b**).

While the 221 sporocarps of the AFLP dataset resolve into 160 unique genotypes, a plot of number of loci randomly sampled versus number of genotypes (Supporting Information Fig. **S1**) suggests 103 AFLP markers do not fully resolve sporocarp identity (Grünwald *et al*., 2003). SNP data provided greater resolution of genotypes, and both conventional and stringently conservative filtering criteria resolved each sporocarp as a unique genotype (Supporting Information Fig. **S1**). The different datasets result in different interpretations, for example, SNPs delineate each sporocarp from population Drake 3 2004 as a unique individual whereas AFLP markers resolve only two genets from the same sporocarps (Fig. **2**, Fig. **3**).

Most sporocarps of Amanita phalloides *resolve into unique genotypes*

Regardless of genotyping strategy, the dominant pattern emerging across California, the Northeast U.S. and Europe is of populations consisting of multiple genets, most of which are made up of a single sporocarp. Thus, we infer that movement across landscapes is mediated by sexual basidiospores, and not asexual vegetative growth and fragmentation (Dahlberg & Stenlid, 1994). Moreover, populations sampled over time do not transition from being composed of smaller and more numerous genets to being dominated by larger and less numerous genets (Fig. **4**). The temporal succession of genotypes suggests individuals are not long lived and reproduce shortly after establishing.

In fact, the majority of genets in both the AFLP and SNP datasets consist of a single sporocarp (Table **1**). Genets encompassing more than one sporocarp were found only within the AFLP dataset. The 221 sporocarps genotyped by AFLP resolve into 160 unique genotypes, 72.40% represent unique genotypes and resolve into small genets consisting of either a single sporocarp, or two to three mushrooms (Fig. **3**, Supporting Information Fig. **S2a,b,** Fig. **S3**). The longest distance between two identical sporocarps was calculated from Jake's Landing (7.51 m), the longest in California was found in Heart's Desire 2 (5.14 m), and the longest in Europe was found in Serbia (3.20 m). The median length of genets consisting of more than one sporocarp is 1.70m (1.12m in European populations, 2.20m in East Coast populations, 1.73m in Californian populations; Table **1**). However, we hypothesize any approach with greater resolving power would distinguish many of these sporocarps as distinct genets.

Only one genet was found in more than one year: an AFLP genotype found at CESAC in 2002 was also found at CESAC in 2006 (specimens CESAC 54 and CESAC 21, separated by 0.81 meters; Fig. **4**). But once again, we hypothesize any approach with greater resolving power would distinguish these two sporocarps. In Californian populations, genotypes did not persist from year to year and genets were consistently small regardless of genotyping method.

Spores of Amanita phalloides *are dispersal limited between and within populations*

At continental scales, genotypes cluster according to geography (Fig. **5**, Fig. **6**). However, a subset of the genotypes from Europe, the East Coast, and California appear to be closely related. In particular, AFLP genotypes from the East Coast appear closely related to European genotypes from southern France, and to a lesser extent, a small subset of AFLP genotypes from California (especially from Heart's Desire populations) appear closely related to genotypes from both southern France and the East Coast. There is less overlap among SNP genotypes identified from Europe and California; SNP genotypes from Europe were collected mainly from central Portugal, with a few collections also taken from throughout northern and eastern Europe, and from Sardinia and Corsica, but notably not from southern France. But whether populations of *A. phalloides* in North America were in fact introduced from southern France is an hypothesis remaining to be tested.

Surprisingly, closely related genotypes also cluster at local spatial scales. Populations appear genetically distinct and sporocarps collected from the same physical location group together and

apart from other populations. Moreover, sporocarps collected a few centimeters apart from each other within a single population are often genetically more similar than sporocarps collected meters apart (Fig. **5**, Fig. **6**). Within Drake 2 there is a clear subpopulation structure and two distinct groups of genetically related sporocarps are apparent.

To delineate spatial autocorrelations more fully, we used Mantel tests to probe for relationships between the physical and genetic distances separating sporocarps, asking whether mushrooms found nearer each other are also more closely related. Correlations were significant for seven of the 15 populations from which AFLP data were collected, and for two of the eight populations from which SNP data were collected (Supporting Information Fig. **S4a,b**, Fig. **S5**; Table **S3**). Mantel tests corroborate fine scale isolation by distance as a feature of multiple populations (Fig. **7**, Supporting Information Fig. **S4a**,**b**, Fig. **S5**). Moreover, when the data of nearby populations were combined, for example, combining all of the data available from Point Reyes National Seashore (Drake populations), Tomales Bay State Park (Heart's Desire populations), or New York (Rochester populations), correlations were also often significant (Supporting Information Table **S3**). The physical area of a population did not influence whether or not there was a significant Mantel correlation (linear regression of area versus the calculated Mantel statistic: slope near zero and P >0.05 for both within and between populations).

Even across years, closely related genotypes often cluster in space (Fig. 6). For example, individuals of Drake 2 collected in 2004, 2014 and 2015 cluster together and away from individuals of Drake 3 collected in the same years. Subpopulations within single populations persist through time, for example, the subpopulations identified from Drake 2 in 2014 were found again in 2015 (Fig. 6). The data suggest spores fall and germinate within centimeters of their parent sporocarps; even after a decade, Drake 2 and Drake 3 (which are less than 100 m apart) remain genetically distinct.

Calculations of F_{ST} (Weir & Cockerham, 1984) confirm populations sustain a relative degree of genetic differentiation across years (Hartl & Clark 2007; Branco *et al.*, 2015; 2017). F_{ST} calculated using the 2004, 2014, and 2015 data of Drake 2 ranges from 0.0054–0.0138, and for Drake 3 ranges from 0.0003–0.0270. The F_{ST} statistic comparing the adjacent populations, Drake 2 and Drake 3, in 2004 is 0.0376 (0.021 with AFLP data), in 2014 is 0.0523, and in 2015 is 0.0398. F_{ST} comparing Drake 3 to either Drake 2 subpopulation (Cluster 1 or 2) reveals

comparable values to those comparing Drake 2 Cluster 1 to Cluster 2 (0.0729±0.0487 vs. 0.0763±0.0040, respectively).

Discussion

The death cap uses sexual basidiospores to spread in both its native European range, as well as in its introduced ranges on the East Coast and California. Vegetative fragmentation appears rare. Populations consist of many small genets and typically each sporocarp is its own genet. But correlations between geographic and genetic distances, even at small spatial scales (tens of meters), suggest spores travel very short distances, falling near to parent sporocarps. In fact, ordination analyses cluster populations in space, even across time: populations from a single location sampled years apart cluster together and away from populations sampled in the same year from nearby locations. Genets appear to be ephemeral, and except for a single pair of sporocarps within the native range of *A. phalloides* (generated using AFLP data and potentially an artifact), the same genotype was never recovered in more than one year (Fig. **4**). Data suggest frequent sexual reproduction and a high turnover of genets within populations, suggesting short lifespans.

Small body sizes and ephemeral genets are typical of other ECM Basidiomycetes in native ranges, paralleling our findings in an invasive system. The majority of genets of *Hebeloma cylindrosporum*, *Laccaria amethystina*, *Tricholoma terreum*, and *Russula cremoricolor* are small and either consist of single sporocarps or are less than a square meter in size (Gryta *et al*., 1997; Gherbi *et al*., 1999; Redecker *et al*., 2001; Huai *et al*., 2003). A high turnover of genets within populations appears typical of *R. cremoricolor* and *T. scalpturatum* (Redecker *et al*., 2001; Carriconde *et al*., 2008).

Genet sizes across the genus *Amanita* appear variable. The species *A. franchetii* reaches a maximum size of 4.7m, but populations house many singleton genets as well; populations of *A. manginiana* are entirely composed of singleton genets, with no genotype recovered between consecutive years (Sawyer *et al*., 2003; Liang *et al*., 2005). By contrast, *A. conicoverrucosa*, *A. punctata, A. pyramidifera*, and *A. muscaria* appear to form genets made up of multiple sporocarps and may reach tens of meters in size (Sawyer *et al*., 2001; 2003). However fingerprinting techniques with low resolution may bias inferences by binning unique genetic individuals together, as we discovered by comparing our AFLP and SNP datasets.

The example of *A. muscaria* is a particularly interesting comparison; like *A. phalloides*, it has been introduced and is invasive outside of its native range (Richardson *et al.*, 2000; Dickie & Johnston, 2008; Dunk *et al*., 2012). The fungus now grows in Australia and New Zealand and is invasive in Colombia (Sawyer *et al*., 2001; Bagley & Orlovitch 2004; Orlovitch & Cairney, 2004; Vargas *et al.*, 2019). Colombian populations of *A. muscaria* associate with native *Q. humboldtii* and are spreading through Colombian oak forests but Australian populations appear confined to commercial plantations of introduced *Pinus radiata* (Sawyer *et al*., 2001). In contrast to the death cap, available data suggest *A. muscaria* is found primarily as large genets in Australia and New Zealand, apparently capable of steady vegetative growth below ground (Sawyer *et al*., 2001). Whether large genets would be detected using modern fingerprinting techniques or at sites outside of Australia and New Zealand remains to be tested, but if *A. muscaria* does grow as large genets, data would suggest frequent sexual reproduction and copious spore production are not required to facilitate invasions by ECM fungi.

Our nascent understanding of the mode and tempo of the death cap's invasion dynamics is likely to facilitate an understanding of the species elsewhere, and of the genus *Amanita* as a source of invasive ECM species. *A. phalloides* has also been introduced to South America (Singer, 1953; Takacs, 1961), East and South Africa (Walleyn & Rammeloo, 1994), Australia (Talbot, 1976; Shepherd & Totterdell, 1988; Wood, 1997) and New Zealand (Taylor, 1982; Ridley, 1991). Other species in the genus, including *A. rubsescens*, *A. thiersii*, and *A. inopinata*, are introduced or invasive elsewhere (Bougher, 1996; Pegler & Shah-Smith, 1997; Sawyer *et al*., 2003; Wolfe *et al.*, 2012). The genus *Amanita* emerges as a developing model for work with invasive ECM fungi, offering the potential for comparisons among closely related species invasive in geographically distant ranges.

The life history of *A. phalloides* appears similar to the life history of many herbaceous weeds (Baker, 1965; Grime, 1977; Roché & Thill, 2001). Basidiospores may give rise to relatively small mycelia that persist for short periods of time before reproducing sexually. Life history evolution among fungi is poorly described (a stark contrast to traditions within the plant literature; Harper 1977; Grime 1977) but *A. phalloides* emerges in stark contrast to the "humongous fungus," epitomized by the pathogens *Armillaria gallica* (Anderson *et al*., 2018) and *A. ostoyae* (Shaw & Roth, 1976; Ferguson *et al*., 2003). The death cap appears to persist in habitats as a small bodied, ephemeral, potentially ruderal species.

494 However, dispersal is clearly not the only control on *A. phalloides* invasion dynamics. While
495 *A. phalloides* uses spores to establish in both California and on the East Coast, in California *A.*
496 *phalloides* is invasive while in New Jersey *A. phalloides* appears confined within planted forests
497 of *Pinus strobus* (Thompson *et al*., 2000; Wolfe & Pringle, 2011). In California, *A. phalloides* is
498 spreading in association with a native oak, a distant relative of the oaks in its native range (Hipp
499 *et al*., 2018). In New Jersey, *A. phalloides* grows at sites slightly outside of the southern range of
500 *P. strobus*. The associations of the death cap with different hosts, within and outside of hosts'
501 ranges, may be a key influence on its spread, but any potential mechanism mediating the
502 dynamic remains unknown (Richardson *et al.*, 2000; Dickie *et al.*, 2017).
503 The paradox of a dispersal limited invasive fungus suggests *A. phalloides* will move slowly
504 as it continues to spread through its habitats in California. An earlier estimate of approximately
505 five km yr^{-1} is almost certainly wrong (Pringle *et al.*, 2009). Although, establishment from spores
506 does explain the death cap's dominance in local habitats given the magnitude of its spore
507 production: reproduction measured for multiple sporocarps during a 48 hour incubation period
508 ranged from 2.22×10^7 to 1.58×10^8 spores per sporocarp (mean: $8.66 \times 10^7 \pm 1.90 \times 10^7$; Wolfe
509 & Pringle unpublished). At the Drake sites of Point Reyes National Seashore (labeled "Drake's
510 Landing" in Wolfe *et al*., 2010) sporocarps of *A. phalloides* average more than half (and up to
511 85%) of the total biomass of ectomycorrhizal sporocarps collected in a single season (Wolfe *et*
512 *al.*, 2010). As *A. phalloides* spores germinate and mycelia quickly reproduce to generate more
513 spores, a local feedback likely enables populations to grow rapidly.
514 The potentially ruderal niche of the death cap also suggests an effective management strategy
515 (Dickie *et al*., 2016). The mushrooms of the death cap are deadly, and each year in California
516 there are poisonings (Zevin *et al*., 1997; Bonacini *et al*., 2017; Vo *et al*., 2017). An increasingly
517 public discussion has focused attention on whether the fungus can be eradicated from local
518 landscapes (Dickie *et al*., 2016; Quirós 2016; Childs 2019). In fact, our data suggest a control
519 strategy: as a sporocarp of the death cap develops, its spore-bearing surface is covered by a
520 partial veil. As the sporocarp matures, the veil ruptures to release spores. Collecting and
521 destroying immature sporocarps before veil rupture would prevent spore release and the
522 subsequent establishment of mycelia. If spores are also short lived (an untested hypothesis),
523 mycelia would be unable to germinate from a spore bank. Because spores can't travel very far,

thoroughly collecting at a site for just a few years would potentially break the life cycle of the fungus and rid a habitat of an invasive and deadly poisonous fungus.

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788

789 **Author contributions**

790 JG, CAA, HE, BW, FR, and AP conceived and designed the experiments; JG, CAA, HE, MG,
791 SIG, SCG, FR, BW and AP collected and processed samples; HC generated AFLP data, HE,
792 YWW and JH assembled genomes and generated SNP data. JG wrote a first version of the
793 manuscript in collaboration with AP, and all authors contributed substantially to the last version
794 of the manuscript.

795

796 **Figure Legends**

797 **Fig. 1:** Map of collections. Circles are proportional to the number of sporocarps sampled.
798 Collections used to generate AFLP data are labelled in purple, and collections used to generate
799 SNP data are labelled in orange. If locations were used to generate both AFLP and SNP data,
800 labels are both purple and orange. Singleton collections are marked with an asterisk. Collections
801 are numbered as a guide to the more detailed information found in Table 1.

802

803 **Fig. 2:** Numbers of AFLP markers (top) or SNPs (bottom) differentiating pairs of sporocarps
804 within a given population. Genetically identical sporocarps are marked with an arrow. To
805 accommodate missing data, SNP differences are scaled up proportionally to m $(m\text{-}x)^{-1}$, where m
806 is the total number of loci and x is the number of missing sites.

807

808 **Fig. 3:** Sporocarp maps and the genets identified in eight populations (data of all populations
809 found in Fig. **S2a,b** and Fig. **S3**). Sporocarps genotyped using AFLP data plotted as squares,
810 sporocarps genotyped using SNP data plotted as circles, and sporocarps genotyped with both
811 plotted as diamonds. Within each map, sporocarps belonging to the same genet are labelled using
812 a single color and surrounded by an arbitrarily shaped, transparent polygon of the same color.
813 Most genets are made up of a single sporocarp, regardless of technology. A dotted green polygon
814 groups sporocarps of Drake 3 2004 identified as a single genet using AFLP data but resolved as
815 distinct genotypes using SNP data. Note that the sporocarp at the top left was not included in the
816 SNP dataset and whether it would resolve into its own genet using SNP data is unknown.

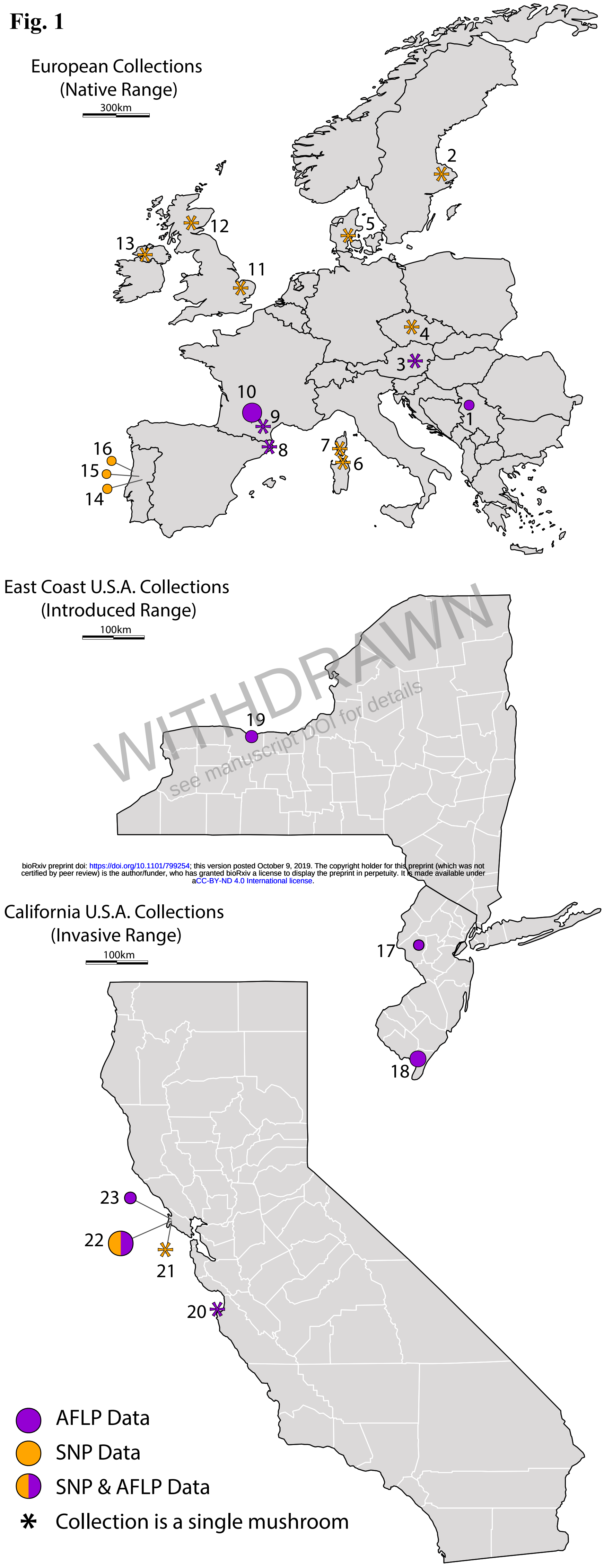
817

818 **Fig. 4:** Sporocarp maps and genets identified from two sites sampled over time: CESAC
819 (France) sampled in 2002 and 2006; and Drake 2 (California) sampled in 2004, 2014 and 2015.
820 Sporocarps genotyped using AFLP data plotted as squares, sporocarps genotyped using SNP data
821 plotted as circles, and sporocarps genotyped with both plotted as rhombuses. Within each map,
822 sporocarps belonging to the same genet are labelled using a single color and surrounded by an
823 arbitrarily shaped, transparent polygon of the same color. Asterisks mark a single AFLP genotype
824 from 2002 found again in 2006.
825
826 **Fig. 5:** Principle coordinate analysis (PCoA) of genetic relationships among sporocarps
827 genotyped using AFLP markers reveals genotypes cluster geographically, with three clusters
828 associated with populations from Europe, the East Coast, and California. Data are color coded by
829 population. Ellipses enclose genotypes from each geographic region within a 0.95 confidence
830 interval.
831
832 **Fig. 6**: Closely related genotypes cluster in space, even through time: for example, a
833 genotype collected from Drake 2 Cluster 1 in 2014 is more closely related to other sporocarps
834 from Cluster 1 collected in 2004, 2014, or 2015, and not to other sporocarps of Drake 2 Cluster 2
835 collected in 2014. a). principle coordinate analysis (PCoA) of genetic relationships among
836 sporocarps genotyped using genome-wide SNPs. b-c) Polygons surrounding clusters of closely
837 related genotypes from Drake 2 2014 and Drake 2 2015 in plot (a) translate to distinct clusters in
838 the physical landscape.
839
840 **Fig. 7:** Mantel correlations between genetic and physical distances for three geographically
841 distinct populations genotyped with AFLP (left), and three populations sampled from the same
842 location in California over time and genotyped with SNPs (right). Grey shading marks a 95%
843 confidence interval around fitted linear models. Plots list the Mantel statistic r using Pearson's
844 correlation method, and asterisks mark significant Mantel correlations (* = $p < 0.05$, ** = $p <$
845 0.005). Data for all populations provided in Fig. S**4a**,**b**, and Fig. **S5**.
846
847

Location	Point on Map (Fig. 1)	Pop. Name	Latitude	Longitude	Collection Date	Technology	No. Mushrooms	No. Individual Genets
Serbia, Obrež, Obedska Bara Nature Reserve	1	Serbia	44.745700	20.010400	2007 October 6	AFLP	14	7
Sweden, Uppland, Palsundet	2	[singleton]	59.8586	17.6389	1978 September 14	SNP	1	1
Austria	3	[singleton]	N/A	N/A	N/A N/A	AFLP	1	1
Czech Republic, Český Šternberk Na Stříbrné Nature Reserve	4	[singleton]	49.8109	14.9282	2006 September 29	SNP	1	1
Denmark, Farum, Nørreskov	5	[singleton]	55.916667	9.533333	2003 September 17	SNP	1	1
Italy, Catala, Calangianus	6	[singleton]	40.9211	9.193	2006 November 13	SNP	1	1
France, Corsica, Fango	7	[singleton]	42.0396	9.0129	n/a November 5	SNP	1	1
Spain, Girona	8	[singleton]	41.9794	2.8214	2006 December 2	AFLP	1	1
France, Arfons	9	[singleton]	43.430054	2.168282	2007 October 7	AFLP	1	1
France, Toulouse, CNRS	10	CESAC	43.578554	1.463026	2002 November 25	AFLP	25	24
France, Toulouse, CNRS	10	CESAC	43.578554	1.463026	2006 November 10-19	AFLP	37	28
England, West Sussex, Mildenhall Woods	11	[singleton]	52.3614	0.4866	2000 October 15	SNP	1	1
Scotland, Pitlochry, Black Sprout Wood	12	[singleton]	56.7044	-3.7297	1991 September 13	SNP	1	1
Northern Ireland, Fermanagh, Inishmakill	13	[singleton]	54.4795	-7.7315	2000 October 14	SNP	1	1
Portugal, Lousã, Vilarinho	14	Vilarinho	40.122204	-8.209709	2015 November 17	SNP	5	5
Portugal, Coimbra, Escola Superior Agrária de Coimbra	15	Agraria	40.2125	-8.450278	2015 November 16	SNP	2	2
Portugal, São Jacinto, Dunas de Mira	16	Mira	40.4575	-8.768611	2015 November 18	SNP	4	4
USA, NJ, Lebanon, Round Valley Resevoir	17	Round Valley	40.6179	-74.8474	2006 October 7	AFLP	11	7
USA, NJ, Dennis, Jake's Landing Rd	18	Jake's Landing	39.189940	-74.853539	2006 November 21	AFLP	44	27
USA, NY, Rochester, Durand Eastman Park	19	Rochester 1	43.233019	-77.554686	2007 September N/A	AFLP	13	12
USA, NY, Rochester, Durand Eastman Park	19	Rochester 2	43.232747	-77.554917	2007 September N/A	AFLP	7	5
USA, NY, Rochester, Durand Eastman Park	19	Rochester 3	43.232896	-77.554851	2007 September N/A	AFLP	2	2
USA, CA, Monterey	20	[singleton]	36.600238	-121.894676	2006 December 14	AFLP	1	1
USA, CA, Point Reyes National Seashore	21	[singleton]	38.0525	-122.852778	1993 October 5	SNP	1	1
USA, CA, Point Reyes National Seashore, Limantour Rd	22	Drake 1	38.0545	-122.83343	2004 November 16	AFLP	19	12
USA, CA, Point Reyes National Seashore, Limantour Rd	22	Drake 2	38.054785	-122.833232	2004 November 16-17	AFLP SNP	13 [AFLP] / 13 [SNP]	13 [AFLP] / 13 [SNP]
USA, CA, Point Reyes National Seashore, Limantour Rd	22	Drake 2	38.054785	-122.833232	2014 November 17	SNP	25	25
USA, CA, Point Reyes National Seashore, Limantour Rd	22	Drake 2	38.05471	-122.83328	2015 December 14	SNP	11	11
USA, CA, Point Reyes National Seashore, Limantour Rd	22	Drake 3	38.055212	-122.834134	2004 November 16-17	AFLP SNP	8 [AFLP] / 5 [SNP]	2 [AFLP] / 5 [SNP]
USA, CA, Point Reyes National Seashore, Limantour Rd	22	Drake 3	38.055212	-122.834134	2014 November 17	SNP	9	9
USA, CA, Point Reyes National Seashore, Limantour Rd	22	Drake 3	38.05514	-122.83418	2015 December 14	SNP	3	3
USA, CA, Point Reyes National Seashore, Limantour Rd	22	Drake 4	38.054675	-122.836545	2004 November 16	AFLP	6	5
USA, CA, Point Reyes National Seashore, Johnstone Trail	23	Heart's Desire 1	38.131216	-122.88885	2004 November 18	AFLP	7	7
USA, CA, Point Reyes National Seashore, Johnstone Trail	23	Heart's Desire 2	38.12871	-122.88869	2004 November 18	AFLP	7	4
USA, CA, Point Reyes National Seashore, Johnstone Trail	23	Heart's Desire 3	38.12513	-122.88891	2004 November 18	AFLP	4	2

Table 1

Fig. 1



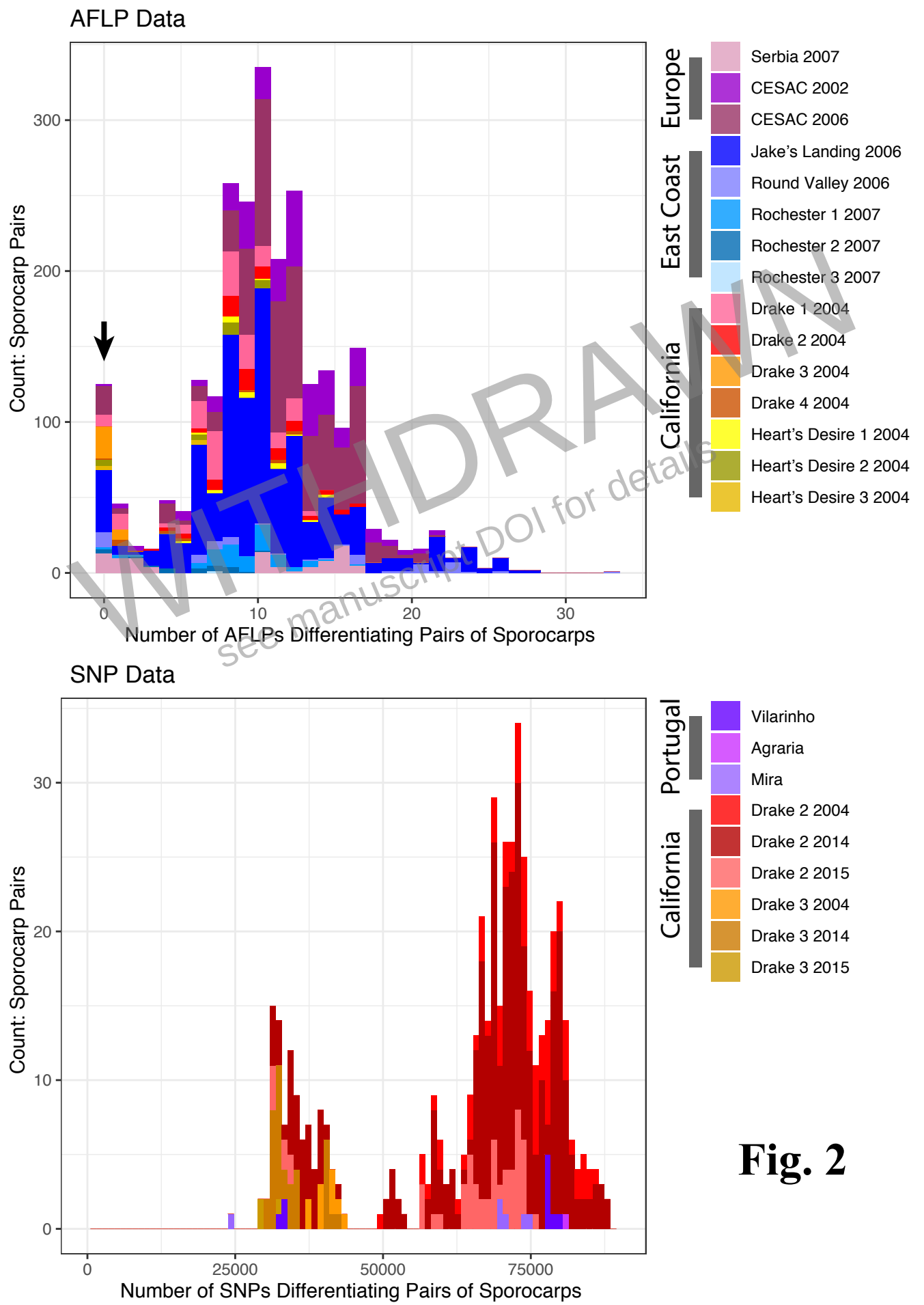


Fig. 2

Fig. 3

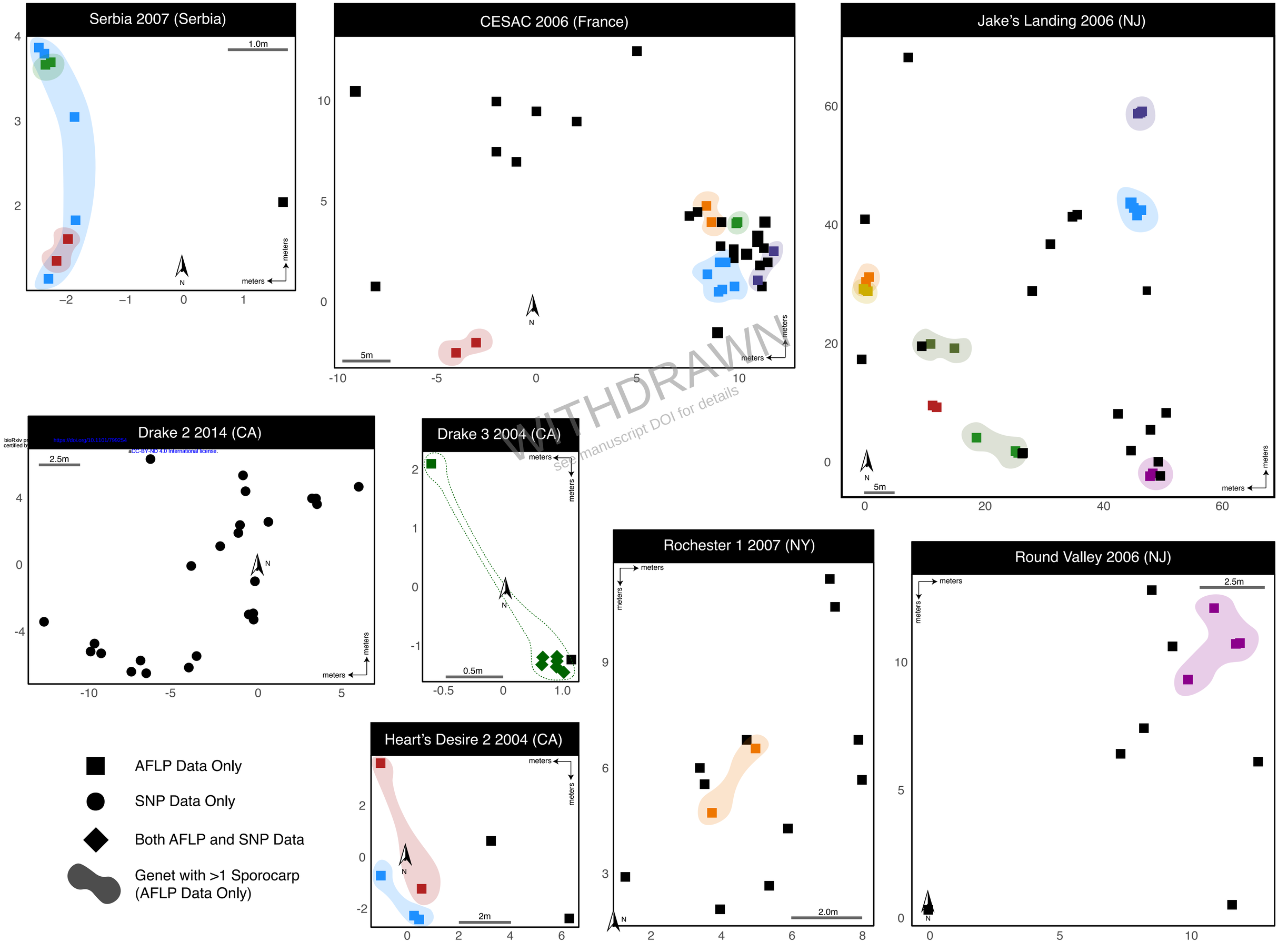
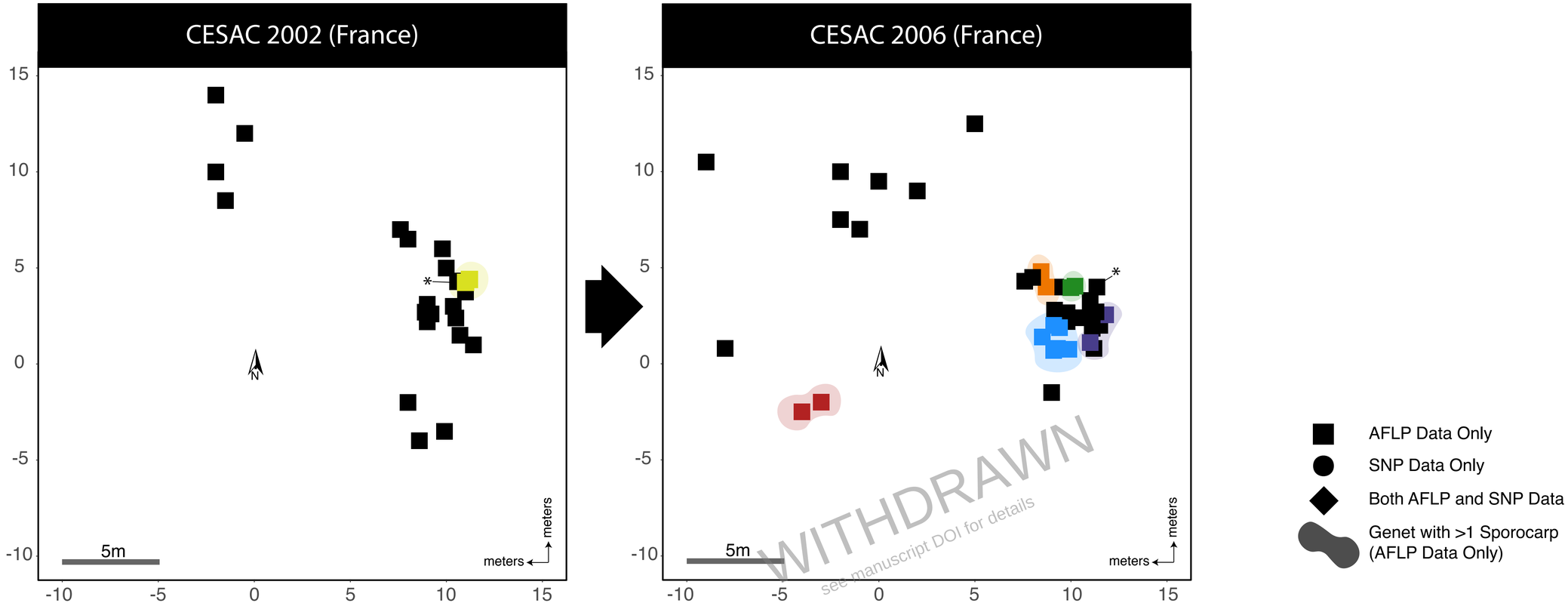
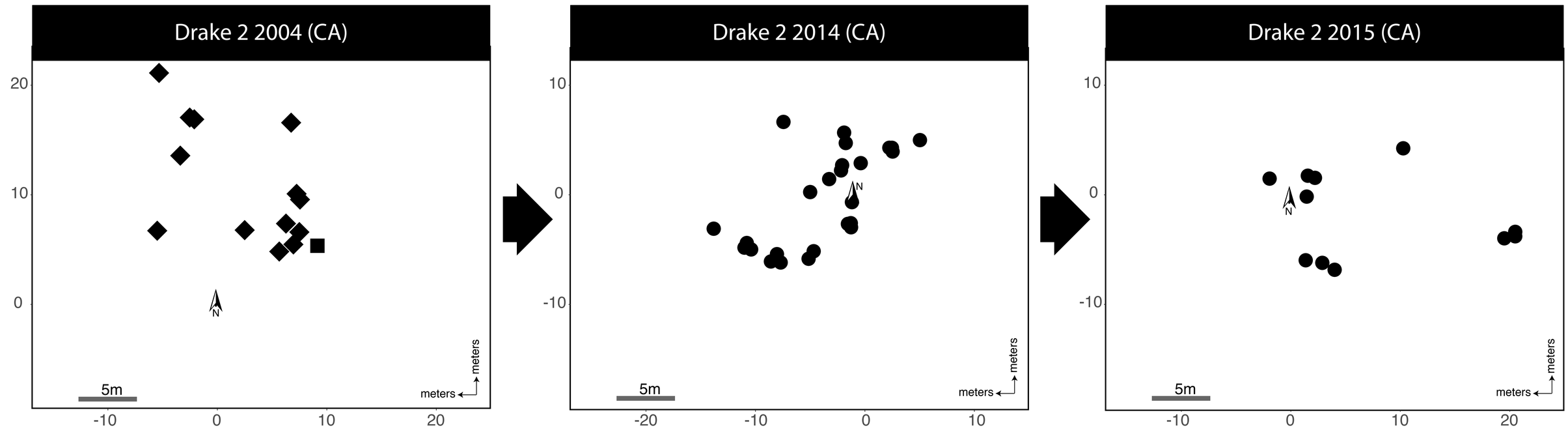


Fig. 4





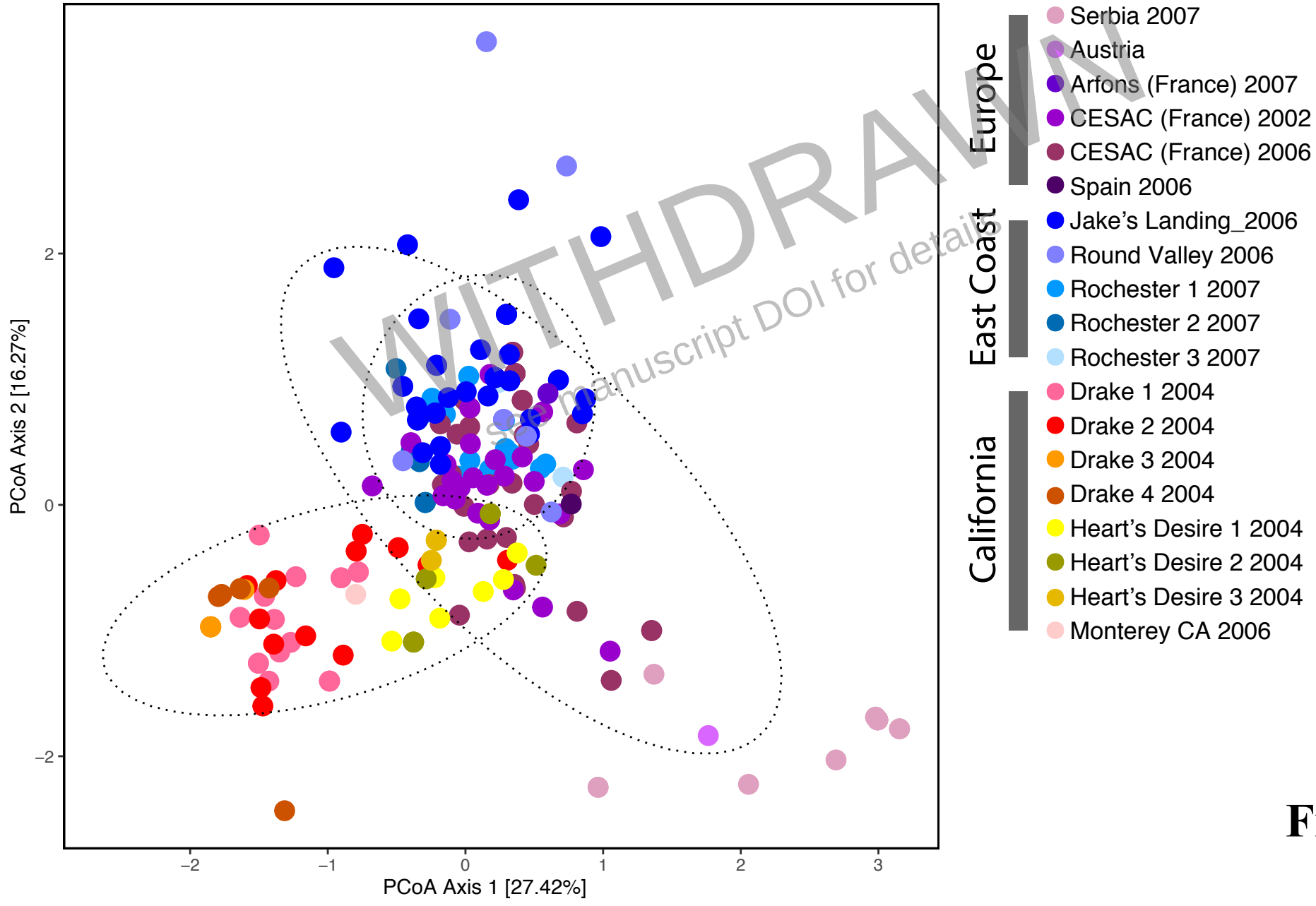
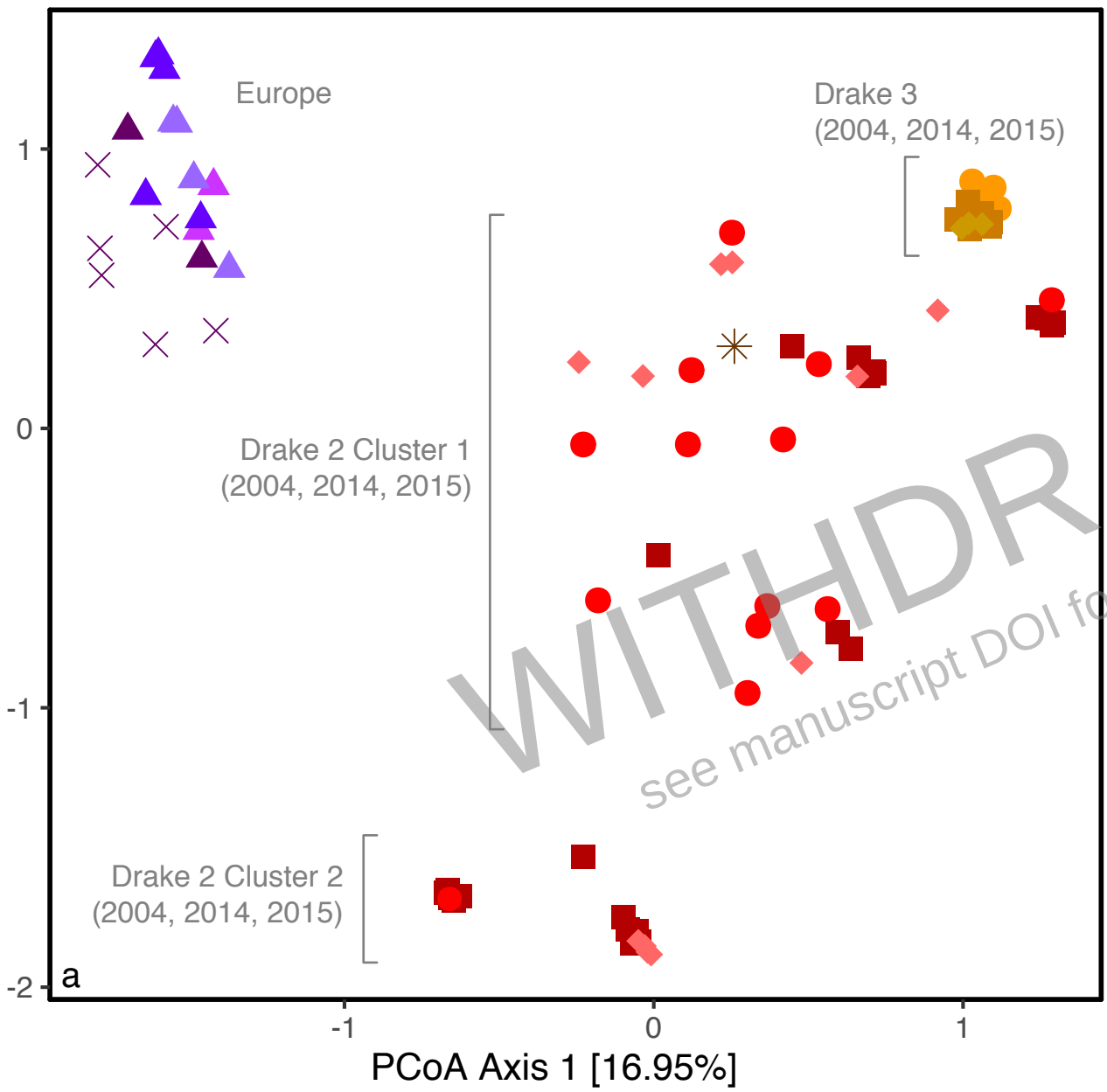
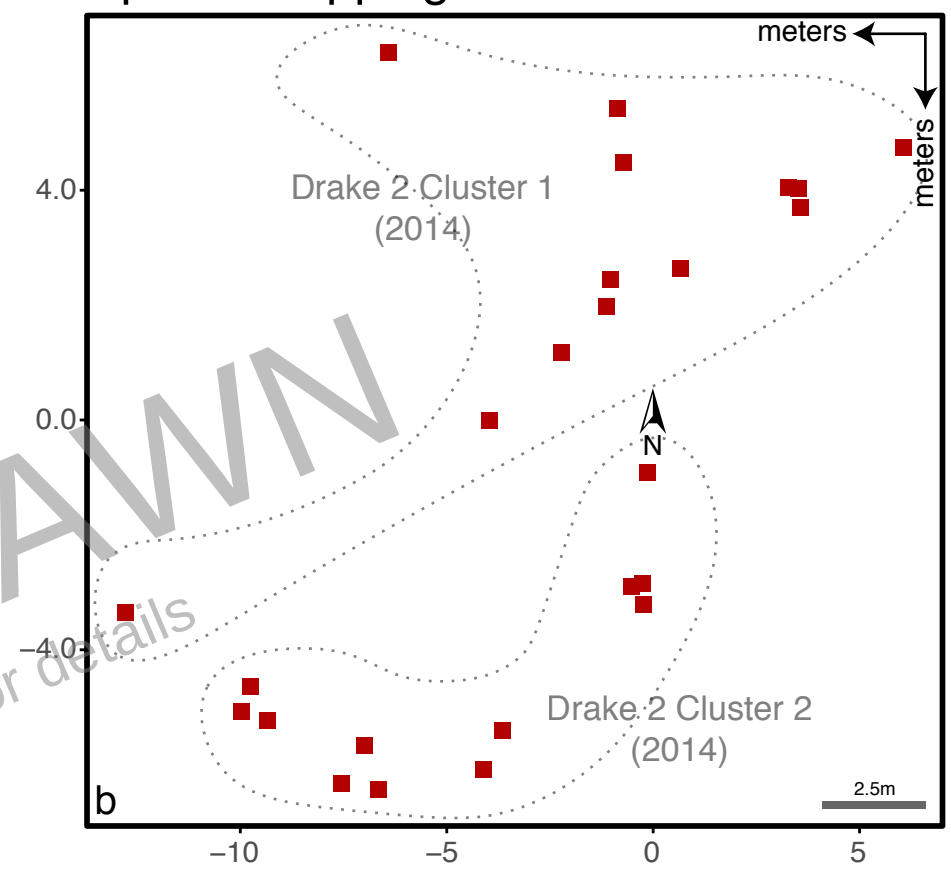
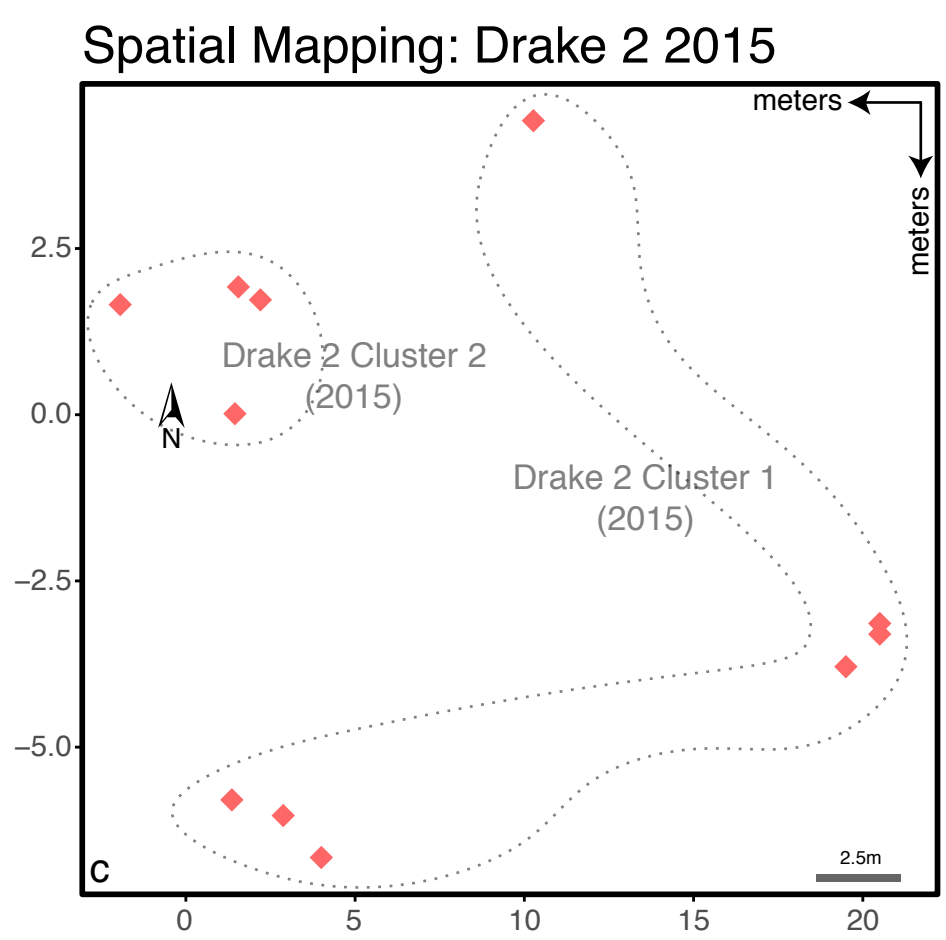


Fig. 5







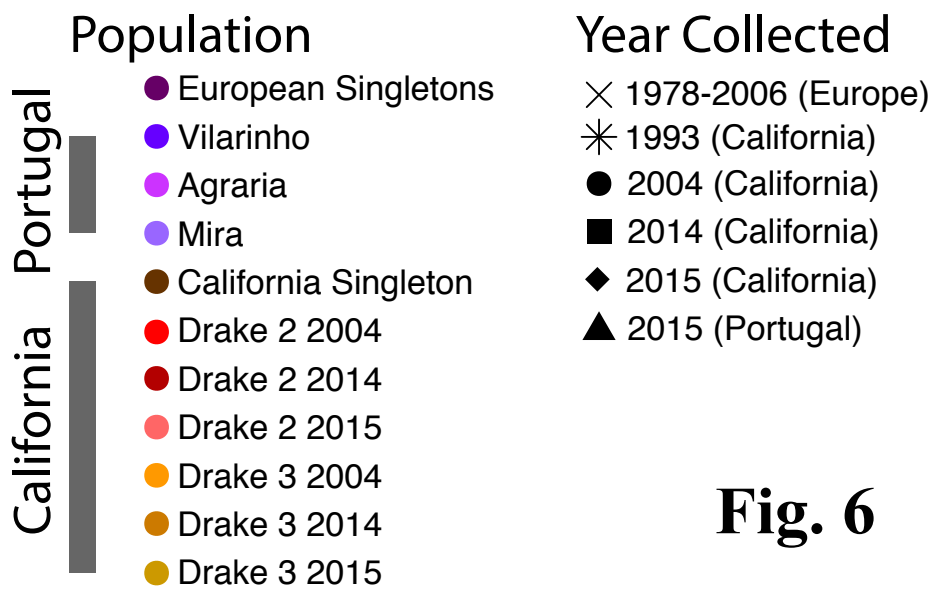


Fig. 6

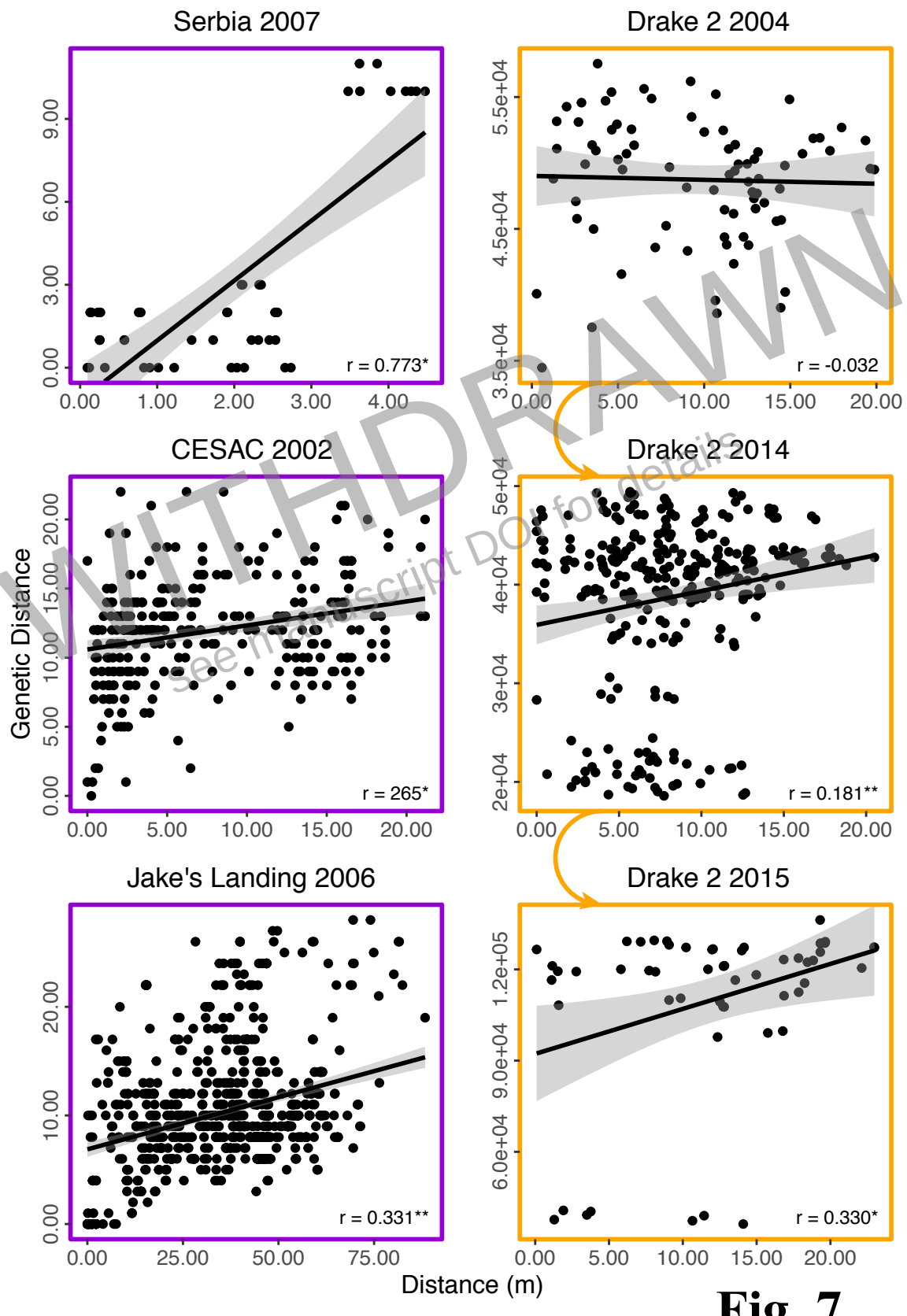


Fig. 7

Supporting Information

Article title: Native and invasive populations of the ectomycorrhizal death cap *Amanita phalloides* are highly sexual but dispersal limited

Authors: Jacob Golan, Catharine Adams, Hugh Cross, Holly Elmore, Monique Gardes, Sydney I. Glassman, Susana C. Gonçalves, Jaqueline Hess, Franck Richard, Yen-Wen Wang, Benjamin Wolfe, Anne Pringle

The following Supporting Information is available for this article:

Methods S1 Sporocarp Mapping Methods

Mapping

At CESAC (in 2002 and 2006) and Drake 2-3 (in 2014 and 2015) transects were measured from an arbitrary center point within each population (at CESAC, a planted *Cedrus libani*) to each sporocarp. The transect angle with respect to north and its distance from the center point were recorded and data later converted to Cartesian coordinates.

Populations from Portugal (Vilarinho, Agraria, and Mira [collected in 2015]) were mapped by measuring the distance between all pairs of sporocarps. Hand drawings of each population were used to arbitrarily choose a single sporocarp as the population center. Cartesian coordinates were then calculated for each sporocarp using the spatial distances between all pairs.

Populations from Drake 1-4 (in 2004), Heart's Desire 1-3 (in 2004), Serbia (in 2007), Round Valley (in 2006), Jake's Landing (in 2006), and Rochester 1-3 (in 2007) were mapped using transect lengths measured from each of two poles placed within each population to each sporocarp. The distance between the two poles was also measured, allowing the length of each transect to be considered as a radius in the mathematical equation of a circle. Using the transect lengths as two intersecting radii, equations for each of two circles were algebraically solved to obtain the Cartesian coordinates of each sporocarp. Using a rough sketch of each population, we identified which point was the correct physical location of any given sporocarp.

After mapping, entire sporocarps were extracted from soil and stored individually in labelled bags. Each sporocarp was assigned a unique five digit specimen number (Table **S1a,b**) tied to an in-house database named AmanitaBASE.

Within 24 hours of sampling, fresh sporocarps were stored using a variety of protocols including drying on a dehydrator, air drying at approximately 35ºC, flash freezing in liquid nitrogen and then lyophilizing, and/or cutting apart and placing in CTAB (Table **S1a.b**, see "Method of Preservation"). Exact protocols depended on the year, location, and the tools available at the time of sampling. Specimens are stored in the Pringle laboratory herbarium unless otherwise noted (Table **S1a,b**).

Single sporocarps were also collected opportunistically, sent to us by colleagues or loaned from herbaria. We received permission to destructively sample herbarium specimens whose original herbarium barcodes are listed in Supporting Information Table **S1a,b**.

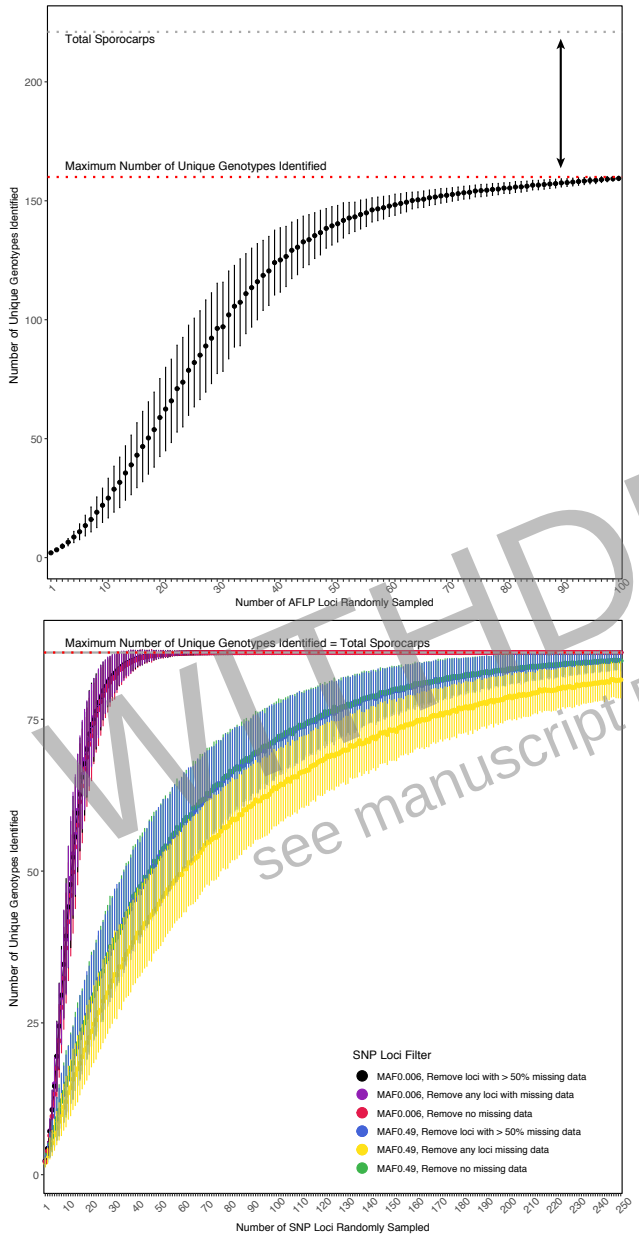
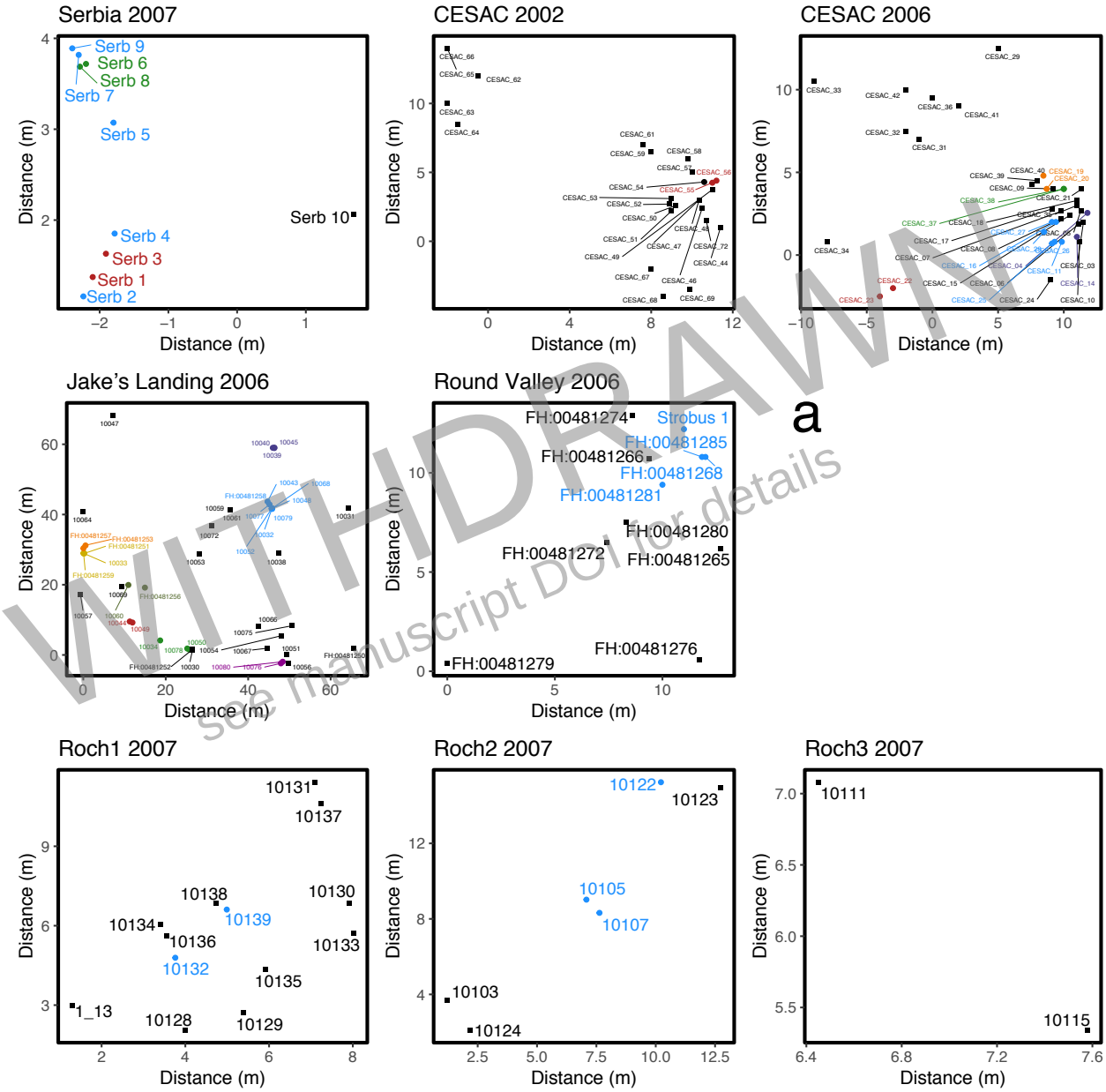


Fig. S1 Genotype accumulation curves are in essence rarefactions of the numbers of genetic markers needed to recover 100% of unique genotypes (Kamvar *et al*., 2014). One to 100 loci were randomly sampled without replacement 1,000 times, and the raw counts of genotypes observed from each random sampling used to generate means and standard errors. The curve plateaus and variance is minimized at about 90 AFLP markers. Using VCFtools (Danecek *et al*., 2011) we tested a variety of different VCF filters to gauge how each filter discriminated among unique genotypes. Loci with a minor allele frequency lower than 0.006 ($[2*N]^{-1}$, where N = 86 diploid sporocarps), or 0.49 (as an arbitrary and overly conservative filter), with a sequencing depth below 60 (the approximate mean depth across all loci and individuals), and with missing data thresholds per locus less than 0%, 50%, and 100%, were used in different combinations to filter the raw VCF file. Dotted red lines mark the maximum number of genotypes recovered with either AFLP or SNP data; dotted grey lines mark the total number of sporocarps used to generate either AFLP or SNP datasets.



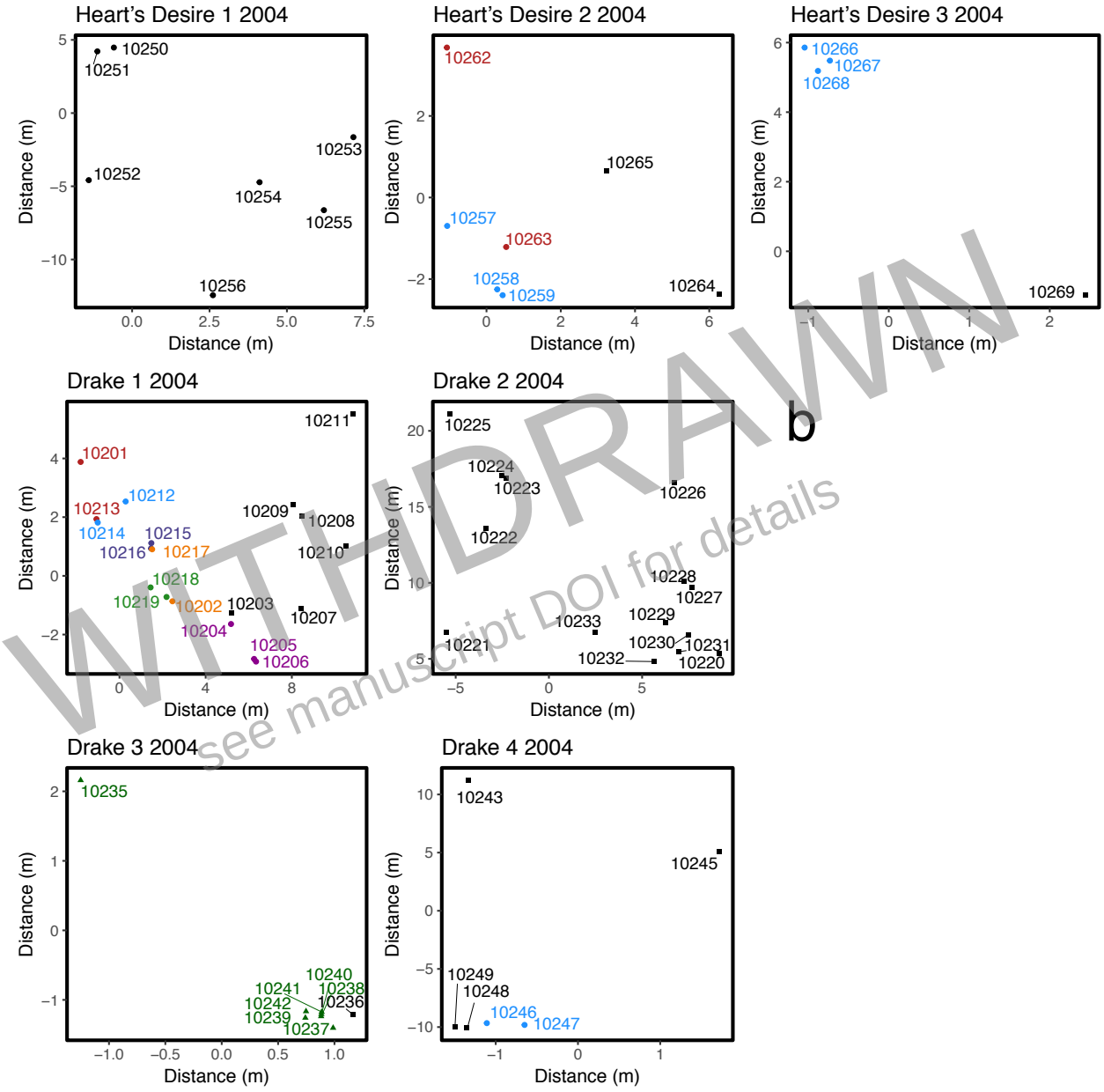


Fig. S2 Sporocarp maps and genets of every population genotyped using AFLP fingerprints. Within each map, sporocarps of the same genotype are labeled using a single color. Black squares mark genotypes represented by a single sporocarp. Panel (a) shows European and East Coast populations, and panel (b) shows Californian populations.

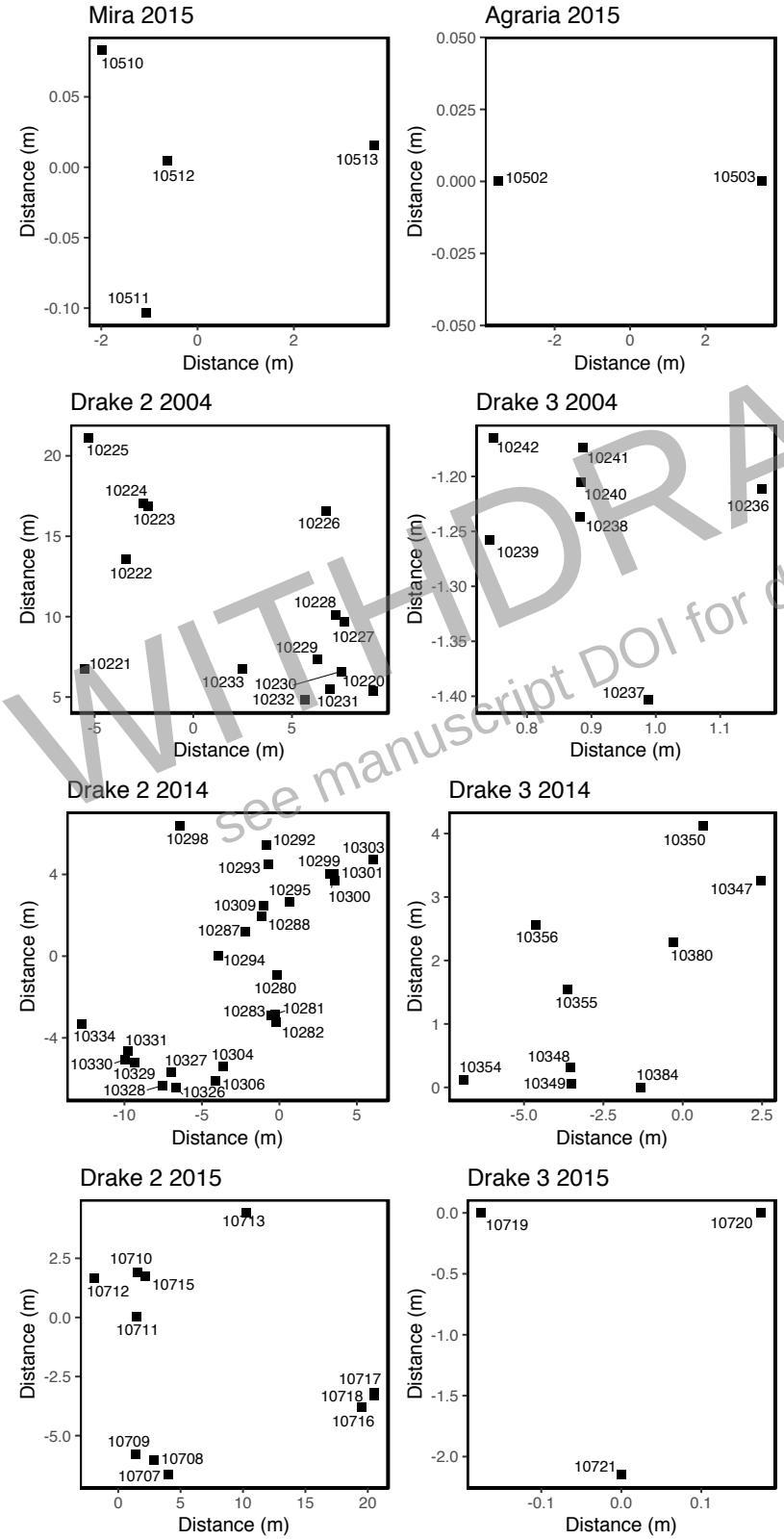
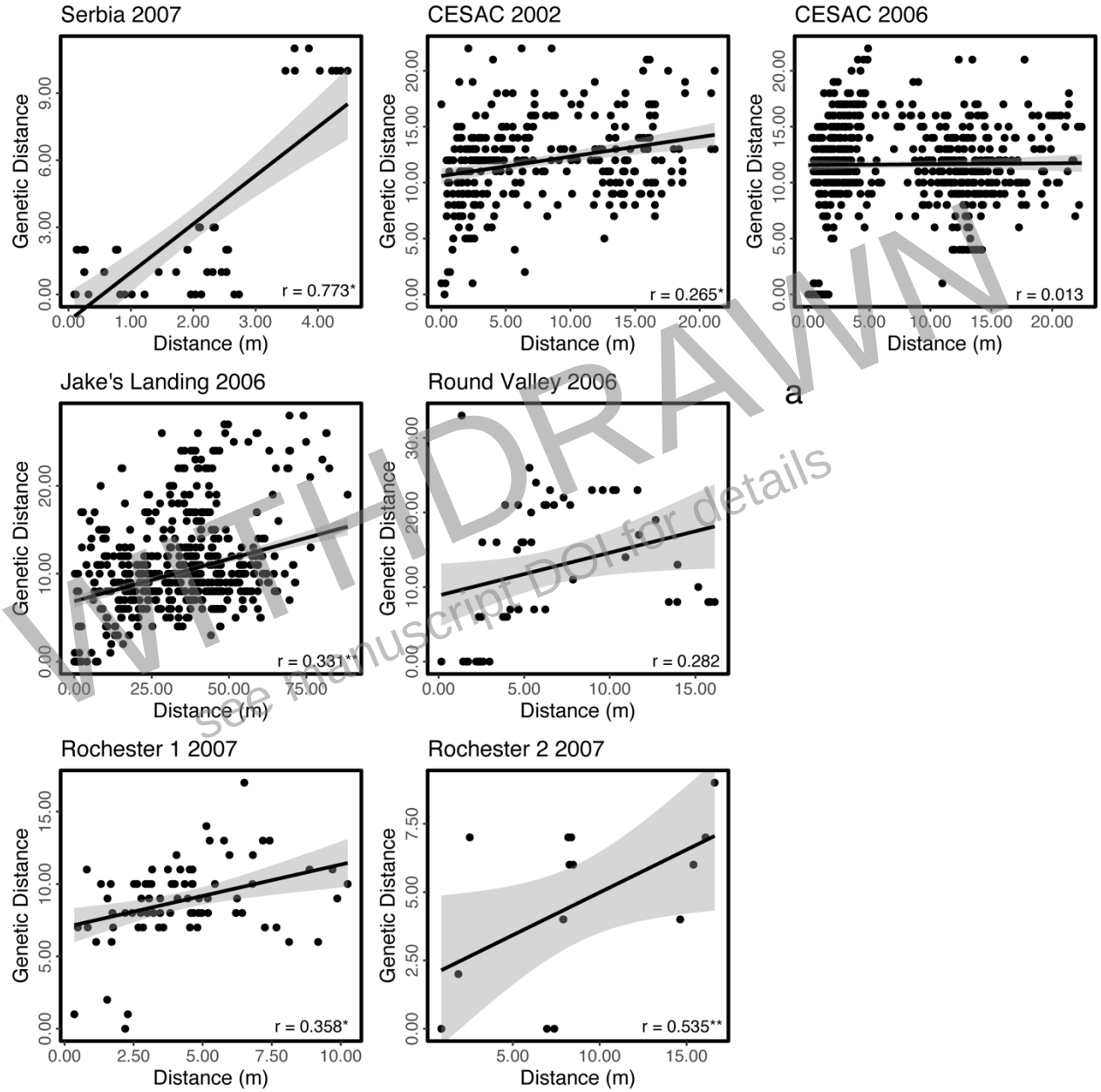


Fig. S3 Sporocarp maps and genets of every population delineated using genome-wide SNPs. Black squares mark genotypes represented by a single sporocarp; no genet encompassed more than one sporocarp.



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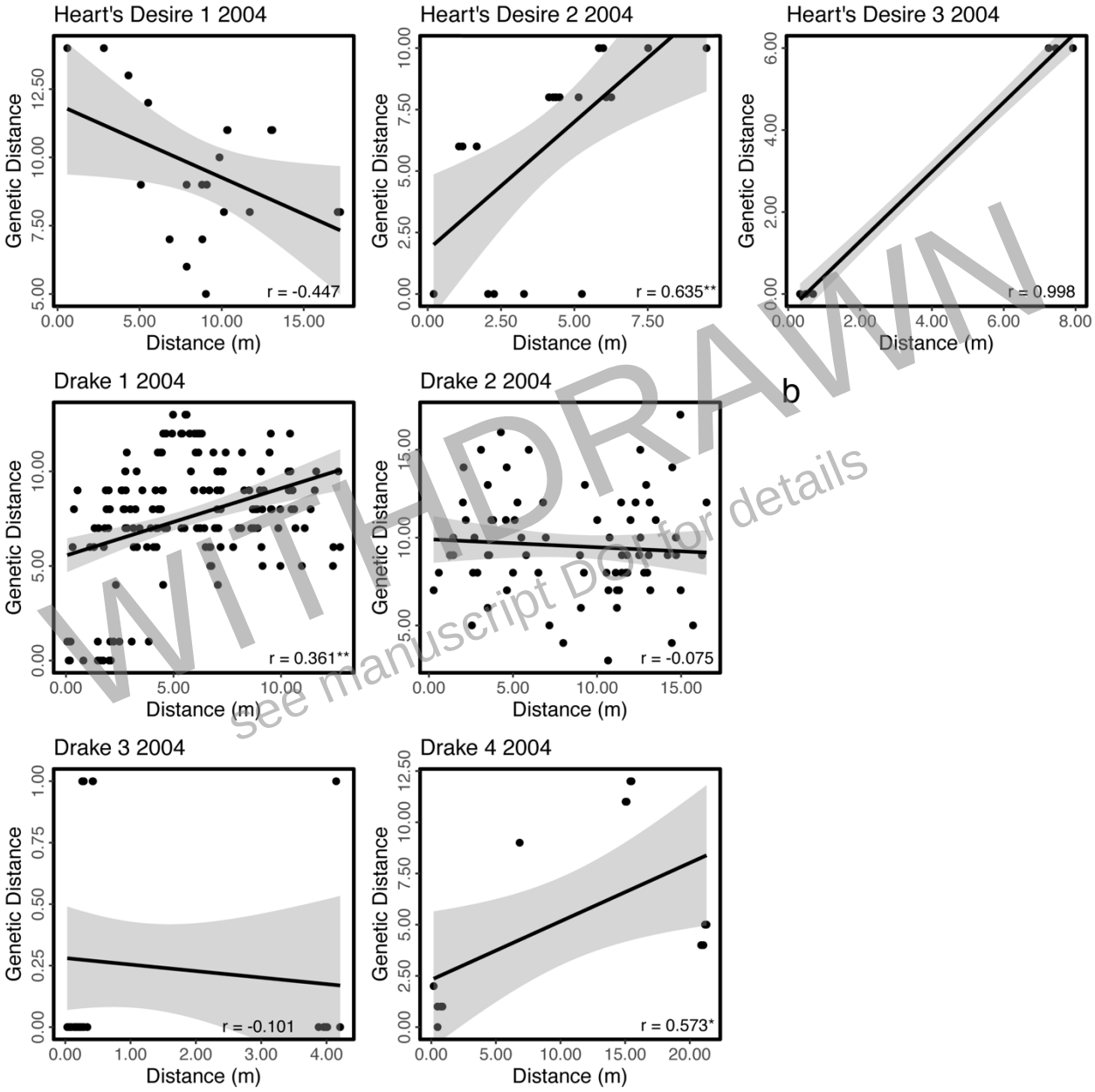

Fig. S4 AFLP data: Correlations between genetic distances and physical distances. Grey shading marks 95% confidence intervals around fitted linear models. Each plot includes the Mantel statistic r using Pearson's correlation method, and asterisks mark significant Mantel correlations (* = $p < 0.05$, ** = $p < 0.005$). Panel (a) shows European and East Coast populations, and panel (b) shows California populations.

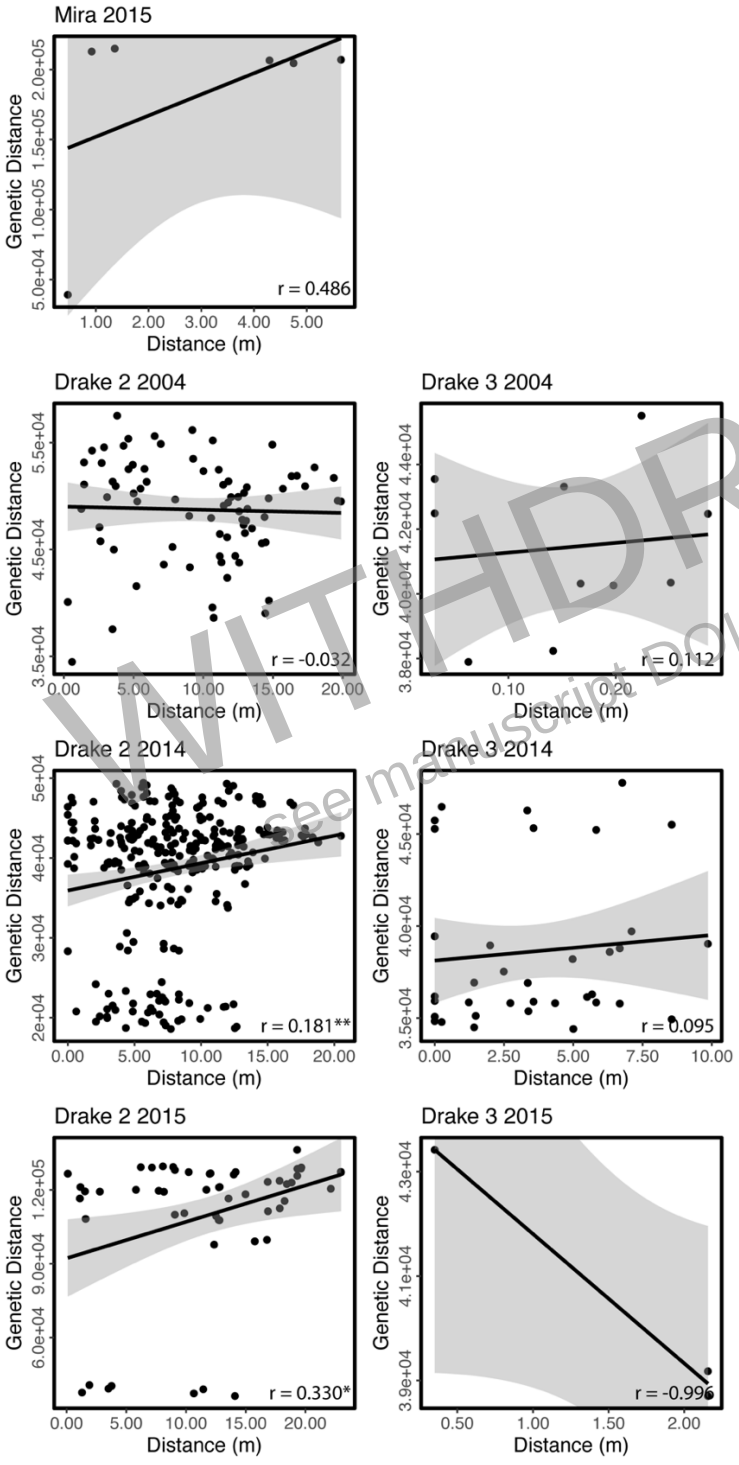

Fig. S5 SNP data: Correlations between genetic distances and physical distances. Grey shading marks 95% confidence intervals around fitted linear models. Each plot includes the Mantel statistic r using Pearson's correlation method and asterisks mark significant Mantel correlations (* = $p < 0.05$, ** = $p < 0.001$).