RESEARCH ARTICLE

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The use of soil pollen to determine the sex of overhead individuals of a temperate dioecious shrub[1]

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PREMISE OF THE STUDY: In dioecious species, determining the sex of individual plants from one-time phenological observations is rarely feasible when some individuals capable of reproducing are not flowering or fruiting at the time of observation. Currently, sexing those individuals requires long-term phenological data on individuals and populations, but such data are rarely available or feasible to collect. We tested the hypothesis that differences in soil pollen concentrations beneath the crowns of female and male plants would exist and be sufficient to reliably determine the sex of the individual plant overhead in a dioecious species. We predicted that soil pollen concentrations beneath male plants would be significantly higher than beneath female plants because only males produce pollen and pollen should accumulate in the soil underneath the male plants over repeated flowering events.

METHODS: We collected samples from surface soil under both sexes of the insect-pollinated dioecious shrub, *Aucuba japonica* (Garryaceae).

KEY RESULTS: Pollen grains were present in surface soil in both Oe and A horizons, and mean pollen concentration under males was significantly higher than under females. Pollen concentrations beneath males were positively correlated with male plant height, potentially reflecting greater pollen production by larger individuals.

CONCLUSIONS: Considering the small plant size and relatively low pollen production of *A. japonica*, this method may hold promise for sexing other dioecious species in the absence of direct phenological data. Our phenology-free and relatively low-cost method for sexing dioecious plants may be especially useful in tropical forests where many species are dioecious.

KEY WORDS Brown forest soil (andosol); dioecious species; female; insect-pollinated species; male; pollen concentration; sex identification; soil pollen analysis; spatial distribution; temperate shrub

Field studies of plant populations often require determining the sex of individuals to understand where fruits and seeds are being produced. In dioecious species, in which individual plants are either female or male, production of fruits/seeds and pollen is spatially separated by the locations of individual plants of one sex or the other. In some dioecious species, female and male plants may differ in morphology, various physiological traits, and consequent performances (Jing and Coley, 1990; Dawson and Ehleringer, 1993; Obeso et al., 1998; Obeso, 2002; Cornelissen and Stiling, 2005). However, even when these traits are found, they are highly plastic and temporally variable (Cox, 1981; Hultine et al., 2013) such that either flowering or fruiting must be observed to accurately determine the sex of individual plants. This is not always feasible because flowering and fruiting periods are temporally limited. This could be advantageous because one could expect the reproductive period to occur at a certain time of the year, however, some individuals may reproduce outside the normal reproductive season. In the tropics, where the proportion of dioecious species is known to be relatively high (Bawa, 1980), many plants do not reproduce annually in a predictable manner (Newstrom et al., 1994). Thus, if one relies on observing flowering or fruiting, determining the sex of these species, especially in long-lived tree species, requires long-term phenological data collected on an individual tree-by-tree basis. Such data are rarely available or feasible to collect, therefore, it would be useful if there was a way one could sex individuals in dioecious species at any time, independent of the phenology of the species.

To address the question of sexing individuals that are not in flower or fruit in dioecious species, we test the hypothesis that there

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are differences in pollen concentrations beneath the crowns of female and male individuals, and that the pollen concentration differences can be used to determine the sex of the plant overhead. Because only males produce pollen and pollen should accumulate in the soil underneath male individuals over time, pollen concentrations in soil samples under males should be higher than those under females. Pollen grains are often preserved for thousands of years, and pollen analyses are often conducted on samples from lake or swamp sediment cores to study past vegetation composition near the deposition site (Godwin, 1934; Ledru, 2001; Chauhan, 2005). However, pollen analyses using soil samples are far less common because of vertical soil mixing and often, poor preservation (Goldstein, 1960; Webb et al., 1981; Moore et al., 1991).

Pollen grains are well preserved in moist (Miyake and Nakagoshi, 1998; Zhao et al., 2009) and acidic soil below pH 5 (Dimbleby, 1957; Miyake and Nakagoshi, 1998). In surface soil, pollen is often considered corroded, in which the pollen exine is perforated because of microbial attack from fungi and bacteria, degraded from chemical oxidation—leading to thinning of the exine, and alternatively, the pollen could be mechanically damaged from structural changes in soil, which leads to crumpling or rupturing of the exine (van Mourik, 2001). Despite these pollen losses, the number of identifiable pollen grains remaining in the soil may be as high as in swamp sediments under some environmental conditions (Miyake and Nakagoshi, 1998). Even in dry acidic soil, the absolute number of pollen grains can be high (Miyake and Nakagoshi, 1998), and losses may not be rapid or large (Dimbleby, 1957), especially on relatively short time scales. Despite its degradation in soil, pollen should be expected to accumulate, and even < 10 yr of pollen deposition will often be sufficient to span one or more reproductive events for species that do not reproduce every year. Representation of species from surface soil is also more local than that from lake sediment (Zhao et al., 2009), which may increase the usefulness of our approach.

Pollen preservation varies among species and depends strongly on the structure of the exine (Miyake and Nakagoshi, 1998). Thus, species composition of pollen in a soil sample will be biased toward species that preserve well with pollen that has a thick exine with high sporopollenin content (van Mourik, 2001; Zhao et al., 2009; Lisitsyna et al., 2012; Quamar and Bera, 2014; Zhang et al., 2016) and those that produce large amounts of pollen (e.g., wind-pollinated species; van Mourik, 2001). For studies of a single species, the difference in representation of soil pollen resulting from the difference in exine structure should not be an issue, assuming that the soil environment of the sampling site is fairly homogeneous. However, there is little empirical evidence on intraspecific spatial variation in pollen concentrations in the soil. In this study, we tested two hypotheses: (1) pollen grains of our focal species are present in surface soil, and (2) pollen concentrations from soil under male individuals are higher than those from soil under female individuals in a dioecious species. We then explore the potential of using the difference in pollen concentrations under females and males to determine the sexes of individuals in a dioecious species.

MATERIALS AND METHODS

Focal species—As our focal species, we used *Aucuba japonica* Thunberg (Garryaceae), an evergreen dioecious shrub that is insect pollinated (Abe, 2001) and grows in low-light understory in temperate forests in Japan. Females start producing fruits in November and maintain the fruits until the following spring unless they are dispersed by birds (Fig. 1A). Males produce flower buds earlier than females in March and both have a flowering peak in April (Fig. 1B). We chose *A. japonica* for five reasons: (1) *A. japonica* is fully dioecious, and not partially so (e.g., it is not gynodioecious). (2) *Aucuba japonica* was fruiting when we conducted our study, which allowed us to sex individuals, and it flowered at least six months before the fruiting, allowing some time for potential pollen corrosion and degradation if they are going to occur in the surface soil. (3) *Aucuba japonica* individuals were spatially separated from one another. (4) The pollen of *A. japonica* was not known to be poorly preserved in sediment samples as for some other candidate species, and (5) pollen from *A. japonica* was readily identifiable and distinguishable from other species.

Pollen grains of *A. japonica* are about 50 μm in diameter and mature pollen grains are tricolporate, with a clavate exine, and are

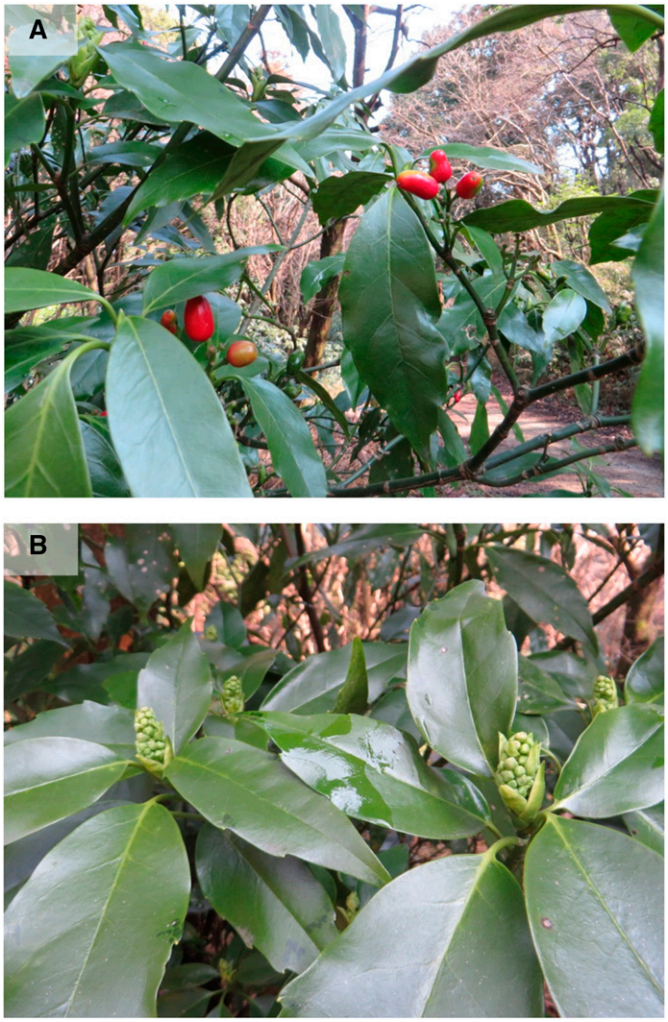

FIGURE 1 Pictures of (A) female, and (B) male *A. japonica* plants, respectively. Both pictures were taken by A. Sugiyama on March 15, 2016 at Ushiku Nature Sanctuary Forest, Ibaraki, Japan when the sexes of plants were confirmed. Female plants started producing fruits in November 2015 and still maintained fruits in March 2016, but did not yet have any flower buds. Male plants were confirmed by the presence of flower buds.

covered by adhesive pollenkitt (Takahashi, 1995), characteristic of zoophilous species (Miyoshi, Fujiki, and Kimura, 2011). Insect-pollinated species often produce fewer pollen grains than wind-pollinated species, and they are often infrequent in peat or sediments (Faegri and Iversen, 1989) and under-represented (van Mourik, 2001). Although an insect-pollinated shrub may not produce as much pollen as wind-pollinated trees, pollen may be found in large quantity in the soil under the plants (Faegri and Iversen, 1989).

Field site—We collected soil samples in the Ushiku Nature Sanctuary Forest, Ibaraki, Japan (35°57′ N 140°10′ E). The topography of the sampling site was flat and elevations of the sampling sites ranged from 9–36 m. The Sanctuary has a mean annual temperature of 14.4°C (max. 34.6°C: min. −10.5°C, the Ushiku Nature Sanctuary Forest 2015 data [unpublished]) and annual precipitation of 1226.5 mm (2015 Ibaraki prefecture data). The forest is dominated by Japanese cedar (*Cryptomeria japonica*) and hinoki cypress (*Chamaecyparis obtusa*), with several other broadleaf tree species (e.g., *Quercus*, *Prunus*) and understory shrubs, such as *A. japonica*. Portions of the forest were logged around 25 yr ago. Thinning, clearing, and weeding of understory vegetation have been conducted periodically since then. The soil is brown forest soil (andosol) and the humus type is moder.

Collection of soil samples—On December 15 and 16, 2015 we collected soil samples under the crowns of 10 female and 10 male *A. japonica* individuals in the nonlogged or unweeded areas of the Ushiku Nature Sanctuary Forest. We identified all the females based on the presence of fruits. Although the presence of fruit confirms a female, the absence of fruits could potentially be a female without any fruit (e.g., removed by dispersers, nonreproductive). To confirm the sex of all the individuals, we revisited each focal individual on March 15, 2016. None of the female individuals had produced flower buds. We collected flower buds from plants presumed to be male to check for pollen and confirmed that each was in fact male. Males with more flower buds may produce more pollen and accumulate more pollen in the soil. Therefore, we also counted the number of flowers (estimated to the nearest 5 when the number was above 40) from the presumed males.

Larger males may produce more flowers, possibly leading to a higher pollen concentration in the soil underneath. To assess the effect of individual size on the accumulation of pollen in the soil, we collected soil samples beneath individuals of a wide range of sizes, from 0.8–5.5 m tall. For each individual, we recorded height, diameter at breast height (dbh) when >1.3 m tall, sex, and the distance to the sampling site from the stem (all of the sampled plants were single-stemmed), which reflected the size of the crown.

To minimize the potential for overlapping pollen shadows from nearby conspecific plants, we selected focal individuals whose crowns were at least 1.5 m (when ≤1 m tall) or 2.5 m (when >1 m tall) away from another *A. japonica* crown. We collected samples from the periphery of the three farthest extensions of the crown because the crowns did not always extend the same distance in all directions and we expected to find greater pollen depositions at these crown-edge locations. At each sampling location, we collected from Oe and A horizons (N = 2 horizons × 3 directions × 10 focal individuals × 2 sexes = 120 samples). We first delimited the sampling area of 10 × 10 cm and removed twigs and leaves to expose the Oe horizon. Using a pair of pruning scissors and a knife, we collected the Oe horizon (considered to reflect accumulation from the past 2–3 yr) in a resealable plastic bag. Then we used a 5 cm diameter 100 mL stainless cylinder (DIK-1801, Daiki Rika Kogyo Co., Ltd., Japan) to sample the top 2 cm of the A horizon (considered to reflect accumulation of up to about 10 yr). We kept the samples refrigerated until we processed them for pollen extraction.

Pollen extraction and pollen counts—We extracted pollen from the soil samples between January and February 2016. We weighed 4 g and 2 g of samples from the Oe and A horizons, respectively, and sieved them through 250 μm sieves. To create a standard for calibrating pollen concentrations, we added ~20,000 grains of microspheres/mL (25 μm diameter) to the samples (Ogden, 1986). We followed a standard pretreatment procedure for pollen analyses (Moore et al., 1991) using 10% KOH solution to remove basic substances, 10% KCl solution to remove carbonate, $ZnCl_2$ solution of specific gravity ~1.8 for heavy liquid separation, and acetolysis mixture procedures to dissolve the surface of the exine to make the surface patterns conspicuous for pollen identification.

After extraction, we mounted the pollen extract on microscope slides using glycerin jelly for counting pollen (Shichi et al., 2013). For each individual sample, we prepared two slides and counted at least 200 microspheres using a transmitted light microscope (Axio ImagerA1, Zeiss, Oberkochen, Germany) with magnifications of 200× or 400× to calculate pollen concentration. When we did not find any *A. japonica* pollen grains, we inspected the second slide. We report pollen concentration as the number of pollen grains in the 100 cm^2 sampling area for each horizon.

Statistical analyses—To compare the difference in pollen concentration in soil beneath females and males, we used generalized linear mixed model (Poisson distribution and log link) with three sampling directions nested within each individual, and each individual was a random effect. To predict the sex from pollen concentration, we ran logistical regressions. We used linear regression to analyze the relationship in males between (a) the number of flower buds and pollen concentration, and (b) individual height and pollen concentration or the number of flower buds,. We used SAS 9.4 software (SAS Institute Inc., 2013) for all the analyses and report adjusted R^2 values.

RESULTS

Pollen grains were present in both Oe and A horizons (Fig. 2). Surface soil samples included exceptionally large amounts of *Cryptomeria japonica*, *Chamaecyparis obtusa*, two-needle *Pinus*, and *Quercus* pollen, reflecting the forest vegetation dominated by conifers. Despite occurring at a much lower concentration, *A. japonica* pollen was present.

Supporting our hypothesis, pollen concentration from soil under males was significantly higher than that from soil under females (Oe horizon: $F_{1,58}$ = 25.0, P <0.0001, A horizon: $F_{1,58}$ = 24.1, P <0.0001; Fig. 3). The logistic regressions for predicting male plants from pollen concentration were $\log[p / (1 - p)] = -0.90 + 0.0063 \times$ number of *A. japonica* pollen grains per 100 cm^2 (P = 0.0066) for Oe horizon, $\log[p / (1 - p)] = -0.77 + 0.0043 \times$ number of *A. japonica* pollen grains per 100 cm^2 (P = 0.0078) for A horizon, and $\log[p / (1 - p)] = -0.83 + 0.0051 \times$ number of *A. japonica* pollen

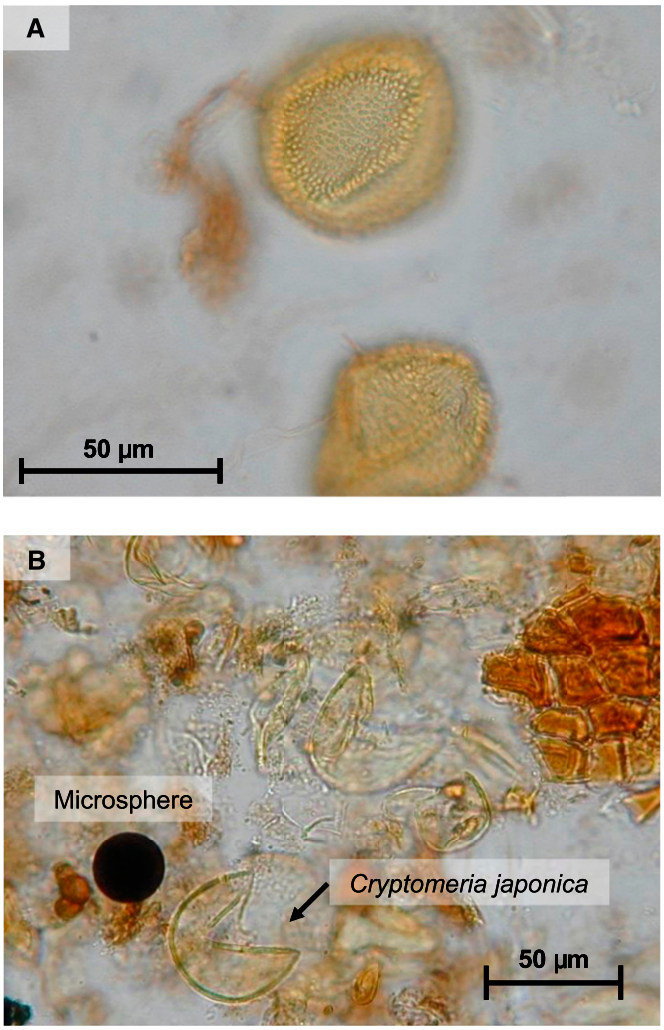

FIGURE 2 Pictures of (A) *A. japonica* pollen grains with a characteristic clavate exine, and (B) microsphere (25 µm) and *Cryptomeria japonica* pollen grains that were included in the soil sample. Both pictures were taken by K. Shichi.

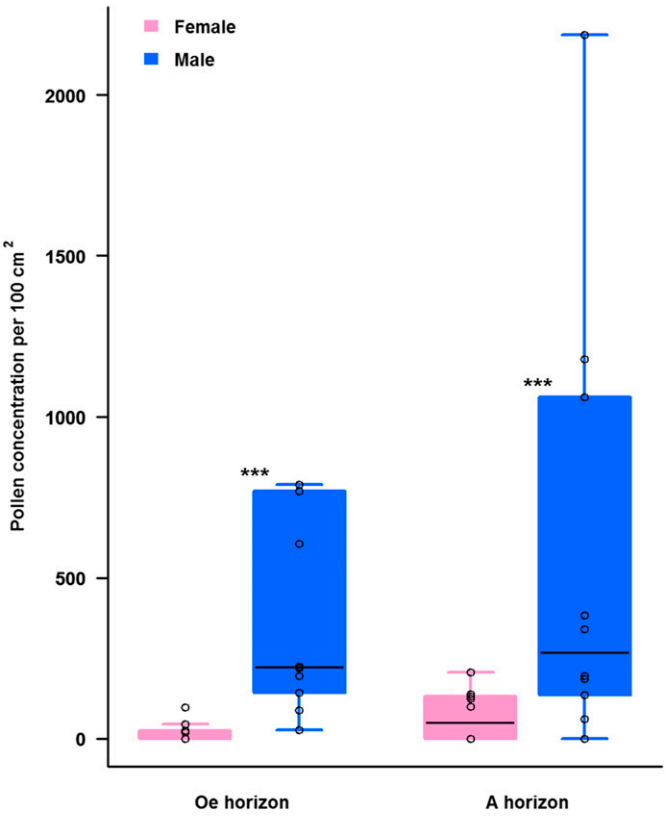

FIGURE 3 Box plots of pollen concentrations comparing female and male individuals. The boxes represent the median (black horizontal line), 1st, and 3rd quartiles. The open circles plotted on each box ($N = 10$ each) are actual data showing the distribution. One male plant with extremely high pollen concentration from the Oe horizon was omitted from this figure to better show the rest of the data. Asterisks indicate differences in the mean between female and male for each horizon at $\alpha = 0.0001$.

grains per 100 cm^2 ($P < 0.0001$) for both horizons combined. At the individual level, pollen concentrations under some females were higher than those under some males for both horizons. Regardless, the error rates were relatively low (Fig. 4).

The number of male flower buds was not positively correlated with pollen concentration (Oe horizon: $R^2 = 0$, $P = 0.45$; A horizon: $R^2 = 0.083$, $P = 0.22$). However, male height was positively correlated with the number of flower buds ($R^2 = 0.46$, $P = 0.0006$). Individual height and pollen concentration were positively correlated, although weakly, for the Oe horizon ($R^2 = 0.37$, $P = 0.036$), but it was not significant in the A horizon ($R^2 = 0.22$, $P = 0.096$). In females, we did not find such a relationship (Oe horizon: $R^2 = 0$, $P = 0.76$; A horizon: $R^2 = 0$, $P = 0.83$).

DISCUSSION

Our results are consistent with the findings of Miyake and Nakagoshi (1998), who reported that pollen was preserved in abundance in the surface soil of the Oe and A horizons of brown forest soil in Japan. We found sufficient pollen of our focal species underneath males in a conifer-dominated forest, supporting our first hypothesis that pollen grains of our focal species are present in surface soil. Furthermore, we showed that pollen concentrations from soil under males were higher than those from soil under females, also supporting our second hypothesis. Although pollen concentration was not correlated with female plant size, pollen concentration increased with male plant size. This positive relationship in males supports the interpretation that we were detecting individual differences in pollen productivity in larger vs. smaller male plants. This interpretation is also supported by our observation that taller and larger individuals had more flower buds in the following spring, assuming the productivity was fairly constant in the past few years.

Our main result is that the mean concentration of pollen was orders of magnitude higher in soil beneath males than beneath females of *A. japonica*. At the individual level, there was a small overlap in the distribution of pollen concentrations between the sexes. One male had no pollen in the A horizon, and there were some females that had greater pollen concentrations than some males with very little or no pollen. The quantity of pollen a male produced should have had a major effect on how much pollen was retained in the soil. The male with no pollen in the A horizon was the smallest

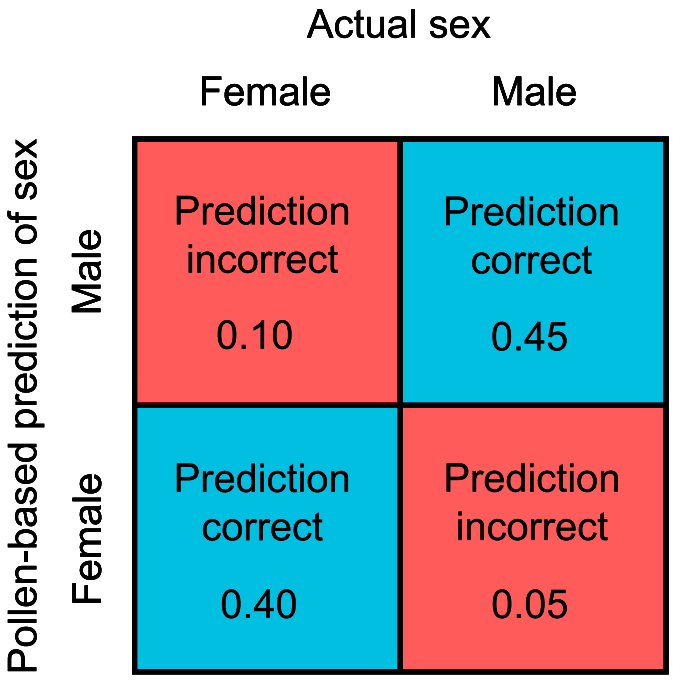

FIGURE 4 Probabilities of correctly and incorrectly predicting the sex of *A. japonica* plants based on pollen concentration using our method. When the probability of being a male was ≥0.5 from logistic regression models for both horizons combined, we assigned the plant to be a male, and when the probability of being a male was <0.5, we assigned the plant to be a female. The probability of being a male ranged from 0.30–0.59 in actual females and 0.48–1.00 in actual males.

shrub, and perhaps the youngest. Pollen that accumulated in the deeper A horizon was likely deposited at least three years ago, and the individual may not have been reproductive then. Yet, some pollen is still expected under females because some pollen is dropped in the course of pollination and dispersed in the air (Zhang et al., 2016). Similarly, pollen grains can be moved across the soil surface by rain, especially on slopes (Dimbleby, 1957), although this effect was likely small because our sampling sites were flat.

In employing this method to determine the sex of a dioecious individual, there are several considerations. First, our method does not allow 100% identification of individual plant's sex because of reasons that we have discussed so far, but it provides a quick and low-cost way to infer the sex of a dioecious plant with a certain likelihood. In this study, predictions based on pollen concentrations using probabilities generated by logistic regressions were 85% correct overall. When we considered the probability of 0.4–0.6 to be 'uncertain' (50% of females and 20% of males), the predictions for the remaining plants were 100% correct. Second, it is unlikely that this method will be applicable for all dioecious species because pollen preservation is highly variable among species (van Mourik, 2001; Zhao et al., 2009; Lisitsyna et al., 2012; Quamar and Bera, 2014; Zhang et al., 2016). Once pollen of the focal species is confirmed to be not particularly susceptible to corrosion or degradation, the productivity of males over the years becomes important because soil samples reflect pollen depositions from multiple years. We expect clearer patterns and higher success using this method with larger, long-lived tree species that become reproductively mature at a larger size. Besides well-preserved pollen and high male productivity, our method is likely to work best for species that are not wind pollinated. We expect more spatial structure in insect-pollinated species than wind-pollinated species because of localized pollen deposition around males, although wind-pollinated species produce much more pollen (Faegri and Iversen, 1989). Our method is best used in a population with no or few co-occurring congeners with similar pollen grains to ensure accurate pollen identification. Finally, the accuracy of inferring the sex of a plant would also be higher in isolated or less-dense populations where there is no or little overlapping pollen shadows from nearby conspecific plants.

As for choosing the soil horizon to sample, we recommend using both the Oe and A horizons for sexing individuals because the overlap in the distribution of pollen concentrations between the sexes became smaller and provided more reliable results when both horizons were combined. This likely resulted from vertical mixing between Oe and A horizons (Moore et al., 1991). Using soil samples from horizons deeper than the A horizon is likely not to be informative because of a rapid decrease in pollen below the organic horizon (Russell, 1993). Humus in soil from which we collected soil samples was moder, a transitional form between mor and mull (Ponge, 2003), and pollen preservation is considered to be moderate. Considering the result we obtained from our small insect-pollinated shrub species with relatively low pollen production, our method appears promising in application to taller (e.g., trees) and more pollen-productive species, although more among-species trends need to be explored to identify species where this method can be employed.

Besides using flowering and fruiting, sex chromosomes and genetic markers have been used to determine the sex of individuals for some species (e.g., Joseph et al., 2014; Wang et al., 2015; Wu et al., 2015). One major advantage of these methods is that the sex can be determined for prereproductive individuals, as early as seedlings (e.g., Joseph et al., 2014; Sun et al., 2014). However, plant species with sex chromosomes are fairly limited in number (Charlesworth, 2002; Ming et al., 2007, 2011). Even when sex chromosomes exist, using them to sex plants requires sophisticated equipment (Filatov, 2005), and genetic markers need to be developed on a per-species basis, which can be expensive and time-consuming.

If the validity of our method is demonstrated for a broader range of dioecious species, our method will be relatively low-cost, and could be used at any time of the year, independent of the phenology of given trees, once they are reproductively mature. The soil samples we collected reflect accumulated pollen productivity of each male, so it is less affected by interannual variation in pollen production or reproduction. This method could also be used to sex trees that recently died. Our method may be particularly valuable in the tropics, where many species are dioecious (Bawa, 1980) and reproductive events are unpredictable (Newstrom et al., 1994). Pollen is generally well preserved in moist and acidic soil (Miyake and Nakagoshi, 1998), common in the wet tropics. Many tropical trees also develop pollen-trapping moss on their trunks, which may allow more reliable and feasible pollen collection. The use of pollen traps may improve the resolution of pollen maps and the measurement of pollen accumulation years (Lisitsyna et al., 2012), analogous to seed traps. Many tropical plant species are also insect-pollinated (Regal, 1982) and occur in sparse, low-density populations, where the pollen shadows should be more spatially separated than in the temperate shrub species, improving the accuracy of this approach.

Beyond determining female and male individuals in dioecious species, our method may be used to answer other questions. Pollen accumulations beneath males can potentially be used to identify males that are especially productive over time. To date, sex-specific effects on soil was predominantly from females, but females and males may differentially affect the soil environment. In females, accumulation of species-specific pathogens occurs around females and nearby progeny (Mangan et al., 2010), which may have community-level effects on species diversity via negative conspecific density-dependent mortality (Harms et al., 2000; Johnson et al., 2012). In males, *N* deposition via pollen can exceed *N* deposition through litter fall (Webster et al., 2008), which may alter nutrients in soil under females vs. males. Differences in pollen accumulation may also be used to detect soil nutrient conditions. Answering questions about dioecious species that requires knowing the sex of individuals may be facilitated by our time-insensitive, relatively low-cost method using soil pollen beneath individual plants.

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DATA ACCESSIBILITY

The data set for this study is uploaded as online supplemental material in Appendix S1.

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