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ORIGINAL PAPER

Non-invasive multi-species monitoring: real-time PCR detection of small mammal and squirrel prey DNA in pine marten (*Martes martes*) scats

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Abstract DNA identification of mammal species occurring in the diet of a predator is potentially a useful approach to remotely monitor the distribution of multiple species. This is important in Ireland, where it has been shown that the combined presence of the introduced bank vole and greater white-toothed shrew impact the distribution of the indigenous small mammals, the wood mouse and pygmy shrew. Direct monitoring of these species and their interactions requires trapping, a labour-intensive and costly approach. In this study, we applied an indirect method by genetically testing the presence of small mammals in pine marten scats collected during the National Pine Marten Survey (2005–2007) to map their distribution. We also included additional scats to investigate if less common prey items, the red squirrel and grey squirrel, could also be detected. This study demonstrates that all target species were genetically detected from pine marten scats. This strategy could be implemented as a monitoring programme for indigenous and introduced mammal species.

Keywords Molecular scatology · Mammals · Invasional meltdown · Ireland · *Apodemus sylvaticus* · *Sorex minutus* · *Myodes glareolus* · *Crocidura russula* · *Sciurus vulgaris* · *Sciurus carolinensis*

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Introduction

As an island situated on the westernmost periphery of Europe, Ireland has a limited assemblage of flora and fauna in comparison with its nearest neighbour Great Britain, and even more so compared with mainland Europe (Yalden 1999; Searle 2008). Irish mammals consist of a few natural colonisers and many naturalised species that are thought to have been introduced over thousands of years between the arrival of humans and AD 1500 (Searle 2008). A number of more recent introductions of mammal species have occurred over the last 100 years. In some cases, these recent arrivals have threatened the distribution of the current indigenous species' range, especially in the case of small mammals and squirrels (O'Teangana et al. 2000; Carey et al. 2007; Montgomery et al. 2012).

Indigenous small mammal species are limited to the wood mouse (*Apodemus sylvaticus*) and pygmy shrew (*Sorex minutus*). The rat (*Rattus norvegicus*) and house mouse (*Mus musculus*), both historically introduced, are also well

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established (Yalden 1999). Recently introduced small mammal species include the bank vole (*Myodes glareolus*) and greater white-toothed shrew (*Crocidura russula*). The bank vole now occupies almost a third of the island in the southwest, and the greater white-toothed shrew is found in Counties Tipperary and Limerick in the south-central part of Ireland (Tosh et al. 2008; Montgomery et al. 2012). The bank vole is believed to have been accidentally introduced into Limerick (west of Ireland) during the 1920s (Stuart et al. 2007). The greater white-toothed shrew was first identified in 2007 in owl pellets collected from County Tipperary, although its origins are not clear (Tosh et al. 2008). Montgomery et al. (2012) found that the combined presence of the bank vole and greater white-toothed shrew had negative impacts on the pygmy shrew distribution, resulting in its replacement, and local extinction; a term coined for this is the 'invasional meltdown.' The indigenous Eurasian red squirrel (*Sciurus vulgaris*) has also suffered declines in recent decades due to the introduction of the North American grey squirrel (*Sciurus carolinensis*) just over 100-years ago (O'Teangana et al. 2000; Carey et al. 2007).

The current distribution maps of all species can be accessed through the National Biodiversity Data Centre's mapping system, 'Data from the Atlas of Mammals in Ireland 2010–2015' (www.biodiversityireland.ie). Small mammals have, in the past, been greatly under-recorded in Ireland, and consequently very few records for wood mouse exist prior to 2010, despite the general assumption that the species is widespread across Ireland. Similarly, the pygmy shrew was also under-recorded prior to a nationwide population genetic study by McDevitt et al. (2009). More visible species such as the red and grey squirrel have been better recorded through public participation surveys (Carey et al. 2007). Consequently, disparities exist in our current ability to monitor less visible species such as small mammals compared with larger species, and such inabilities are now of conservation concern given the recent discovery that introduced species may be displacing indigenous ones.

A possible solution to this may be the wide-scale dietary analysis of carnivore scats such as the pine marten, as its diet in Ireland has been described as generalist and opportunistic (Lynch and McCann 2007). Wood mouse is typically a favoured small mammal in pine marten diet, mostly in the Mediterranean region, whereas vole (bank and field vole) consumption tends to be higher in northern latitudes (de Marinis and Marsseti 1995; Zalewski 2004; Zhou et al. 2011). Greater white-toothed shrew occurred in the pine marten diet in Spain (de Marinis and Marsseti 1995; Rosellini et al. 2008). In Ireland, Lynch and McCann (2007) found wood mouse, pygmy shrew and bank vole in the pine marten diet, and Warner and O'Sullivan (1982) found wood mouse and pygmy shrew (bank vole was not present in the study site). Both studies found a low occurrence of red squirrel in the diet, but grey squirrel and the greater white-toothed shrew were not present in either study site. As pine marten are now distributed across a large proportion of Ireland (O'Mahony et al. 2012), they may well be a suitable predator to remotely study the distribution of indigenous, introduced and invasive species.

Although technically possible, hard part analysis of a large number of predator scats over a large sampling area to infer mammal distribution may not be feasible taking constraints (labour, time, specialist training and overall cost) into consideration. However, over the last number of years, molecular scatology has emerged as a reliable tool to study the diet of mammals (Deagle et al. 2005; Murray et al. 2011; Shehzad et al. 2012; Zarzoso-Lacoste et al. 2013).

When dietary background information is known from previous hard part or molecular dietary analysis, specific species can be targeted using primers designed only to amplify the intended species. This can be addressed using real-time PCR, a technique well established for diagnostic studies (Dooley et al. 2004). Such diagnostic tools have useful applications in dietary studies, as the primers are designed to target short regions of DNA, suitable for detection of low quantity or degraded DNA, an inherent problem in dietary studies due to the breakdown of prey DNA in the predator's gut and environmental degradation. Real-time PCR is also suitable for the detection of prey items that are less abundant in the predator's scat and the use of diagnostic primers enables the detection of low quantity target DNA from mixtures containing more abundant DNA from other items. Successful applications include studies by Matejusová et al. (2008); Bowles et al. (2011), and Murray et al. (2011), where real-time PCR was applied to mammal and bird dietary studies to target species of interest. All studies found that real-time PCR was an accurate and cost-effective approach to address specific dietary questions.

In this study, we used real-time PCR to detect the presence of small mammals and squirrels using DNA extracted from pine marten scats collected during the National Pine Marten Survey (NPMS) 2005–2007 (O'Mahony et al. 2012). As squirrels were expected to be a minor component of the diet, we also collected additional scats from sites where red squirrels were known to occur and tested scats from a captive pine marten where grey squirrel was a food item. The primary purpose of this study was to map the distribution of target prey species, wood mouse, pygmy shrew, bank vole, and greater white-toothed shrew, and to assess if the technique was suitable for the detection of species that were expected to occur at low frequency in the diet, i.e., red squirrel and grey squirrel.

Materials and methods

Sample collection

A set of 252 pine marten scats were used to test for the presence of small mammals and squirrels (Table 1). These

Table 1 Pine marten (PM) sample collection information and detection of red squirrel (RS), grey squirrel (GS), wood mouse (WM), pygmy shrew (PS), bank vole (BV) and greater white-toothed shrew (GWTS) from pine marten scats. Known presence of target species in sample collection (+); known absence of target species (−)

Small mammal and squirrel detection					Squirrel detection				
Target	NPMS		Waterford		Midlands		Captive marten		Total
PM	168		84		223		23		498
RS	1	+	2	+	7	+	0	−	10
GS	0	+	0	−	0	−	12	+	12
WM	30	+	7	+					37
PS	26	+	9	+					35
BV	8	+	0	+					8
GWTS	6	+	0	−					6

samples were collected for the NPMS during the summer months (June–September) 2005–2007 (N=168) and from County Waterford (N=84). These samples originated from 153 10-km^2 sites across Ireland. The NPMS sample locations are mapped in O'Mahony et al. (2012) and Fig. 1. Samples collected in Portlaw, County Waterford (southeast Ireland) were collected in August 2010. As the occurrence of red squirrel in the diet was expected to be low, additional samples were collected from Counties Laois and Offaly (Midlands) (N=223), where the presence of red squirrels and the absence of grey squirrels were confirmed by a squirrel trapping study that took place during the scat collection, from March 2010 to

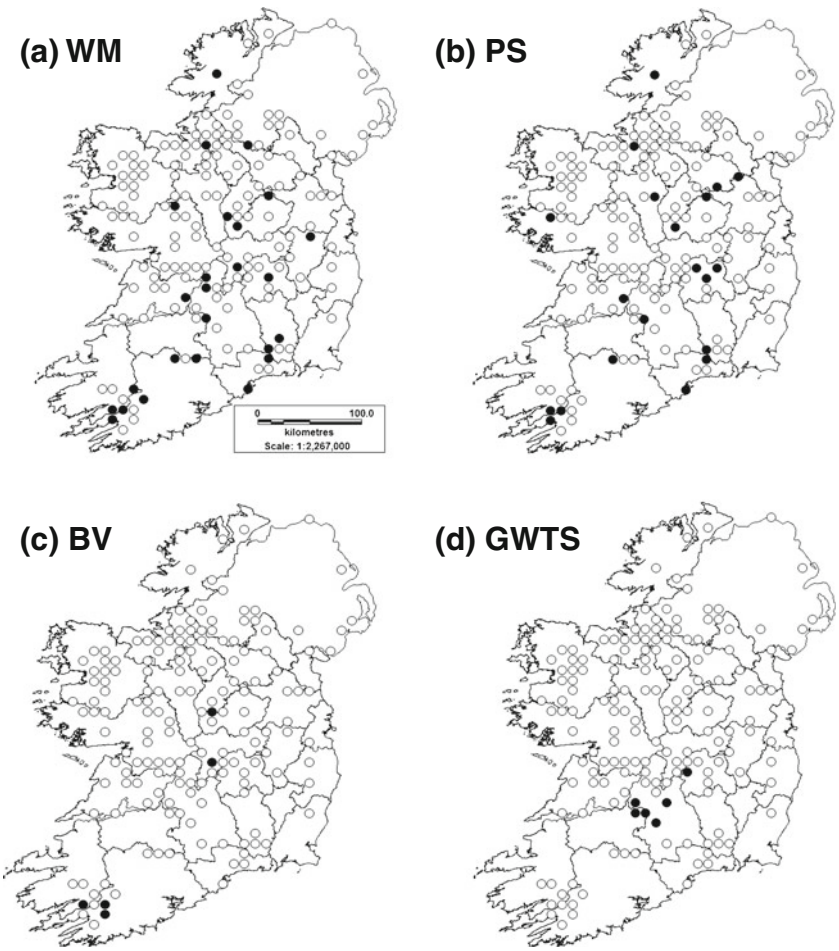

Fig. 1 Distribution of genetically identified small mammal species from DNA extracted from pine marten scats. Sample information can be found on Table 1. *Black circles*: positive detection, *white circles*: negative detection. Wood mouse (WM), pygmy shrew (PS), bank vole (BV), greater white-toothed shrew (GWTS)

September 2011 by E. Sheehy (Table 1). Grey squirrels have not been previously found to occur in the pine marten diet, and to establish a positive control, scats were collected from a captive pine marten that had been fed culled grey squirrels as part of its diet in Wales, UK (Table 1).

DNA analysis

Samples from NPMS were extracted and tested for pine marten DNA as part of the study of O'Mahony et al. (2012). For consistency, the same approach was taken with the remaining samples in this study. In brief, the DNA extraction was consistent with the study of O'Reilly et al. (2008), taking a small section (0.2 g) of faecal material from the outside of the scat. One DNA extract was isolated from each pine marten scat sample and genetically identified (Mullins et al. 2010). The samples from Table 1 (N=252) were tested for the presence of small mammal DNA (wood mouse, pygmy shrew, bank vole and greater white-toothed shrew) using the assays described by Moran et al. (2008), and the greater white toothed shrew assay was designed as part of this study. All scats (N=475) were tested for red and grey DNA using the assays described by O'Meara et al. (2012). Real-time PCR reactions were conducted as described by the associated studies, with the exception that DNA extracts were not diluted (1 μl of neat DNA). All real-time PCR reactions were performed singly, i.e., each target species was tested in isolation due to expected low quantities of target DNA that may be more accurately detected in singleplex real-time PCR reactions. Species were identified based on positive amplification with the species-specific assay and the corresponding Ct value was recorded (see Information box S1). Positive results were replicated at least twice for verification.

Greater white-toothed *shrew assay* design and sequencing

Conventional PCR primers (CrocF-482: 5′-CGCTTCTTCG CATTTCACTTT-3′, CrocR: 5′CATGTTAATGTAAAGAG GTCCGCTAC-3′) for the greater white-toothed shrew were designed to amplify and sequence a 482-bp region of mitochondrial cytb gene using haplotypes published by Brändli et al. (2005) (GenBank accession numbers: AY918341–AY918400). An internal primer (CrocF: 5′-ATAAGCCAAT GCATATTCTGAATTTTAG-3′) was subsequently designed to work in conjunction with CrocR to target a short 52-bp product using real-time RCR (designed to amplify degraded DNA). Primers were designed using Primer Express 2 software (Applied Biosystems) and ordered from Eurofins MWG Operon.

Conventional PCR consisted of 5 μl of GoTaq Hot Start Green Master Mix (Promega), 2 μM of each primer and 1 μl of DNA extract in a total volume of 10 μl. Negative controls contained water instead of DNA. The PCR programme consisted of 95 °C for 5 min followed by 40 cycles of 94 °C for 60 s, 56 °C for 60 s and 72 °C for 60 s followed by 5 min elongation at 72 °C. PCR products were separated and visualised on 2 % agarose gel stained with ethidium bromide. The PCR products were purified using DNA Clean and Concentrator-5 Kit (Zymo Research) and sequenced in both directions with BigDye Terminator Cycle Sequencing Kit 3.1 (Applied Biosystems). Sequences were obtained by running the products on an ABI 310 DNA sequencer (Applied Biosystems) and analysed using BLAST software at http://www.ncbi. nlm.nih.gov/BLAST/ (Altschul et al. 1997). The real-time PCR reaction followed the protocol described in Moran et al. (2008) using 1 μl of undiluted genomic DNA extract.

All genetically identified small mammal species were mapped onto Ordnance Survey Ireland (OSI) maps using MapInfo 11.0 GIS software using the same 10-km^2 grid as O'Mahony et al. (2012). Negative sites were also included for each small mammal species. We used the sample collection from the NPMS (N=168) to assess the occurrence of multiple small mammals in the diet, as this collection covered the largest geographical area and contained the highest number of species.

Results

In the field study, scat samples collected at all sites were firstly tested for the presence of pine marten DNA using real-time PCR and samples with a Ct value ≤33 were deemed positive and suitable for subsequent dietary analysis. This value was selected as a threshold above which it would be difficult to detect prey DNA. Prey DNA extracted from predator scats is known to be of lower quantity and quality than the species that deposited the scat (Matejusová et al. 2008). Genetically testing the pine marten scats also confirmed that the scats were of pine marten origin. The range of positive Ct values for the small mammal and squirrel assays were similar across species 22–37 (Table S2). Grey squirrel DNA was not detected in any of the field collected samples but occurred in 12 of the 23 scats collected from the captive pine marten. The Ct values varied both within and between species, indicating that scats contained varying quantities of target prey DNA ranging from abundant to moderate (see Information box S1).

The greater white-toothed shrew assay that was designed for this study was found to be species-specific. The presence of greater white-toothed shrew was further confirmed by PCR amplification with species-specific primers and DNA sequencing two positive samples. Samples with the lowest Ct values were selected for sequencing, as they contained the highest quantity of target DNA and were more likely to

produce the most accurate sequencing results. The 457-bp consensus sequence obtained from both samples was identical and was a new haplotype to those in the current database and showed maximum homology (99.8 %) to haplotypes from France and Switzerland (Brändli et al. 2005). The haplotype has been deposited in GenBank (accession number: JX424288).

Wood mouse was found to be the most common small mammal encountered in pine marten scats, followed by pygmy shrew, bank vole, greater white-toothed shrew, and red squirrel (Table 1). When mapped, wood mouse was detected in 25 of the 10-km^2 sites, pygmy shrew was found in 26 sites, bank vole in five sites, and greater white-toothed shrew in six sites (Fig. 1).

Based on the NPMS scats, prey DNA was detected from at least one small mammal species (i.e., wood mouse, pygmy shrew, bank vole or greater white-toothed shrew) in 32.74 % of scats. One species of mammal was detected in 25 % of scats, 6.55 % contained two species and 1.79 % contained three species. Wood mouse was most commonly found in single scats, with both wood mouse and pygmy shrew most often found to co-occur in the same scat (N=10), although geographically there was further overlap as some 10-km^2 sites contained more than one scat. The scats that contained DNA from three species contained wood mouse, pygmy shrew and greater white-toothed shrew in one scat, and the second scat contained wood mouse, pygmy shrew and bank vole. The greater white-toothed shrew was found to occur singly in the remaining samples (N=5). Bank vole occurred in the same scat as wood mouse on a single occasion, and bank vole was found not to co-occur with other species (pygmy shrew and wood mouse) in the remaining samples (N=6). Small mammals were also found to overlap geographically in different scats from the same site in several instances, especially in County Waterford.

The distribution of the bank vole found in pine marten scats in this study (Fig. 1) was within the known distribution of the species from 2010 (Montgomery et al. 2012), and despite the widespread distribution of the species, it did not feature prominently across its range in the pine marten diet. However, the distribution of the greater white-toothed shrew was larger than previously described for the species range during 2008 (Tosh et al. 2008; Montgomery et al. 2012). The results show that the greater white-toothed shrew occurred in a sample collected in County Laois, further north than previously described. Although the sample size is small, in the area where greater white-toothed shrew was detected, its occurrence appears not to cluster with other small mammal species more commonly detected elsewhere, and wood mouse and pygmy shrew did not prominently feature.

The red squirrel was detected as a low frequency item but was detected more often in the sample collection from the Irish midlands than in the samples from NPMS and County Waterford. Samples collected from the captive pine marten in Wales showed that grey squirrel could be detected in the pine marten diet, if it was present, but only between one and two days postfeeding. Grey squirrel did not occur in the field-collected scats in this study. Grey squirrels were not known to occur in the scat collection sites in the Irish Midlands or County Waterford, although there was a possibility of overlap between grey squirrel and pine marten ranges in the NPMS sample collection.

Discussion

This study aimed to develop a protocol for the detection of six species in the diet of pine marten, small mammals (wood mouse, pygmy shrew, bank vole and greater white-toothed shrew) and squirrels (red squirrel and grey squirrel), by exploiting a set of previously developed non-invasive species-specific assays; this study also designed two new assays for the detection and sequencing of the recently discovered greater white-toothed shrew. This offers two new approaches for the non-invasive identification of the greater white-toothed shrew, using real-time PCR and conventional PCR. The latter is useful for wildlife labs that do not have real-time PCR facilities, but the sensitivity of that assay is lower due to the larger size DNA fragment. The objective of this work was to investigate a non-invasive method that could be used to detect target species DNA in pine marten scats. This study reports the first successful application of real-time PCR to detect small mammals and squirrels in the diet of genetically identified pine marten scats. It is also the first study to use predator dietary analysis to indirectly map the distribution of indigenous and introduced small mammal prey species.

Small mammal detection

The detection of target small mammal DNA in scats was quite low; 67.23 % of samples from NPMS did not contain target small mammal DNA. However, previous dietary studies of the pine marten in Ireland have shown that other food items such as fruit, birds, reptiles and invertebrates form a significant part of the diet (Warner and O′Sullivan 1982; Lynch and McCann 2007). The occurrence of small mammals in the diet of pine marten in this study was found to be broadly comparable with other Irish studies (Warner and O′Sullivan 1982; Lynch and McCann 2007), with the exception of the pygmy shrew and red squirrel (detected more often in this study) that may be due to the increased sensitivity of real-time PCR (Matejusová et al. (2008).

The wood mouse and pygmy shrew were the most commonly detected species in this study (Table 1, Fig. 1). The pygmy shrew may be perceived to be an unpalatable prey item due to the presence of scent glands, but the species was detected relatively frequently in this study. Other carnivore

dietary studies have also found shrews to be an important item (e.g., Baltrūnaitė 2002). The bank vole presented at a low level in the pine marten diet, despite a greater possibility of it appearing in the diet than the greater white-toothed shrew, as that featured prominently in the pine marten diet within its known range.

It is likely that the greater white-toothed shrew has been longer established in Ireland than the time of its initial discovery in Counties Tipperary and Limerick in 2007 (Tosh et al. 2008). We have shown that the greater white-toothed shrew was present in those counties as well as in County Laois during 2005–2007, Fig. 1). Although the number of samples in this study that contained greater white-toothed shrew were low, it is worth noting that that pine marten may be preferentially predating on the greater white-toothed shrew, or alternatively, other potential prey items such as the pygmy shrew were already displaced by the presence of greater white-toothed shrew and bank vole, as described by Montgomery et al. (2012). The results in this study possibly support the latter. If that is the case, such displacement had occurred as early as 2005–2007. Although this theory needs further investigation, the tools developed in this study can be used to test this.

Squirrel detection

There was no evidence of grey squirrel DNA in any of the field collected scats, but as already mentioned, the majority of scats were collected from sites that were either outside the known range of the grey squirrel or where grey squirrel had been confirmed as absent during sample collection. However, we have shown that grey squirrel can only be detected one to two days after consumption, leaving a short opportunity to detect a species that rarely occurs in the diet. There was also no evidence of grey squirrel in the samples from the NPMS despite the potential for some overlap between the species (Carey et al. 2007; O'Mahony et al. 2012). Red squirrel was detected more often in the samples from the Midlands, but these samples were collected throughout the year and previous studies have shown that the consumption of red squirrel by pine marten is more likely to occur in the winter months in Ireland and Scandinavia (Warner and O'Sullivan 1982; Helldin 1999; Helldin 2000). Samples from NPMS and County Waterford were collected during the summer months and that may explain the slightly lower detection of red squirrel in that sample set. More intensive studies at a finer spatial scale in areas where both pine marten and grey squirrels are confirmed to be present are required to investigate any relationship between the species, in terms of predation or impacts on species range.

Advantages of a molecular approach

The increased throughput of samples with molecular analysis enables efficient large-scale sampling of small mammals and to infer mammal distribution. The detection of mammalian prey items in this study were comparable with previous hard part analysis studies, implying that the DNA extraction protocol and subsequent molecular detection techniques used in this study were found to extract both the host and mammalian prey DNA efficiently.

The primer selection criteria in molecular scatology studies have been described as a critical component for detecting low quantity DNA targets (Zarzoso-Lacoste et al. 2013), but the real-time PCR primers used in this study were developed to be species-specific and to amplify a short amplicon, thus making their application to molecular scatology studies very useful. This study demonstrates an efficient approach to facilitate high-throughput dietary analysis and could be used to screen large numbers of scats prior to additional quantitative hard part analysis, making this technique particularly useful for species that occur in low frequencies such as squirrels.

Warner and O'Sullivan (1982) reported that almost 3 % of the mammal prey items could not be identified to species. Traditional techniques used to identify prey remains from domestic cats in Poland note that intact skulls from small mammals are rarely found in carnivore scats, making identification of small bone remains more difficult (Krauze-Gryz et al. 2012). The visual identification of prey remains can be difficult due to the fragmentation process that takes place during digestion, making molecular detection a practical (and possibly necessary) alternative (Zarzoso-Lacoste et al. 2013).

Both Lynch and McCann (2007) and Warner and O'Sullivan (1982) identified scats by relying on the smell only, a method even expert field pine marten surveyors agree can be misleading (Ruiz-González et al. 2007; Balestrieri et al. 2011; Caryl et al. 2012). Subsequently, the overall dietary content could contain errors if the species had originally been misidentified. In this study, the scats were all identified to species using DNA identification, and the same DNA extract was used for dietary analysis, a cost-effective approach to increase the information gained from a single DNA extraction.

Conclusion

This study demonstrated the use of a predator diet to monitor small mammals in Ireland and has the potential to be used as a technique to monitor the spread and decline of small mammals. This technique can potentially be applied to the dietary analysis of other predators in Ireland including fox (*Vulpes vulpes*), American mink (*Mustela vison*) and even bird of prey pellet analysis. The protocol promises to greatly improve our understanding of multi-species systems and will be valuable for researchers, wildlife conservationists and the overall management of indigenous and introduced species.

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