

IDENTIFYING AVIAN SEED DISPERSERS THROUGH DNA BARCODING AFTER RAPID FIELD SAMPLING

IDENTIFICACIÓN DE AVES DISPERSORAS DE SEMILLAS MEDIANTE MUESTREO DE CAMPO RÁPIDO Y CÓDIGOS DE BARRAS DE ADN

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SUMMARY.— Avian seed dispersers are mostly identified by direct observations of fruit consumption or by analysis of seeds ejected by mist-netted birds. However, these methods typically require many fieldwork days to provide initial insight into which bird species disperse the seeds of a plant species or community of interest. Here, we highlight the advantages of DNA-barcoding analysis after rapid field sampling to uncover unknown seed dispersers. By rapid field sampling we refer to searching and sampling bird-dispersed seeds during a short period of time (just a few hours) in places where the probability of seed deposition is high. This ‘one-day’ sampling strategy relies on a major advantage of DNA barcoding: it allows identifying disperser species during a relatively long period after they defecate or regurgitate seeds, over a time frame of several weeks. We showcase this methodological approach with three emblematic and threatened plant species for which there is little or no information on avian seed dispersal, either from their entire range or in certain populations. The approach can be cost-effective and very valuable for pilot studies aimed at providing initial information on poorly known systems, or in locations where periodical fieldwork is not feasible owing to accessibility or logistic constraints.—González-Varo, J.P., Rumeu, B., Traveset, A. & Illera, J.C. (2022). Identifying avian seed dispersers through DNA barcoding after rapid field sampling. *Ardeola*, 69: 279-290.

Key words: DNA barcoding, *Ephedra fragilis*, frugivory, *Juniperus cedrus*, *Juniperus macrocarpa*, pilot sampling, *Turdus torquatus*.

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RESUMEN.—Las aves dispersoras de semillas se identifican principalmente mediante observaciones de consumo de frutos o mediante capturas con redes de niebla para el análisis posterior de las semillas expulsadas. Sin embargo, estos métodos generalmente requieren muchos días de trabajo de campo para proporcionar una primera idea de qué especies de aves dispersan las semillas de una especie de planta o una comunidad vegetal de interés. Aquí, destacamos las ventajas del análisis de códigos de barras de ADN después de un muestreo de campo rápido para descubrir dispersores de semillas desconocidos. Con muestreo de campo rápido nos referimos a la búsqueda y muestreo de semillas dispersadas por aves durante un período corto de tiempo (de tan sólo unas horas) en lugares donde la probabilidad de deposición de semillas es alta. Esta estrategia de muestreo ‘en un único día’ se basa en una gran ventaja de los códigos de barras de ADN: permiten identificar especies dispersoras durante un período relativamente largo desde que estas defecan o regurgitan las semillas, cubriendo un marco temporal de varias semanas. Mostramos este enfoque metodológico con tres especies de plantas emblemáticas y amenazadas para las cuales hay poca o ninguna información sobre la dispersión de semillas por aves, ya sea para toda su área de distribución o en determinadas poblaciones. El enfoque puede ser rentable y muy valioso para estudios piloto destinados a proporcionar información inicial sobre sistemas poco conocidos, o en lugares donde el trabajo de campo periódico no es factible debido a limitaciones logísticas o de accesibilidad.—González-Varo, J.P., Rumeu, B., Traveset, A. e Illera, J.C. (2022). Identificación de aves dispersoras de semillas mediante muestreo de campo rápido y códigos de barras de ADN. *Ardeola*, 69: 279-290.

Palabras clave: códigos de barras de ADN, *Ephedra fragilis*, frugivoría, *Juniperus cedrus*, *Juniperus macrocarpa*, muestreo piloto, *Turdus torquatus*.

INTRODUCTION

Seed dispersal by frugivorous animals constitutes an essential process for the regeneration and dynamics of many plant populations and communities (Howe & Smallwood, 1982; Herrera, 2002). Indeed, up to 94% of woody species in tropical rainforests and up to 64% in Mediterranean woodlands produce fleshy fruits (or cones) and, thus, rely on seed dispersal by frugivores (Jordano, 2014). In this mutualism, frugivores digest the edible pulp of fruits, transport the seeds internally and eject them in conditions that are generally suitable for germination (Herrera, 2002). Birds play a central role in providing this ecological function by being the main seed dispersers of most fleshy-fruited plants, both in terms of taxonomic diversity (Jordano, 2014) and quantitative contribution to community-wide seed dispersal (e.g. Rumeu *et al.*, 2020).

A key step when studying avian seed dispersal is identifying who is dispersing the

seeds of the plant species being studied (González-Varo *et al.*, 2014). Avian seed dispersers have been mostly identified through direct observation at fruiting plants (e.g. Snow & Snow, 1988; Vitorino *et al.*, 2022) and mist netting for subsequent analysis of seeds ejected by captured birds (e.g. Herrera, 1984; Rey *et al.*, 2021). These methods sample interactions during the seed-dispersal process, either at the fruit-consumption stage or at the seed-transport stage (Figure 1) (see also Quintero *et al.*, 2022). Therefore, they typically require many fieldwork days to provide an initial insight on the identity of the seed dispersers of a plant species or community of interest (Heleno *et al.*, 2013), especially when bird abundance is low (e.g. Rumeu *et al.*, 2011).

DNA-barcoding techniques have been applied recently to identify the frugivore species that mediated dispersal by extracting and amplifying small fragments of its DNA (228-464 bp of the mitochondrial region COI) from the surface of defecated or regurgitated

seeds (González-Varo *et al.*, 2014; González-Varo *et al.*, 2017). An important advantage of this method is that it allows seed sampling at deposition sites, once internal dispersal by the frugivore has occurred (González-Varo *et al.*, 2014). So far, studies using DNA barcoding to identify seed dispersers have been based on periodic and systematic sampling of frugivore-dispersed seeds in seed traps or fixed transects (González-Varo *et al.*, 2017; Parejo-Farnés *et al.*, 2018; Acosta-Rojas *et al.*, 2019; González-Varo *et al.*, 2019a; González-Varo *et al.*, 2019b; Martínez-López *et al.*, 2019; Rumeu *et al.*, 2019; González-Varo *et al.*, 2021; Schlautmann *et al.*, 2021; García-Rodríguez *et al.*, 2022; Quintero *et al.*, 2022). Another major advantage of this technique is that allows identifying seed dispersers during a relatively long period after seed deposition (Figure 1). Hence, when we sample dispersed seeds on a given day, we are actually sampling dispersal events that

et al., 2019; González-Varo *et al.*, 2019a; González-Varo *et al.*, 2019b; Martínez-López *et al.*, 2019; Rumeu *et al.*, 2019; González-Varo *et al.*, 2021; Schlautmann *et al.*, 2021; García-Rodríguez *et al.*, 2022; Quintero *et al.*, 2022). Another major advantage of this technique is that allows identifying seed dispersers during a relatively long period after seed deposition (Figure 1). Hence, when we sample dispersed seeds on a given day, we are actually sampling dispersal events that

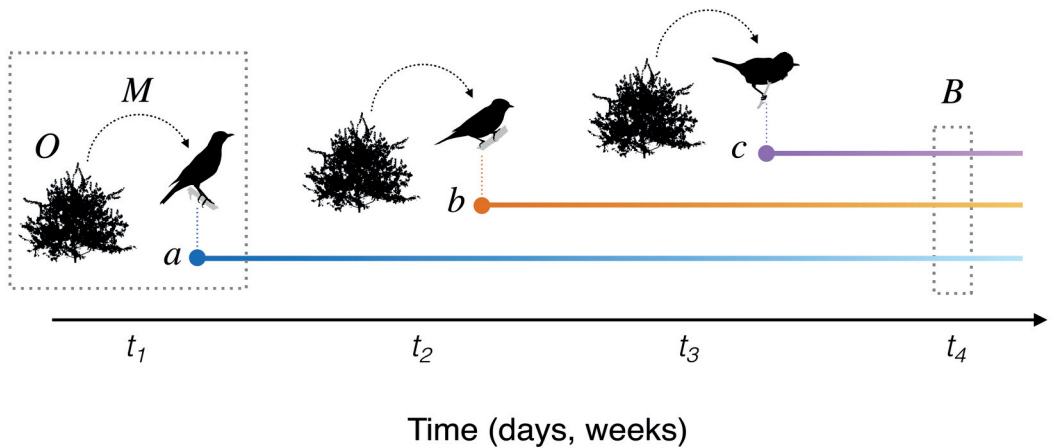


FIG. 1.—Conceptual diagram illustrating three seed-dispersal events mediated by distinct bird species (*a*, *b* and *c*) at different times (t_1 , t_2 and t_3). Traditional sampling methods such as direct observations (*O*) or mist netting (*M*) can only identify seed dispersers during the dispersal events, either during fruit consumption (*O*) or seed transport (*M*). Thus, the disperser responsible for dispersal event *a* could only be identified at t_1 and, in the same way, dispersers responsible for the events *b* and *c* could only be identified at t_2 and t_3 , respectively. In contrast, DNA barcoding (*B*) allows identifying the three seed dispersers during a relatively long interval following the dispersal events (e.g. at t_4), a period that may span from weeks (González-Varo *et al.*, 2014) to several months (see Results). The colour intensity gradient of lines *a*, *b* and *c* illustrates progressive environmental degradation of avian DNA.

[Diagrama conceptual que ilustra tres eventos de dispersión de semillas mediados por distintas especies de aves (*a*, *b* y *c*) en diferentes momentos (t_1 , t_2 y t_3). Los métodos de muestreo tradicionales como las observaciones focales (*O*) o las redes de niebla (*M*) solo pueden identificar a los dispersores de semillas durante los eventos de dispersión, ya sea durante el consumo de frutos (*O*) o el transporte de las semillas (*M*). Así, el dispersor responsable del evento de dispersión *a* solo podría identificarse en t_1 y, de la misma forma, los dispersores responsables de los eventos *b* y *c* solo podrían identificarse en t_2 y t_3 , respectivamente. Por el contrario, los códigos de barras de ADN (*B*) permiten identificar a los tres dispersores de semillas durante un marco temporal relativamente amplio después de los eventos de dispersión (por ejemplo, en t_4), un período que puede extenderse desde semanas (González-Varo *et al.*, 2014) hasta varios meses (véase Resultados). El gradiente de color de las líneas que parten de las semillas dispersadas ilustra una degradación ambiental progresiva del ADN aviar.]

have happened over a long period, typically covering several weeks (Figure 1).

Here, we highlight the advantages of DNA barcoding for uncovering unknown seed-dispersal interactions through rapid, directed field sampling. By ‘rapid’ we refer to sampling surveys of a few hours within a single day at a particular site. By ‘directed’ we refer to searches in non-random places but in areas where the probability of finding bird-

dispersed seeds is high, such as under fruit-bearing plants or perching sites nearby (González-Varo *et al.*, 2014). Bird-dispersed seeds may be found as scats containing seeds or as individual regurgitated seeds without pulp remains (Figure 2). The final aim of this paper is to illustrate the advantages of using DNA-barcoding analysis after rapid directed field sampling to provide information on poorly known seed-dispersal systems. Camera

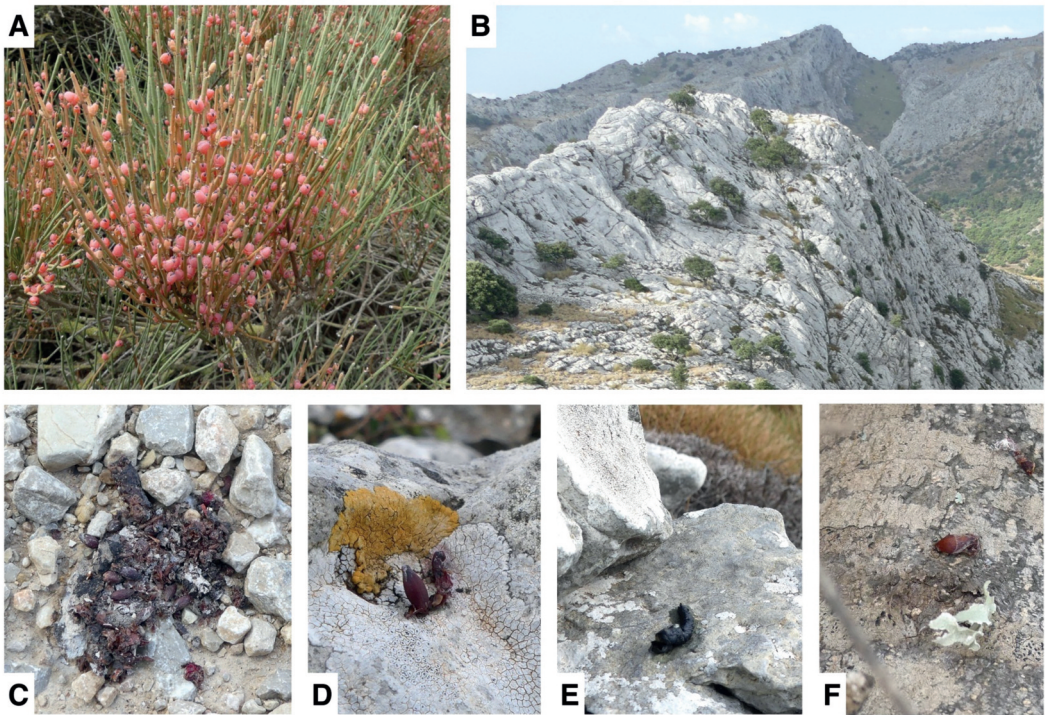


FIG. 2.—Photographs taken during the rapid, directed field sampling of *Ephedra fragilis* seeds dispersed by birds in the Tramuntana Mountains, near the Cúber reservoir in Mallorca. (A) Female *E. fragilis* plant bearing fleshy cones. (B) Landscape occupied by the study plant population, dominated by rocky outcrops with isolated trees and shrubs. (C) Large bird dropping on the ground with many seeds. (D) Single-seeded and (E) multi-seeded droppings deposited over rocks. (F) Single-seeded dropping deposited on the bark of a tree branch. Photos: Juan P. González-Varo.

[Fotografías tomadas durante el muestreo de campo rápido y dirigido de semillas de *Ephedra fragilis* dispersadas por aves en la Sierra de Tramuntana (cerca del embalse de Cúber, en Mallorca). (A) Planta hembra de *E. fragilis* con conos carnosos. (B) Paisaje de la población de plantas de estudio, dominado por afloramientos rocosos con árboles y arbustos aislados. (C) Deposición grande de ave en el suelo con muchas semillas. (D) Depositiones con una o (E) varias semillas sobre rocas. (F) Deposition con una semilla sobre la corteza de la rama de un árbol. Fotos: Juan P. González-Varo.]

traps can also be useful for the same purposes but outside restricted areas they are highly susceptible to theft (Meek *et al.*, 2019) and require revisiting to obtain the data.

We use data from three gymnosperm species that produce fleshy cones for endozoochorous seed dispersal to exemplify the type of information obtainable through this approach: the joint pine *Ephedra fragilis* in Mallorca (Balearic Islands), the Canary juniper *Juniperus cedrus* in La Palma (Canary Islands) and the large-fruited juniper *J. macrocarpa* in coastal Cádiz Province (southern Iberian Peninsula). These are emblematic and threatened gymnosperms for which there is little or no information on avian seed dispersal, either for their entire range or within certain populations. Regarding the joint pine, there is a lack of information on ornithochory although seed dispersal by Balearic Lizards *Podarcis lilfordi* has been thoroughly studied on Dragonera islet (~3km²), only 1km off Mallorca (Rodríguez-Pérez *et al.*, 2012; Neghme *et al.*, 2017; Fuster & Traveset, 2019). These lizards are extinct on the main island of Mallorca (see Traveset *et al.*, 2012), where the dispersers of joint pine remain unknown. In the case of the Canary juniper, we know that its dispersal is highly dependent on Ring Ouzels *Turdus torquatus* wintering in El Teide National Park (Tenerife), where they mostly feed on juniper cones (Rumeu *et al.*, 2009). As yet, there is no information on the avian seed dispersers in La Palma, the other island in the Canarian archipelago with high-mountain ecosystems, occurrence of this endangered juniper, and where Ring Ouzels have seldom been reported (Martín & Lorenzo, 2001). Finally, although the large-fruited juniper is known to be dispersed by mammals –mainly Red Foxes *Vulpes vulpes* and Badgers *Meles meles*– (Fedriani & Delibes, 2009), we have found no data on avian seed dispersal for this coastal species.

METHODS

Study species and sampling details

We sampled dispersed seeds of joint pine at two locations in Mallorca: Cap Blanc on the coast (39.364°, 2.788°; 60m a.s.l.) and a site close to Cúber reservoir in the Tramuntana Mountains (39.767°, 2.762°; 880–900m a.s.l.). We sampled at Cap Blanc on 13 July 2018 and at Cúber on 13 August 2018; a month apart because the joint-pine fruiting peak on the mountain is later than on the coast. Sampling consisted of searching for dispersed seeds (both scats and regurgitated seeds) dropped on or beneath structures used by birds for perching close to joint pines bearing fleshy cones; including trees, shrubs, rocks, and the wooden railings and walls of the Cap Blanc lighthouse (Figure 2).

We sampled dispersed seeds of Canary juniper at two nearby sites in the high mountains of La Palma: the ‘Observatorio Roque de Los Muchachos’ (28.764°, –17.894°; 2,155m a.s.l.) and a site close to the ‘Pico de la Cruz’ (28.751°, –17.862°; 2,150m a.s.l.). Both sites were sampled on 8 August 2020. At the observatory, we sampled bird scats beneath planted Canary junipers around the research buildings. At the other location, we sampled scats beneath wild junipers growing close to the road.

We sampled dispersed seeds of large-fruited juniper at two coastal locations of Cádiz Province: La Breña (36.189°, –5.982°; 120–130m a.s.l.) and La Algaida (36.551°, –6.206°; 5m a.s.l.). At each site, we placed five seed traps beneath the canopy of female junipers bearing cones in early February 2021. Seed traps were 0.22m² plastic trays covered with wire mesh to prevent post-dispersal seed removal (González-Varo *et al.*, 2014). We checked the content of the seed traps at La Breña and La Algaida on 26 and 28 April 2021, respectively, and found fallen cones and some seeds without pulp inside

them. We sampled these seeds as they seemed to have been regurgitated by birds.

Following González-Varo *et al.* (2014), we sampled each individual seed or scat with seeds for subsequent DNA-barcoding analysis by putting it into a 1.5ml or 2.0ml sterile tube with a minimum of handling. Capped tubes were labelled and stored in a freezer at -20°C until DNA extraction.

DNA-barcoding analysis

We used DNA-barcoding analysis (mitochondrial COI: cytochrome c oxidase subunit I) to identify the bird species responsible for each seed-dispersal event, as DNA of animal origin can be extracted from the surface of defecated or regurgitated seeds (González-Varo *et al.*, 2014). For PCR amplification, we used primers ‘COI-fsd-degF’ and ‘COI-fsdR’ to amplify a fragment of 272-bp that comprises a variable part within the longest 464-bp COI region (González-Varo *et al.*, 2017). Detailed laboratory protocols for DNA extraction, PCR, sequencing and species identification are available in González-Varo *et al.* (2014), González-Varo *et al.* (2017) and González-Varo *et al.* (2021) (Supplementary Methods). Only three of the 59 samples analysed (~5%) were re-amplified owing to previous PCR failure. Resulting sequences were identified at the species level based on best sequence matches in the ‘BARCODE OF LIFE DATA’ identification system (BOLD SYSTEM v4: <https://v4.boldsystems.org>; Ratnasingham & Hebert, 2007), typically at a 98-100% similarity. For one sequence that did not match sequences from BOLD, we used BLAST (www.blast.ncbi.nlm.nih.gov; Altschul *et al.*, 1997) to match it to sequences from GenBank. DNA-barcoding analysis was conducted at the University of Oviedo (Research Institute of Biodiversity molecular laboratory). All barcoding sequences obtained in the present study are available in the Sup-

plementary Material, Appendix 1; our sequences are not appropriate for publication in GenBank not only because they are sometimes shorter than 200bp (so not accepted by GenBank), but also because our samples can include a number of unknown repetitions of the same individual bird contributing to multiple seed dispersal events.

The costs of DNA-barcoding analysis can be very disparate depending on whether the analysis is fully conducted by researchers at a laboratory of molecular ecology (~10€ per sample in our case) or fully outsourced to a private company (~90€ per sample). Regarding working time, a full-time analyst may be able to process 48 samples (from DNA extraction to disperser species identification) within three working days.

RESULTS AND DISCUSSION

We identified six avian disperser species from 30 out of the 32 droppings (~94%) containing joint pine seeds sampled at the two Mallorcan locations (see Table 1). One sample identified the Black Rat *Rattus rattus* (a seed predator) and PCR amplification failed in the other sample. Despite the limited sample size and the rapid fieldwork, we were able to detect some differences between the two joint pine populations. We identified only two disperser species, the Sardinian Warbler *Curruca melanocephala* and the Eurasian Blackbird *Turdus merula*, on the coast (Cap Blanc). However, we identified six disperser species on the mountain (Cúber); the Sardinian Warbler, the Blackbird, the Carrion Crow *Corvus corone*, the Common Raven *Corvus corax*, the Blue Rock-thrush *Monticola solitarius* and the Eurasian Blackcap *Sylvia atricapilla*. Whereas the Sardinian Warbler was identified in most samples from Cap Blanc (69%), it was only identified in one sample from Cúber (7%), where the Blackcap accounted for most samples (57%)

(Table 1). Importantly, our results uncover a set of avian seed dispersers of the joint pine, demonstrating that the species is ornithochorous like other *Ephedra* species with fleshy bracts (Hollander & Vander Wall, 2009). Our findings are also consistent with the finding that Blackbirds have consumed joint pine cones in feeding experiments in

captivity (Rodríguez-Pérez *et al.*, 2005). Our Carrion Crow record is surprising and would have involved the hooded subspecies, *C. c. cornix*, the only form on the Balearic archipelago where it is only known as a rare visitor (Gil-Velasco *et al.*, 2017, 2018; Gil-Velasco *et al.*, 2019). Even with these small sample sizes (16 samples from Cap Blanc and 14

TABLE 1

Bird species identified through DNA barcoding from droppings containing seeds of the three plant species of interest. Numbers denote the quantity of samples (i.e. droppings) with successful bird species identification at each study site (total and by bird species); values in parentheses denote the total number of seeds found in those samples. For example, at Cap Blanc, *Turdus merula* was identified in five samples that contained 22 seeds of *Ephedra fragilis*.

[Especies de aves identificadas mediante códigos de barras de ADN en deposiciones que contienen semillas de las tres especies de plantas de interés. Los números indican la cantidad de muestras (i.e. deposiciones) con identificación exitosa de especie de ave en cada sitio de estudio (total y por especie de ave), mientras que los números entre paréntesis indican el número total de semillas encontradas en esas muestras. Por ejemplo, en Cap Blanc, se identificó *Turdus merula* en cinco muestras que contenían 22 semillas de *Ephedra fragilis*.]

Plant species	Bird species	Sampling sites	
		Cap Blanc: 16 (33)	Cúber: 14 (37)
<i>Ephedra fragilis</i>	<i>Corvus corax</i>	—	1 (14)
	<i>Corvus corone cornix</i>	—	1 (5)
	<i>Curruca melanocephala</i>	11 (11)	1 (2)
	<i>Monticola solitarius</i>	—	1 (2)
	<i>Sylvia atricapilla</i>	—	8 (8)
	<i>Turdus merula</i>	5 (22)	2 (6)
<i>Juniperus cedrus</i>		Observatorio: 4 (9)	Pico de la Cruz: 5 (12)
	<i>Turdus merula</i>	4 (9)	—
	<i>Turdus torquatus</i>	—	5 (12)
<i>Juniperus macrocarpa</i>		La Breña: 2 (2)	La Algaida 3 (3)
	<i>Cuculus canorus</i>	—	1 (1)
	<i>Saxicola rubicola</i>	—	1 (1)
	<i>Turdus merula</i>	1 (1)	1 (1)
	<i>Turdus torquatus</i>	1 (1)	—

samples from Cúber; Table 1), we can apply rarefaction and extrapolation analysis to get an idea of the completeness of this type of sampling in characterising a given disperser assemblage. We provide an example of this type of analysis in Supplementary Material, Appendix 2.

Regarding the seeds of Canary juniper sampled at altitude on La Palma, we successfully identified two bird species from the nine scats analysed (see Table 1). The Blackbird was identified in the four scats sampled beneath planted junipers around the observatory buildings, whereas the Ring Ouzel was identified in the five scats sampled beneath wild junipers (see Table 1). As pointed out above, the key role of Ring Ouzels in dispersing Canary juniper seeds is well known from El Teide National Park in Tenerife (Rumeu *et al.*, 2009; Rumeu *et al.*, 2011) but has not been documented in La Palma, where these birds have seldom been reported (Martín & Lorenzo, 2001). This is thus the first report of dispersal of these seeds by Ring Ouzels in La Palma and one of the few records of the occurrence of this bird species in the island. Remarkably, we were able to unveil this seed-dispersal interaction after sampling during a couple of hours on the same day in summer, a period when Ring Ouzels are completely absent from the Canary Islands, where they only occur in winter (Martín & Lorenzo, 2001; Rumeu *et al.*, 2009). The fact that the samples were collected in late August 2020 and that Ring Ouzels leave the archipelago between March and April (Rumeu *et al.*, 2009), when they return to breed in Europe, means that DNA barcoding allowed us to successfully identify this species in scats that were at least four months old. The checking period for seed traps or transects in DNA-barcoding studies has typically ranged between one and four weeks (e.g. González-Varo *et al.*, 2014; Rumeu *et al.*, 2019; Schlautmann *et al.*, 2021; García-Rodríguez *et al.*, 2022). Therefore, to

our knowledge, the Ring Ouzel scats from La Palma represent the oldest samples for which DNA barcoding has been successfully applied to identify seed-disperser species. This success could be explained by the cool and arid conditions on the high mountains of La Palma (Irl *et al.*, 2020); in contrast, rainy and damp conditions seem to decrease the success of specific DNA amplification (see González-Varo *et al.*, 2014).

Finally, we identified an animal species in 16 out of the 17 individual seeds of large-fruited juniper sampled in the coast of Cádiz Province. However, for 11 seeds the animal was a mammal. Of these 11 seeds, the Wood Mouse *Apodemus sylvaticus* was identified in eight seeds, the Black Rat in two seeds and the Common Rabbit *Oryctolagus cuniculus* in one seed. The high frequency of rodent identification probably resulted from seeds falling into the seed traps when rodents handled cones for seed predation on juniper branches above. These results along with those from the joint pine reveal that the primers 'COI-fsd' and 'COI-fsd-deg' (González-Varo *et al.*, 2014, 2017) can be used to identify mammalian frugivores through DNA barcoding; although the species identified here most probably acted as seed predators and pulp consumers rather than as legitimate seed dispersers. The other five seeds identified bird species: two by Blackbirds and single seeds by Ring Ouzel, Common Cuckoo *Cuculus canorus* and European Stonechat *Saxicola rubicola* (see Table 1); hence thrushes (*Turdus* sp.) dispersed most seeds (3/5). Blackbird and Ring Ouzel were identified in two seeds from La Breña, whereas Blackbird, Cuckoo and Stonechat were identified in three seeds from La Algaida. Our results thus reveal avian seed dispersers of the large-fruited juniper, demonstrating that this plant species is also ornithochorous. Further research is needed to elucidate whether the contribution of birds is quantitatively significant and effective for

seedling recruitment, particularly in relation to mammalian seed dispersal (considered the main dispersal mode for large-fruited juniper; Fedriani & Delibes, 2009). Our results included another unexpected observation: there are very few records of the Common Cuckoo feeding on fruits and dispersing seeds (Snow & Snow, 1988). Coincidentally, in two records from the literature, cuckoos were eating cones from a juniper species (the Common Juniper *J. communis*) (Cramp, 1998).

FINAL CONSIDERATIONS AND CONCLUSIONS

Obtaining information on the seed-dispersal services provided by different bird species is essential to understand their role in plant population and community dynamics (Traveset *et al.*, 2014). With just a couple of hours of field sampling at each study site, and subsequent DNA-barcoding analysis, we were able to uncover avian seed dispersers of emblematic plants that were previously unknown, either in general or from specific locations (Ring Ouzels in La Palma in this study). However, an important caveat concerning this methodological approach is that we should carefully interpret quantitative differences between disperser species, i.e. their contribution in terms of number of samples or seeds dispersed, particularly, if these are small or moderate. Since different bird species tend to disperse seeds in different microhabitats (Izhaki *et al.*, 1991), certain disperser species may be either under- or oversampled because the probabilities of detecting droppings and seed predation by granivores may vary across the directly sampled microhabitats (García *et al.*, 2005).

This methodological approach can be very cost-effective, particularly when barcoding analysis is fully conducted by the research team at a molecular ecology laboratory. Moreover, it can be very valuable for pilot studies

aimed at providing initial information before planning more ambitious sampling designs for future investigations. Indeed, the identity of the disperser species helps to determine which sampling method is more effective (Schlautmann *et al.*, 2021; Quintero *et al.*, 2022); for example, large birds are generally undersampled in mist-netting studies (Vitorino *et al.*, 2022). Finally, the methodology presented here can be very valuable where periodical fieldwork is not feasible owing to accessibility or logistic constraints, for example in remote places that can only be visited occasionally.

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AUTHOR CONTRIBUTIONS.—JPGV conceived the study. JPGV, BR and AT collected the samples in the field. JCI performed the molecular analysis. JPGV wrote the first draft of the paper. All the authors revised and edited the manuscript, and gave final approval for publication.

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Appendix 1. Sequence data.

[Datos de las secuencias.]

Appendix 2: Analysis of sample coverage and sampling completeness.

[Análisis de la cobertura de la muestra y de la exhaustividad del muestreo.]

Table A1. Metrics characterising the robustness of our sampling and the diversity of avian seed dispersers at two *Ephedra fragilis* populations.

[Métricas que caracterizan la robustez de nuestro muestreo y la diversidad de aves dispersoras de semillas en dos poblaciones de *Ephedra fragilis*.]

Figure A1. Rarefaction and extrapolation diversity curves assessing the robustness of our sampling to identify the avian seed dispersers of two *Ephedra fragilis* populations. [Curvas de diversidad de rarefacción y extrapolación que evalúan la solidez de nuestro muestreo para identificar los dispersores de semillas aviarias de dos poblaciones de *Ephedra fragilis*.]

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