Unravelling seed dispersal through fragmented landscapes: Frugivore species operate unevenly as mobile links

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Abstract

Seed dispersal constitutes a pivotal process in an increasingly fragmented world, promoting population connectivity, colonization and range shifts in plants. Unveiling how multiple frugivore species disperse seeds through fragmented landscapes, operating as mobile links, has remained elusive owing to methodological constraints for monitoring seed dispersal events. We combine for the first time DNA barcoding and DNA microsatellites to identify, respectively, the frugivore species and the source trees of animal-dispersed seeds in forest and matrix of a fragmented landscape. We found a high functional complementarity among frugivores in terms of seed deposition at different habitats (forest *vs*. matrix), perches (isolated trees *vs*. electricity pylons) and matrix sectors (close *vs*. far from the forest edge), cross-habitat seed fluxes, dispersal distances, and canopy-cover dependency. Seed rain at the landscape-scale, from forest to distant matrix sectors, was characterized by turnovers in the contribution of frugivores and source-tree habitats: open-habitat frugivores replaced forest-dependent frugivores, whereas matrix trees replaced forest trees. As a result of such turnovers, the magnitude of seed rain was evenly distributed between habitats and landscape sectors. We thus uncover key mechanisms behind 'biodiversity–ecosystem function' relationships, in this case, the relationship between frugivore diversity and landscape-scale seed dispersal. Our results reveal the importance of open-habitat frugivores, isolated fruiting trees, and anthropogenic perching sites (infrastructures) in generating seed dispersal events far from the remnant forest, highlighting their potential to drive regeneration dynamics through the matrix. This study helps to broaden the 'mobile link' concept in seed dispersal studies by providing a comprehensive and integrative view of the way in which multiple frugivore species disseminate seeds through real-world landscapes.

Introduction

Currently, most of the Earth's ice-free terrestrial land is anthropogenic, mainly agricultural fields and urban settlements (Foley *et al.* 2005; Ellis *et al.* 2010). Natural or semi-natural habitats only cover the remaining 45% and a substantial amount of them (~40%) persist as patches embedded in a matrix of anthropogenic land covers (Ellis *et al.* 2010; Driscoll *et al.* 2013; Haddad *et al.* 2015). Under this scenario, dispersal becomes a critical process for community dynamics (Butaye *et al.* 2002; Damschen *et al.* 2008; Montoya *et al.* 2008). Species must be able to disperse through the matrix for the connectivity of their populations, the colonization of vacant habitats after disturbance, or to shift their ranges in response to climate change (Trakhtenbrot *et al.* 2005; Corlett & Westcott 2013; González-Varo *et al.* 2017).

Frugivorous animals provide seed dispersal services for a substantial proportion of woody plant species across many vegetation types (> 40%; especially in tropical forests: > 70%), playing a central role in their regeneration (Jordano 2013). Frugivores ingest fleshy fruits, transport the seeds in their guts, and drop them in conditions that are generally suitable for germination, generating spatial templates for early plant recruitment (Nathan & Muller-Landau 2000; Wang & Smith 2002). The ability of these plants to disperse through the matrix relies therefore on the spatial behaviour of the frugivore species that feed on their fruits (Carlo & Yang 2011; Morales *et al.* 2013). Organisms that actively move across the landscape and transfer propagules towards and within disturbed habitats are termed 'mobile links' (Lundberg & Moberg 2003), and are considered essential for ecosystem resilience after disturbance (Folke *et al.* 2004; Kremen *et al.* 2007). Then, how do multiple frugivore species disperse seeds through the matrix operating as mobile links?

Addressing this question deserves an important consideration: the matrix is not an "ecological desert" (Haila 2002; Driscoll *et al.* 2013). On the one hand, native woody species

can in fact occur in the matrix, as isolated single elements (e.g. trees; Guevara & Laborde
74 1993; Duncan & Chapman 1999; Herrera & García 2009) or as part of unmanaged and regenerating areas, such as hedgerows and abandoned lands (Debussche & Lepart 1992;
76 Harvey 2000; Escribano-Avila *et al.* 2012). Notably, non-native plants often occur in these areas after colonization from gardens or crops (Deckers *et al.* 2008; Lenda *et al.* 2012). Thus,
78 the matrix is also a source of plant propagules. On the other hand, frugivores can vary in their response to landscape alteration, a property known as 'response diversity' among species
80 contributing to the same ecosystem function (Elmqvist *et al.* 2003). We know that many frugivore species not only move through anthropogenic land covers (Lenz *et al.* 2011; Pizo &
82 dos Santos 2011), but also use them regularly (Sekercioglu *et al.* 2007; Albrecht *et al.* 2012). The fine-grained vegetation of the matrix, including isolated trees and hedgerows, can act as
84 stepping-stones and corridors, or even as usual foraging sites (e.g. Luck & Daily 2003; Pizo & dos Santos 2011), depending on whether frugivores behave as matrix *avoiders* or *frequenters*.
86 Hence, seed fluxes between habitats are a crucial feature to consider when tackling seed dispersal in anthropogenic landscapes: some frugivore species might foster seed dispersal from
88 remnant vegetation whereas others might promote seed dispersal from matrix elements, as suggested by studies on seed rain composition (e.g. Guevara & Laborde 1993; Duncan &
90 Chapman 1999). Moreover, different frugivore species might foster seed dispersal towards natural or artificial sites of the matrix. For instance, birds can drop seeds in deforested areas
92 beneath different types of perches (Holl 1998), such as isolated trees (Duncan & Chapman 1999) and electricity pylons (Kurek *et al.* 2015).

94 Unravelling how different frugivores contribute to seed fluxes within and between habitats is essential to understand the processes driving plant community dynamics in the
96 Anthropocene (Gosper *et al.* 2005; McConkey *et al.* 2012). Yet, despite increasing advances in our knowledge on frugivory interactions in fragmented landscapes (i.e. *who eats what?*;

(Schleuning *et al.* 2015), there is still a significant gap of empirical information about the comprehensive seed dispersal process (i.e. *who dispersed the seeds, where, and from where?*), especially when diverse animal assemblages and large-scale landscapes are considered (Côrtes & Uriarte 2013). Tackling these questions has been mainly hindered by two methodological constraints that are inherent to the study of animal-mediated seed dispersal: (*i*) the identification of the frugivore species and (*ii*) the identification of the source plant involved in each seed dispersal event (see González-Varo *et al.* 2013; González-Varo *et al.* 2014, and references therein). The first is essential to understand the complementary or redundant roles of multiple mutualists in the seed dispersal process, therefore, the mechanisms driving 'biodiversity–ecosystem function' relationships (García & Martínez 2012; Schleuning *et al.* 2015). The second enables detecting seed fluxes between habitats, measuring contemporary dispersal distances and characterizing landscape features around the dispersal events, therefore, characterizing multiple functional components that determine the role of different frugivore species as mobile links (Jordano *et al.* 2007; González-Varo *et al.* 2013; González-Varo *et al.* 2017).

Here, we address how multiple frugivore species disperse seeds through the matrix acting as mobile links. We combine for the first time two sets of DNA-based molecular markers to identify the frugivore species (DNA barcoding) and the source tree (DNA microsatellites) of frugivore-dispersed seeds directly sampled in the field. We focus on a tree species in a fragmented landscape that occurs both in the remnant forest and in the matrix, both as isolated trees and as a main component of hedgerows. Specifically, we assess whether different frugivore species (*i*) disperse seeds unevenly through the landscape, in different habitats (forest *vs*. matrix), perches (natural *vs*. artificial) and matrix sectors (close *vs*. far from the forest edge); (*ii*) promote contrasting seed fluxes between habitats; (*iii*) produce different seed dispersal distances; and (*iv*) choose differently tree canopies as stepping-stones or corridors

when dispersing seeds through the landscape. According with the 'biodiversity–ecosystem function' relationships reported in plant-animal mutualisms (Klein *et al.* 2003; García & Martínez 2012), we expected to find complementarity among frugivore species across the multiple functional components analyzed.

Materials and methods

The plant-frugivore system

The plant-frugivore system comprised a widespread fleshy-fruited species that is dispersed by a diverse guild of frugivorous birds. The study plant was the wild olive tree (*Olea europaea* var. *sylvestris*, Oleaceae), a main component of mature woodlands and forests in warm areas across the Mediterranean Basin. Its fruits are ellipsoidal drupes with a lipid-rich pulp that ripens during the late autumn (mean diameter = 9.0 mm, mean length = 13.4 mm, n = 60 fruits from 12 plants). Each fruit contains a single seed wrapped in a hard endocarp; hereafter, the whole unit referred as a seed (mean diameter = 5.7 mm, mean length = 11.1 mm). Wild olives are consumed by a diverse guild of small- to medium-sized frugivorous birds belonging to families Sylviidae, Turdidae, Muscicapidae, Columbidae, Sturnidae and Corvidae (Jordano 1987; Rey & Alcántara 2014). Many of these birds are migratory species from Central and Northern Europe that use Mediterranean woodlands as their main wintering quarter (Tellería *et al.* 2005), even those woodlands within highly fragmented landscapes (González-Varo 2010).

Study landscape

We conducted our study in an anthropogenic landscape located in southern Spain (Cádiz province; 36º 39′ N, 5º 57′ W), in a lowland area (40–60 m a.s.l.) devoted to intensive agriculture (Fig. S1). The study landscape, which extends over 280 ha (1.4 km in longitude × 2 km in latitude), includes a forest remnant embedded in an agricultural matrix (Fig. S1). The

remnant is a Mediterranean lowland forest of ca. 120 ha, 80 of which are within the study landscape. Its vegetation consists of large holm- (*Quercus ilex* subsp. *ballota*) and cork- (*Q. suber*) oaks, and an understorey dominated by treelets and shrubs, among which wild olive trees, kermes oaks (*Q. coccifera*, Fagaceae), lentiscs (*Pistacia lentiscus*, Anacardiaceae), evergreen buckthorns (*Rhamnus alaternus*, Rhamnaceae) and rockroses (*Cistus salvifolius*, Cistaceae) are the dominant species. The adjacent matrix is composed of cereal fields where some isolated trees (mean density = 2.1 trees per ha; mean canopy cover = 2.9%), mainly holm oaks and wild olive trees, have been left after forest destruction during the 20th century (aerial digital orthophotos dating from 1956 available at http://www.juntadeandalucia.es/medioambiente/site/rediam). The landscape also has a large hedgerow (ca. 1450 m length) along a water channel in the south, and different types of infrastructures, including roads, a semi-urban area in the west, an industrial park in the south, and two (medium-voltage) power lines with electricity pylons (Fig. S1, S2). The covers of the main land uses within this landscape are as follows: crop fields 52.3%, forest 28.5%, infrastructures 6.5% and tree orchards 2.1%; the remaining 10.6% is accounted by pastures, field margins, hedgerows, small vegetable orchards and gardens. The wild olive tree is present in the forest remnant (mean = 41.0 trees per ha, n = 14 plots of 0.15–0.34 ha) and also in the matrix, as isolated trees in the crop fields (mean = 0.7 trees per ha, in 86, 1-ha grid cells) and as a main component of the hedgerow (~8.3 trees per 100-m length).

Sampling frugivore-dispersed seeds

We sampled wild olive seeds dispersed by birds in the forest and in the matrix of the study landscape. Sampling was carried out during the whole dispersal period of the wild olive (late October to early April) and for two consecutive fruiting seasons (2013–2014 and 2014–2015). We used seed traps placed beneath plant canopies (trees and shrubs) to quantify the magnitude

of seed deposition (seeds per m^2) in each habitat type (details below). Seed traps consisted of plastic trays (40 cm × 55 cm, 8 cm height) with small holes (1 mm diameter) to allow the drainage of rainwater, and covered with wire mesh (1 cm light) to prevent post-dispersal seed predation by vertebrates (Fig. S2). We also used fixed transects to quantify the magnitude of seed deposition in (canopy free) open interspaces, where bird-mediated seed-rain is less likely and post-dispersal seed predation is typically low due the lack of shelters for rodents (see González-Varo *et al.* 2014). Moreover, we used direct searches to increase the total number of seeds for DNA identification of disperser species and seed sources. We conducted sampling surveys fortnightly during each fruiting season. We sampled each bird-dispersed wild olive seed (i.e. defecated or regurgitated) putting it with a minimum of handling into a 2.0-mL sterile tube with the aid of the tube cap (Fig. S2). Tubes were labelled and stored in a freezer at –20ºC until DNA extraction (González-Varo *et al.* 2014). Sampling in the forest and in the matrix was as follows.

In the forest, we sampled bird-dispersed seeds beneath the canopy of different vegetation components and in open interspaces. We monitored a total of 37 and 42 seed traps during the fruiting seasons of 2013–2014 and 2014–2015, respectively, placed beneath different oak trees (11 and 12), treelets/shrubs bearing fleshy fruits (14 and 13) and treelets/shrubs not bearing fleshy fruits (12 and 17). Distance between seed traps ranged from 5 to 530 m. In the 2013–2014 season, we set up six fixed transects (23 to 45-m long and 1-m wide) to sample in open interspaces. In the 2014–2015 season, we considered the route we fortnightly used to survey the seed traps as a single fixed belt-transect (≈ 1550 m length and 1-m wide) where we sampled dispersed seeds in open interspaces. Additionally, we also conducted direct searches of dispersed seeds at under-sampled microhabitats. The sampling area in the forest covered ca. 20 ha in its southwest limit (Fig. S1).

In the matrix, we sampled bird-dispersed seeds beneath the canopy of isolated oaks, beneath electricity pylons and in open areas. We monitored a total of 31 and 35 seed traps during the fruiting seasons of 2013–2014 and 2014–2015, respectively, placed beneath isolated oaks (one trap per oak). These oaks were located in the south of the landscape (Fig. S1), between the forest and the hedgerow, with distances to the forest edge ranging from 5 to 325 m; distances between the target oaks ranged from 10 to 610 m. We also placed plastic mesh rectangles (1.5 × 2.0 m) beneath the target oaks, where we easily found dispersed seeds in direct searches during our periodical surveys (Fig. S2). We considered the route we fortnightly used to survey the isolated oaks as a single fixed (1-m wide) transect to sample dispersed seeds in open interspaces (≈ 1820 and 2250 m length in seasons of 2013–2014 and 2014–2015, respectively). Moreover, we periodically conducted direct searches in the concrete-made base (0.6 m^2) of ten electricity pylons (Fig. S2), five in each of two power lines, one crossing the crop in the north of the landscape and the other parallel to the hedgerow in the south (Fig. S1).

Seed disperser identification through DNA barcoding

We used DNA barcoding to identify the bird species that dispersed the seeds sampled (n = 582), both in the forest (n = 248) and in the matrix (n = 334). DNA of animal origin can be extracted from the surface of defecated or regurgitated seeds (Fig. 1), allowing the identification of the frugivore species responsible of each dispersal event (González-Varo *et al.* 2014). Briefly, disperser species identification was based on a 464-bp mitochondrial DNA region (COI: cytochrome c oxidase subunit I). For DNA extraction, we used a GuSCN/silica protocol, incubating each seed directly in extraction buffer (added to the 2.0-mL tube where the seed was sampled in the field). For PCR amplification, we used the primers COI-fsdF and COI-fsdR following PCR protocol described by González-Varo *et al.* (2014). For a subset of sampled seeds (n = 42) that failed to amplify using COI-fsd primer pair (apparently as a

consequence of DNA degradation after strong rains), we tested additional protocols using other primer sets in order to gain in amplification success for smaller DNA fragments. We designed two new primers to amplify our 464-bp COI DNA region in two fragments (228 and 272 bp): COI-fsd-degR (5'-GTTGTTTATTCGGGGGAATG-3'), to be combined with COI-fsdF, and COI-fsd-degF (5'-GGAGCCCCAGACATAGCAT-3'), to be combined with COI-fsdR. We also tested two primers pairs (BirdF1-AvMiR1 and AWCintF2-AWCintR4; amplicon size 404 and 314 bp respectively) for avian DNA barcode when working with degraded DNA reported in Lijtmaer *et al.* (2012). Nested-PCR reactions using COI-fsd-degF and COI-fsdR primer set on the AWCintF2-AWCintR4 amplicon as template (following Alcaide *et al.* 2009) provided successful results for 22 of these 42 seeds.

We only sequenced one strand (forward primer) of the amplified COI fragments because in most cases the electrophoretic patterns were clear and resulting sequences (length: mean = 364 bp; median = 401 bp; range = 95–417 bp) allowed successful discrimination between species. Sequences (i.e. barcodes) were aligned and edited using SEQUENCHER 4.9, and then identified using the 'BARCODE OF LIFE DATA' identification system (BOLD: http://www. boldsystems.org; Ratnasingham & Hebert 2007). BOLD accepts sequences from the 5´ region of the COI gene and returns species-level identification and assigns a percentage of similarity to matched sequences (for details, see González-Varo *et al.* 2014). In our study system, barcoding is unable to discern between the starlings *Sturnus unicolor* and *S. vulgaris* owing to the low degree of genetic differentiation (<2%) between these species, which in fact are treated as subspecies by some authors (Lovette *et al.* 2008). We assigned our samples to *S. unicolor* based on field observations.

Source tree identification through DNA microsatellites

We used DNA microsatellites to identify the source tree, and thus the source habitat, of the dispersed seeds sampled in the matrix (n = 334). We extracted the endocarp DNA of the seed and analyzed its multilocus genotype since it is a tissue of maternal origin (Fig. 1), with identical DNA copies of its source tree (Godoy & Jordano 2001). We sampled leaves from a total of 283 trees present in the study landscape in order to match their microsatellite genotypes with that of the endocarps. We sampled all adult (>1 m height) wild olive trees present in the study matrix (n = 201), including isolated trees in the crop field (n = 73), trees from the main hedgerow (n = 114) and a few trees growing in the edge of gardens, roads and buildings (n = 14). Besides, we sampled leaves from wild olive trees present in the forest, in the area adjacent to the matrix area where we placed the seed traps (see Fig. S1). These trees (n = 82) accounted for a small proportion (10%) of the estimated number of trees present within our study plot in the forest (~820 trees). However, we targeted our sampling towards very large trees most of which were located along the forest edge (e.g. Fig. S3), aiming to increase the likelihood of detecting 'forest to matrix' seed dispersal events (see Fig. S1). Notably, the crop size of these large trees (~10^5) can be up to four orders of magnitude greater than that of small- and medium-sized trees (10^1–10^4; JPGV *unpubl. data*), accounting for a large fraction of the fruits produced in the forest.

For DNA isolation from dried leaves and endocarps, we followed the protocols described by Pérez-Méndez *et al.* (2016); the single exception was that we also used a modified CTAB extraction method for endocarps. We used a set of 11 polymorphic microsatellite markers (out of 16 tested) developed for the olive tree (*O. europaea* var. *europaea*) that successfully amplified from both seed endocarps and leaves: IAS-oli11, IAS-oli17 (Rallo *et al.* 2000), IAS-oli23 (Díaz *et al.* 2006), ssrOeUA-DCA1, ssrOeUA-DCA3, ssrOeUA-DCA4, ssrOeUA-DCA7, ssrOeUA-DCA8, ssrOeUA-DCA9, ssrOeUA-DCA15, ssrOeUA-DCA18 (Sefc *et al.* 2000). Details on PCR protocols can be found in Appendix S1. DNA fragments

were sized in ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using GeneScan 500 LIZ size standard (Applied Biosystems), and were scored using GENEMAPER v.4.1 software (Applied Biosystems). Each marker presented between five and 29 alleles with an estimated mean number of 16.4 alleles per locus and a paternity exclusion probability of 0.999. Dispersed seeds were assigned to a mother tree by matching the endocarp multilocus genotype with the genotype of sampled trees (Godoy & Jordano 2001). All wild olive trees genotyped had a distinct multilocus genotype, thereby unambiguous source tree assignments can be made. Matches between endocarp and adult genotypes were found using the R package ALLELEMATCH (Galpern *et al.* 2012), which applies a hierarchical clustering method to robustly infer unique individuals (unique genotype profiles) at an optimal threshold of mismatches. In 97.6% of seeds (249 out of the 255) where source trees were successfully identified, there was a perfect matching with their adult genotypes; in the remaining 6 samples, we conservatively applied an allowed mismatch of up to two alleles, below the threshold (*alleleMismatch* = 3) estimated by ALLELEMATCH. The overall missing-data load of our dataset was 1.9%.

Importantly, we discarded that some seeds dispersed in the matrix could come from nearby olive orchards located within and outside the study landscape. We genotyped cultivated olive trees (n = 29) from five different orchards, but found no evidence of such dispersal events, which makes sense considering that these orchards produce very large green olives that are harvested unripe for local consumption (see details in Appendix S2).

Data analyses

All analyses were performed using R v. 3.2.3 (R Development Core Team 2015) and QGIS v. 2.14.0 (Quantum GIS Development Team 2015). We used the R package 'bipartite' version 2.03 (Dormann *et al.* 2009) to plot a weighted seed deposition network between the frugivore

296 species identified through DNA barcoding and the habitats/microhabitats where they dispersed the seeds. We considered 'forest' and 'matrix', differentiating in the latter between seeds
298 deposited beneath natural ('isolated trees') or artificial perches ('electricity pylons').

In order to assess spatial trends in seed rain magnitude, frugivore contributions to seed
300 rain and seed dispersal fluxes between habitats, we classified the sampling sites to belong to the forest or to five different 50-m band distance classes from the forest edge in the matrix (i.e.
302 0: forest; 1: 0–50 m; 2: 50–100 m; 3: 100–150 m; 4: 150–200 m; 5: > 200 m). Such classes represent a gradient of landscape sectors from the most natural (0) to the most anthropogenic
304 and furthest from the forest (5). Number of seed traps per class were as follows: $n_0 = 43$, $n_1 = 8$, $n_2 = 9$ (2), $n_3 = 7$, $n_4 = 9$ (3), $n_5 = 5$ (4); numbers in parentheses denote electricity pylons.

306 We used data from seed traps to assess differences in the magnitude of seed rain in the forest and the different distance classes from the forest edge in the matrix. We pooled both
308 study years (2013–2014 and 2014–2015 fruiting seasons) by averaging data per seed trap, then calculating the average number of seeds per m^2 (i.e. annual seed density). For this analysis we
310 excluded seed traps placed beneath fruiting wild olive trees ($n = 5$) in order to account for actual dispersal events, that is, involving horizontal movement away from the canopies of
312 source trees. We used a Kruskal-Wallis test to assess differences in seed rain density between distance classes.

314 We used DNA barcoding identifications to calculate the relative contributions (%) of different frugivore species to seed rain at different distance classes. We calculated two
316 contributions, first considering only natural microhabitats (i.e. trees, shrubs and open ground) and, secondly, considering all microhabitats, including electricity pylons. We performed χ^2
318 contingency tests to assess significant heterogeneity in relative frugivore contributions across distance classes. We quantified the similarity in frugivore contributions to seed rain between
320 distance classes by calculating a proportional similarity index (PS; Hurlbert 1978): $PS_i =$

$\sum_{i=1}^{n} min(p_{ia}, p_{ib})$; where for n species, p_{ia} is the relative contribution of the species i at
322 distance class a, and p_{ib} is the relative contribution of the species i at distance class b. Hence, the PS ranged from 0 (no overlap in frugivore contributions) to 1 (complete overlap) (e.g.
324 Jordano 1994; González-Varo 2010). We used the nonparametric Kendall's rank correlation coefficient (τ) to test for monotonic associations between the relative contributions of different
326 frugivore species to seed rain and increasing distance classes from the forest edge (i.e. distance classes, 0: forest; 1: 0–50 m; etc.). We hypothesized this relationship to be negative for forest-
328 dependent frugivores while positive for open-habitat frugivores.

We used DNA microsatellite assignments to calculate the relative contributions (%) of
330 different source habitats to seed rain at different distance classes. Seeds were classified into three categories: 'forest' (when the source tree was located in the forest), 'matrix' (when the
332 source tree was located in the matrix) or 'unknown' (when the source tree was not identified). We performed a χ^2 contingency test to assess significant heterogeneity in the contribution of
334 each source habitat to seed rain at different distance classes. We used the nonparametric Kendall's rank correlation coefficient (τ) to test for monotonic associations between the
336 relative contributions of each source habitat and increasing distance classes from the forest edge. We hypothesized this relationship to be negative for 'forest' while positive for 'matrix'.
338 We also assessed these relationships for each of the main frugivore species, in order to assess whether they mediated distinctive seed flows between habitats.

340 We calculated dispersal distances of seeds sampled in the matrix using the UTM coordinates of the microsatellite-identified source trees and the sampling sites (i.e. isolated
342 trees and electricity pylons). Besides, we calculated the canopy cover (including the canopy of both isolated trees and the forest) within a 25-m buffer along each seed dispersal segment (i.e.
344 50-m band; Fig. S1). We chose a 25-m buffer not only because it is a spatial scale that has proved to affect movement patterns of frugivorous birds (e.g. Morales *et al.* 2013), but also

because it provided enough variability to assess frugivores' preferences for specific canopy cover along their movements (range = 0.7–66.2%). We used Kruskal–Wallis tests to assess statistical differences between frugivore species in seed dispersal distances and canopy cover along the seed dispersal events they mediated. We used *post–hoc* Mann–Whitney *U*-tests to assess differences between pairs of species. We also used Mann–Whitney *U*-tests to assess whether the canopy cover along the seed dispersal events mediated by each frugivore species differed from that available in the landscape, within 120, 100 × 100 m cells (see Fig. S1). These cells were the subset of cells that intersected with the buffers, thus including the area within which all seed dispersal events occurred.

Results

We analyzed a total of 582 seeds, 248 seeds sampled in the forest (191 in seed traps, 48 in direct searches and 9 in transects) and 334 seeds sampled in the matrix (114 in seed traps, 137 in direct searches and 83 in electricity pylons). The vast majority of seeds (97.9%) was found beneath perches, either natural or anthropogenic; only 12 seeds (2.1%) were sampled from open interspaces on the ground, all them in the forest. We successfully identified through DNA barcoding a total of nine frugivore species from 532 seeds (91.4%), six species from 218 seeds sampled in the forest and six species from 314 seeds in the matrix (Fig. 2). Three species were identified in seeds dispersed in both habitats (*Sylvia atricapilla*, *Turdus philomelos* and *Columba palumbus*), although their relative contribution varied between habitats (Fig. 2). Three species were only identified from seeds sampled in the forest and other three species from seeds sampled in the matrix (species names in Fig. 2). Yet, only four species accounted for 97.4% of frugivore-identified seeds, referred hereafter by their genus name (*Sylvia*, *Turdus*, *Columba* and *Sturnus*; Fig. 1). Notably, *Sturnus* was the only disperser species identified from seeds sampled under electricity pylons (Fig. 2).

372 *Seed rain density and frugivore contributions*

Seed rain in open interspaces was almost negligible in the forest (mean = 0.03 seeds per m^2)
374 and null in the matrix. We calculated that 99.8% of seeds dispersed per forest hectare were deposited beneath woody plant canopies, and virtually 100% of seeds dispersed per matrix
376 hectare were beneath isolated trees and electricity pylons. Seed rain density beneath natural perches (woody plants) was not significantly different between forest and matrix (mean = 6.3
378 and 7.3 seeds per m^2, respectively; MW *U* test: $P = 0.283$). Moreover, seed rain density beneath electricity pylons (mean =10.8 seeds per m^2) did not differ significantly from that
380 found beneath isolated trees of the matrix (MW *U* tests: $P = 0.088$).

We found non-significant differences in the magnitude of seed rain beneath natural
382 perches between the forest and the different distance classes from the forest edge in the matrix ($\chi^2_5 = 5.53$, $P = 0.355$; Fig. 3A). However, frugivore contributions significantly varied
384 between distance classes, both when considering seed deposition in natural microhabitats ($\chi^2_{40} = 241.2$, $P << 0.001$; Fig. 3B) and, especially, when considering all microhabitats, including
386 electricity pylons ($\chi^2_{40} = 438.5$, $P << 0.001$; Fig. 3C). Such differences reflected a significant decrease in the contribution of *Sylvia* ($\tau = -0.87$, $P = 0.008$) along with a parallel increase in
388 the contribution of *Sturnus* ($\tau = 0.83$, $P = 0.011$) with increasing distance from the forest edge (Fig. 3B, 3C); *Turdus* and *Columba* were identified in all distance classes and their relative
390 contribution was not significantly associated with distance from forest ($|\tau| \leq 0.6$, $P > 0.6$; Fig. 3B, 3C). Indeed, *Sylvia* was not identified in seeds sampled in class '> 200 m', whereas
392 *Sturnus* was not in seeds sampled in classes 'forest' and '0–50 m' (Fig. 3B, 3C). Consequently, frugivore contributions gradually and significantly shifted while moving farther from the
394 forest, as shown by a significant decrease in proportional similarity (PS index) (see detailed

results in Table S1). For example, there was a similarity of 84% in frugivore contribution between 'forest' and the first distance class '0–50 m', but a similarity of 9–32% between the forest and the farthest distance class ('> 200 m'), depending on whether only considering natural microhabitats (32%; Fig. 3B) or all microhabitats, including electricity pylons (9%; Fig. 3C).

Source habitat contributions

We successfully identified the source tree in 76.3% of the seeds sampled in the matrix (255 out of 334); the remaining 23.7% seeds (79) were assigned to 'unknown' source tree. Among seeds with successfully identified source trees, 16.1% (41) came from trees located in the forest and 83.9% (214) from trees located in the matrix. We found significant variation in the contribution of different source habitats to seed rain in the matrix at different distance classes from the forest edge ($\chi^2_8 = 123.2$, $P << 0.001$; Fig. 3D). Such differences reflected a significant decrease with increasing distance from the forest edge in the contribution of forest trees ($\tau = -1.00$, $P = 0.008$; Fig. 3D) along with a parallel increase in the contribution of matrix trees ($\tau = 0.80$, $P = 0.042$; Fig. 3D). Source trees located in the forest trees accounted for 56% of seeds sampled between 0–50 m from the forest edge, for 13–15% between 50–150 m, for 3% between 150–200 m and for 0% at distances farther than 200 m (Fig. 3D). In contrast, source trees located in the matrix accounted for 13% of seeds sampled between 0–50 m from the forest edge, for 49–50% between 50–150 m, and for 81–86% at distances farther than 150 m (Fig. 3D). We found non-significant association between the contribution of unknown sources and distance from the forest edge ($\tau = -0.40$, $P = 0.242$).

At the frugivore species level, *Sylvia* ($\tau = -0.91$, $P = 0.035$) and *Turdus* ($\tau = -1.00$, $P = 0.008$) significantly dispersed less seeds from the forest in the matrix with increasing distance from the forest edge (Fig. 4). Yet, *Turdus* dispersed forest seeds towards the matrix

twice as far than *Sylvia* (Fig. 4). On the other hand, *Turdus* and *Columba* significant dispersed more seeds belonging to matrix trees while moving away from the forest (τ = 0.80, P = 0.042 in both species). We also found that *Columba* significantly dispersed a lower proportion of seeds of unknown source at further distance classes (τ = –1.00, P = 0.008) (see details in Table S2).

Distance and canopy cover along seed dispersal events

We successfully identified both the frugivore species and the source tree in 74.3% of the seeds sampled in the matrix (248 out of 334). Among them, dispersal distances differed significantly between the four main frugivore species (χ^2_3 = 27.4, P << 0.001; Fig. 4). Distances mediated by *Sylvia* and *Turdus* were very similar: they deposited most seeds within 300 m from source trees and very rarely dispersed seeds further (*Sylvia* up to 638 m and *Turdus* up to 1321 m; Fig. 4). On average, *Sturnus* dispersed most seeds at slightly longer distances (up to 559 m), whereas *Columba* did it at distances remarkably longer, with several events above 500 m up to 1224 m (Fig. 4). The two dispersal distances obtained from *Corvus monedula* were 292 m and 942 m, whereas the two from *Phoenicurus ochruros* were 15 m and 63 m.

The canopy cover along these dispersal events also differed significantly between the four main frugivore species (χ^2_3 = 124.3, P << 0.001; Fig. 4). Buffer areas along dispersal events mediated by *Turdus* and – particularly – *Sylvia* had a high canopy cover of isolated trees or forest edge (Fig. 4). In contrast, buffers along dispersal events mediated by *Columba* and, especially, *Sturnus* showed a low canopy cover. Indeed, *Sturnus* was the only species that dispersed seeds along areas having canopy covers non-significantly different from those available in the landscape (MW U-test: P = 0.209; in the other three species all $P \leq 0.016$; Fig. 4).

Discussion

Organisms that actively move across the landscape and transfer propagules from remnant to disturbed habitats, and between elements within disturbed habitats, have been defined as mobile links (Lundberg & Moberg 2003). Here, we reveal seed dispersal across habitats and landscape sectors as a spatially structured process, characterized by turnovers in the contribution to seed rain of both frugivore species and source-tree habitats. Seed rain in the matrix was mostly mediated by matrix-frequenter frugivores, which include matrix visitors from the forest and open-habitat species. Moreover, most seeds dispersed in the matrix came from source trees located there; the contribution of forest trees sharply declined with increasing distances from the forest edge. *Sturnus*, an open-habitat species, provided a unique function by dropping seeds from matrix trees beneath human-made perches. Finally, the most forest-dependent frugivores dispersing seeds in the matrix (*Sylvia* and *Turdus*) did it predominantly along areas of high canopy cover, which potentially acted as stepping-stones or corridors. Taken together, our results demonstrate a remarkable functional complementarity among frugivore species operating as mobile links. In fact, the magnitude of seed rain beneath perches was evenly distributed through the landscape as a result of very unevenly distributed contributions of distinct frugivore species.

Functional complementarity in seed deposition by frugivores through the landscape

We found that seed deposition was virtually confined beneath natural and artificial perches, which reinforces the documented importance of perching sites for bird-mediated seed dispersal, especially in anthropogenic habitats (Guevara & Laborde 1993; Duncan & Chapman 1999; Harvey 2000; Graham & Page 2012; Rey & Alcántara 2014). Our results evidenced a clear spatial turnover in frugivore contributions to seed rain between forest and matrix (Fig. 2). Only

three frugivore species out of the nine identified (*Columba*, *Sylvia* and *Turdus*) deposited seeds in both habitats; the other six species deposited seeds either in the forest or in the matrix. Independent data on bird abundances lead us to discard that the turnover observed in the five species with minor contributions reflected under-sampling; i.e. these species were predominantly abundant either in the forest or in the matrix (see Appendix S3). The turnover between forest and matrix became also evident in terms of the relative contribution by those species that dispersed seeds in both habitats: *Sylvia* mostly dispersed seeds in the forest whereas *Turdus* and *Columba* mostly did it in the matrix, yet at different frequencies. These results allow to rank the forest-dependence of these species as: *Sylvia* > *Turdus* > *Columba* (Fig. 2); which is congruent with their abundances in forest and matrix (Appendix S3). Our findings are in line with studies documenting changes in frugivore assemblages in anthropogenic landscapes not only as a result of species loss, but also of species turnover (Luck & Daily 2003; Pizo & dos Santos 2011; Albrecht *et al.* 2012; Farwig *et al.* 2017). Hence, the matrix acts as a filter for some forest species, but it comprises the usual domains of matrix-frequenter species (e.g. Sekercioglu *et al.* 2007), which can be either forest species that regularly visit the matrix (*Columba* > *Turdus* > *Sylvia*) or open-habitat species (here *Sturnus, C. monedula and P. ochruros*).

We also found such spatial turnover at a finer grain within the matrix, between different distance classes from the forest edge (Fig. 3B-C), and between natural and artificial perches (i.e. isolated trees and electricity pylons; Fig. 2). First, there was a gradual shift in frugivore contributions to seed rain with increasing distance from the forest edge. Secondly, only one species (*Sturnus*) – out of the six identified in the matrix – deposited seeds beneath electricity pylons (Fig. 2). This demonstrates that seed dispersal towards infrastructures can be mediated by a very reduced subset of open-habitat species. The latter is in accordance with observational studies about the use of artificial perches (crossbars) by frugivorous birds in cleared tropical

forests (Holl 1998; Graham & Page 2012). This function can be key for community dynamics since perching infrastructures are very ubiquitous in anthropogenic landscapes and often located in unmanaged lands, where focal plant regeneration is possible (Kurek *et al.* 2015). In fact, it is common to observe young wild-olive trees growing beneath electricity pylons of the study region (see Fig. S4).

Our study provides a good example of how response diversity among frugivore species can translate into functional complementarity in seed deposition patterns, and thereby into resilience of the seed dispersal function across a fragmented landscape (Elmqvist *et al.* 2003; García *et al.* 2013). Functional complementarity in our study system became evident through the similar seed rain densities sampled in the forest and at different distance classes from the forest edge in the matrix (Fig. 3A), beneath natural perches and electricity pylons. Importantly, such evenly distributed seed rain densities resulted from unevenly distributed frugivore contributions in different habitats, landscape sectors and perching sites. That means that losing a frugivore species from this system, especially any of the four main species (*Columba*, *Sylvia*, *Turdus* or *Sturnus*), would impact only specific parts of the landscape. Our findings align with correlational evidence of functional complementarity in seed deposition by thrushes (*Turdus* spp.) in a fragmented landscape (García & Martínez 2012; García *et al.* 2013). In the study landscape, seedling establishment beneath most isolated trees and electricity pylons is virtually prevented by the current management practices, mainly, ploughing for cropping and livestock grazing. However, the observed seed dispersal patterns are expected to generate recruitment patterns whenever these perching sites are located in abandoned lands or unmanaged matrix sectors (Debussche & Lepart 1992; Escribano-Avila *et al.* 2012; Rey & Alcántara 2014).

Differential contribution of source habitats to seed deposition through the landscape

520 The evenly distributed seed rain densities through the landscape also resulted from turnovers of source-habitat contributions, illustrating how the landscape-scale seed rain is structured on seed
522 shadows of individual trees located in different habitats. We found that wild olive trees located in the forest were the predominant sources of seeds deposited within the first 50-m of the
524 matrix. However, their contribution declined sharply at further distances from the forest edge, where most seeds came from matrix trees, especially at distances further than 150 m (Fig. 3D).
526 Our results are consistent with previous studies suggesting that most seeds arriving to deforested lands might not come from the forest but rather from nearby disturbed sites (Duncan
528 & Chapman 1999; Pizo & dos Santos 2011; Graham & Page 2012). The fact that the contribution of unknown sources was not associated with the distance from forest edge
530 strongly suggests these non-genotyped trees were located both in the forest and in the matrix, outside the study landscape. Interestingly, these general patterns emerged from frugivore-
532 specific differences in seed dispersal from – and towards – the different habitats. For instance, *Sylvia* and especially *Turdus* dispersed seeds from the forest towards nearby isolated oaks
534 during their incursions into the matrix (Fig. 4), which were much more frequent in the latter (Fig. 3B-C). In contrast, *Sturnus* mainly dispersed seeds from the matrix and towards the
536 furthest sectors from the forest. Thus, the seeds from unknown source trees dispersed by *Sturnus* likely belonged to trees located in anthropogenic habitats outside the study landscape.
538 Finally, the fact that most seeds dispersed by *Columba* came from unknown sources, particularly at closer distances from the forest, along with the long-dispersal distances
540 mediated by this species, suggests that such unknown sources were probably located in the forest (Fig. 4).

542 It is not difficult to envisage how these seed dispersal patterns might occur under distinct landscape configurations, for example, within a landscape with smaller forest patches
544 at distances of a few hundred meters from each other. Our results suggest that *Turdus* and,

especially, *Columba*, would play a major role dispersing seeds between patches (Fig. 4). Yet, they also suggest that most immigrant seeds arriving to a particular forest patch would belong to nearby fruiting trees located in the matrix (Fig. 3D), whenever these are present.

Features of seed dispersal events emerging from frugivore behaviour

We found a remarkable heterogeneity among frugivore species in dispersal distances for the seeds they deposited in the matrix as well as in the canopy cover along these dispersal events (Fig. 4). *Sylvia* and *Turdus* dispersed most seeds at distances below 300 m and through areas harbouring high canopy cover of isolated trees and forest edge, which would have acted as stepping-stones and corridors (Damschen *et al.* 2008; Herrera & García 2009). In contrast, *Columba* and *Sturnus* dispersed seeds over longer distances (especially *Columba*) and using the most open areas of the matrix. These findings support the idea that seed dispersal events arise from the interaction between landscape features and frugivore traits, including behaviour (Morales *et al.* 2013). First, the larger frugivores (*Columba* ≈ 500 g; *Sturnus* ≈ 85 g) dispersed seeds further than smaller ones (*Sylvia* ≈ 17 g; *Turdus* ≈ 70 g), as found in several systems (e.g. Jordano *et al.* 2007; González-Varo *et al.* 2013; Pérez-Méndez *et al.* 2016). On the other hand, frugivores dispersed the seeds though areas varying in canopy cover, according with their forest-dependence (i.e. *Sylvia* > *Turdus* > *Columba*; null in *Sturnus*). This is in line with observational studies documenting variability in spatial behaviour and response to forest loss among frugivorous birds (García *et al.* 2013; Morales *et al.* 2013).

But why did forest frugivores enter the matrix? Evidence from the observed seed dispersal patterns and frugivore densities (Appendix S3) suggests that *Turdus* and *Columba* actively left the forest, searching for the large crops of isolated wild olive trees of the matrix, on average ~5 times larger than crops from trees located in the forest (mean ≈ 115,000 and 25,000 fruits per tree, respectively; JPGV *unpubl. data*). Hence, seed dispersal in the matrix by

570 *Turdus* and *Columba* appeared to be driven by fruit-resource tracking (see García & Ortiz-Pulido 2004; e.g. Albrecht *et al.* 2012; García *et al.* 2013). In contrast, the patterns observed in
572 *Sylvia* suggest that seed dispersal in the matrix arose mostly from a passive spillover from the forest, during the nomadic displacements of this superabundant wintering bird (see Tellería *et*
574 *al.* 2005; González-Varo 2010).

576 ## *Applicability and generalization of the approach*

The use of microsatellite makers to identify the source plants has proven to be a milestone in
578 our understanding of seed dispersal patterns generated by animals (Godoy & Jordano 2001; Jordano *et al.* 2007). However, the identification of the animal species that dispersed the seeds
580 has been, until very recently, a pervasive constraint that has hindered a comprehensive characterization of the dispersal events generated by different frugivore species (see González-
582 Varo *et al.* 2014). Here, we combine for the first time DNA barcoding and DNA microsatellites to identify, respectively, the frugivore species (*who*) and the source plant (*from*
584 *where*) of individual seeds sampled in the field (*to where*), characterizing comprehensively how multiple frugivores disperse seeds through the landscape.

586 Our approach, based on two distinct DNA sources (Fig. 1), can be applied to many other systems, such as those in which microsatellite markers have already been used to identify
588 source plants (Jordano *et al.* 2007; Pérez-Méndez *et al.* 2016). Yet, source plant identification is not always feasible, particularly in very large populations where thousands of individuals
590 must be genotyped to obtain a decent number of maternal assignments. Two different approaches have dealt with this problem by providing statistical tools to characterize the
592 compositional diversity of seeds within and between sampling sites (e.g. seed traps). Seed clumps within and between sites can be characterized according to their genetic relatedness
594 (reviewed in García & Grivet 2011) or, alternatively, according to diversity indices (alpha, beta

and gamma) applied to the composition of seed sources (Scofield *et al.* 2012). Importantly, both approaches do not require identifying the location of the source trees and have proven useful to disentangle the spatial scale of seed dispersal by animals. Therefore, our approach can be generalized by combining DNA barcoding with microsatellite genotyping to obtain these statistics of compositional diversity of seed sources.

Concluding remarks

As far as we know, the patterns reported here constitute the most comprehensive direct empirical evidence (i.e. non-correlational) of how multiple frugivore species disseminate seeds through an anthropogenic landscape, from and towards different habitat types. Our findings provide novel insights into the role of frugivorous animals as mobile links (Lundberg & Moberg 2003; Kremen *et al.* 2007), uncovering in an unprecedented way key mechanisms behind 'biodiversity–ecosystem function' relationships (García & Martínez 2012; Schleuning *et al.* 2015). They also suggest that different (non-mutually exclusive) mechanisms may determine the role of different frugivore species as mobile links, including habitat-specificity, spatial behaviour and fruit-resource tracking (see also Albrecht *et al.* 2012; Morales *et al.* 2013).

Although long distance seed dispersal from forest trees and towards the matrix was infrequent, our study reinforces the importance of frugivores for the connectivity of plant populations and the colonization of vacant sites far from the forest. However, the fact that most seeds arriving to the matrix came from trees located there not only reveals the pivotal role of matrix plants on vegetation dynamics, it also suggests the potential of open-habitat frugivores to spread invasive fleshy-fruited species (Gosper *et al.* 2005), which typically occur in anthropogenic habitats (e.g. Lenda *et al.* 2012). In fact, open-habitat frugivores used landscape areas far from forest and, unlike forest frugivores, dropped seeds beneath infrastructures where

recruitment is possible (Kurek *et al.* 2015). The latter underscores the importance of addressing mobile-link functions between the natural and human-made elements of the matrix. Our study thus helps to widen the 'mobile link' concept in seed dispersal studies by providing a comprehensive and integrative view of how multiple frugivore species disseminate seeds through fragmented landscapes.

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J.P.G.V. conceived the study; J.P.G.V. and P.J. planned the sampling design, J.P.G.V., C.C. and P.J. collected the data in the field; J.M.A. and C.C. performed lab-work; J.P.G.V. conducted the statistical analyses and wrote the first manuscript draft. All authors contributed substantially to revisions and approved the final manuscript.

Data accessibility

Data associated to this article (seed rain density, seed-level information, barcoding sequences, microsatellite genotypes, coordinates of genotyped trees, and fully identified seed dispersal events) are deposited in Dryad (doi:10.5061/dryad.f9320).

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Aerial photograph and digitalized map of the study landscape showing the sampling sites, the microsatellite-genotyped trees and some seed dispersal events.

Fig. S2 Photographs illustrating different methodological components of this study.

Fig. S3 Example of a large wild olive tree located in the forest edge, the type of tree from the forest for which we targeted sampling for genotyping.

Fig. S4 Example of regeneration of wild olive trees beneath an electricity pylon.

Appendix S1 PCR protocols for leaf and endocarp genotyping.

Appendix S2 Details about the lack of evidence of seed dispersal from nearby olive orchards.

Appendix S3 Frugivore abundances in forest and matrix.

Table S1 Proportional similarity (PS index) in the contribution of different frugivorous birds to seed rain at different distance classes from the forest edge.

Table S2 Nonparametric Kendall's rank correlations (τ) testing for associations between the relative contribution (%) of different source habitats of wild olive trees to seed rain in the matrix at different distance classes from the forest edge, and the ranks of such distance classes.

Fig. 1 Scheme of a transversal section of a wild olive seed dispersed by a frugivore, showing the main DNA sources that can be sampled. Black arrows show the two DNA sources used in this study. The frugivore DNA can be extracted from cell and gut tissue remains present in defecated or regurgitated seeds. The source tree DNA can be extracted from the endocarp, which is the woody and maternally originated tissue surrounding the embryo.

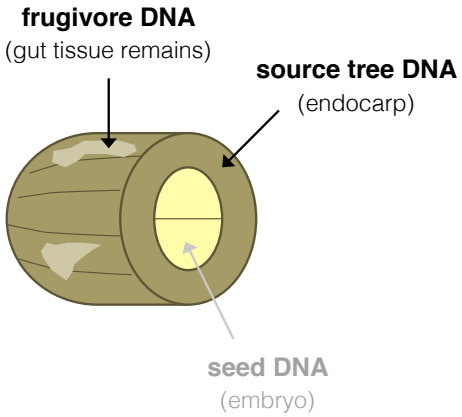


Fig. 2 Seed deposition network connecting frugivore species and the habitat or perch type where they dispersed the seeds (n = 532 dispersed seeds with frugivore identified through DNA-barcoding). Horizontal width of the links is proportional to the frequency of seed deposition by each frugivore species in each habitat (forest or matrix) or perch type (isolated trees or electricity pylons). The full species names of less frequent frugivores are *Erithacus rubecula (Er), Sylvia melanocephala (Sm), Parus major (Pm), Corvus monedula (Cm) and Phoenichuros ochruros (Po)*.

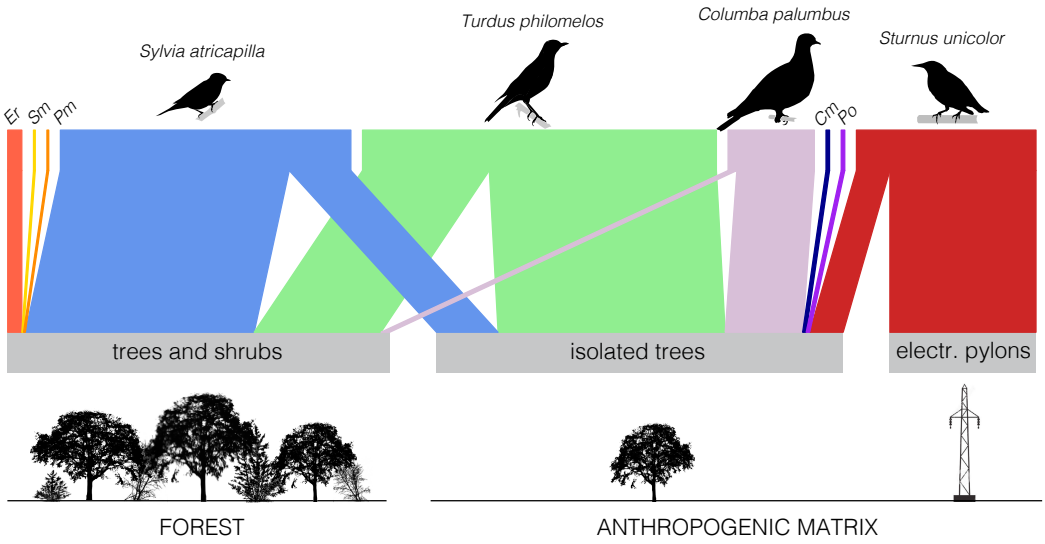


Fig. 3 Seed dispersal patterns (magnitude, vectors and sources) in the forest and at different distance classes from the forest edge. (A) Frugivore-mediated seed rain density measured in seed traps placed under natural perches; boxplot showing median, quartiles, and percentiles 5th and 95th (dots denote mean values). (B) Relative contribution (%) of different frugivore species to seed rain in natural microhabitats (e.g. trees, shrubs). (C) Relative contribution (%) of different frugivore species to seed rain in all microhabitats, including anthropogenic electricity pylons. Colour codes in (B) and (C) as in Fig. 2. (D) Relative contribution (%) of different source habitats (forest, matrix or unknown) to seed rain in the matrix.

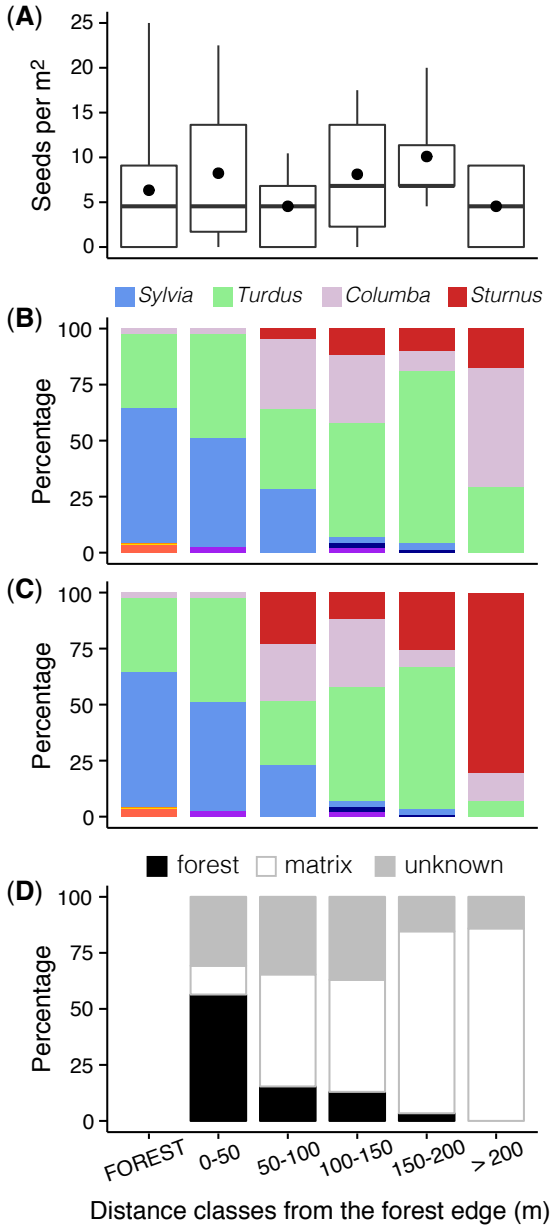


Fig. 4 Seed dispersal patterns in the matrix mediated by different frugivore species (rows). Left panels show the relative contribution (%) of different source habitats (forest, matrix or unknown) to seed rain at different distance classes from the forest edge; *n* = all seeds dispersed by each species (n_{Sylvia} = 35; n_{Turdus} = 129; $n_{Columba}$ = 44; $n_{Sturnus}$ = 102). Central and right panels show, respectively, the relative distribution (%) of seed dispersal distances and canopy cover along the dispersal events (within a 25-m buffer); *n* = all seeds dispersed by each species with identified source trees (n_{Sylvia} = 25; n_{Turdus} = 116; $n_{Columba}$ = 14; $n_{Sturnus}$ = 89); vertical lines denote median values and different letters denote significant differences between frugivores (MW *U*-tests). Canopy cover along seed dispersal events significantly differed from canopy cover in the landscape in all frugivore species but *Sturnus* (see inset; $n_{landscape}$ = 120, 100 × 100 m cells).

