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The long and the short of it: long-styled florets are associated with higher outcrossing rate in *Senecio vulgaris* and result from delayed self-pollen germination

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Background: It has been reported that some plants of the self-compatible species *Senecio vulgaris* produce capitula containing long-styled florets which fail to set seed when left to self-pollinate, although readily set seed when self-pollinated by hand.

Aims: To determine if production of long-styled florets is associated with higher outcrossing rate in *S. vulgaris*, and whether long-styles occur in non-pollinated florets, whereas short-styles are present in self-pollinated florets.

Methods: The frequency of long-styled florets was compared in the radiate and non-radiate variants of *S. vulgaris*, known to exhibit higher and lower outcrossing rates, respectively. In addition, style length was compared in emasculated florets that were either self-pollinated or left non-pollinated.

Results: Long-styled florets were more frequent in the higher outcrossing radiate variant. Following emasculation, long styles occurred in non-pollinated florets, while short styles were present in self-pollinated florets. The two variants did not differ in style length within the non-pollinated or within the self-pollinated floret categories.

Conclusions: A high frequency of long-styled florets is associated with higher outcrossing rate in *S. vulgaris* and results from delayed self-pollination and pollen germination on stigmas.

Keywords: Asteraceae; outcrossing rate; pollination; pollen germination; ray and disc florets; selfing; *Senecio*; style length

Introduction

Understanding the causes, effects and maintenance of variation in outcrossing rate between and within species is of importance in the study of plant evolution (Goodwillie et al. 2005, 2010; Porcher et al. 2009). Most flowering plants are hermaphrodite (Richards 1986) and a large proportion of these are self-compatible and able to reproduce by a mixture of selfing and outcrossing. Although theory predicts that mating systems should evolve towards a stable system of either predominant selfing or outcrossing (but see Winn et al. 2011; Barrett 2014), it has been estimated that ca. 40% of plant species exhibit a mixed mating system where outcrossing rate ranges between 0.2 and 0.8 across species (Goodwillie et al. 2005). A review of the influence of floral display traits on plant-mating system variation (Goodwillie et al. 2010) showed that outcrossing rate was positively associated with the product of flower size and number. Outcrossing rate is also positively associated with pollen–ovule ratio across species (Cruden 1977), although not necessarily within populations of a species (Damgaard and Abbott 1995). Other floral traits that promote outcrossing in self-compatible plants include protandry (anther dehiscence preceding stigma receptivity within a flower), protogyny (stigma receptivity preceding anther dehiscence) and

herkogamy (separation of anthers and stigmas in space) (Ennos 1981; Schoen 1982; Richards 1986).

A particularly interesting system for examining the causes, effects and maintenance of intraspecific variation in outcrossing rate is present within the self-compatible species *Senecio vulgaris* L. (Asteraceae). Plants of this species usually produce non-radiate (discoïd) capitula (flower heads) containing only hermaphrodite disc florets, although radiate variants are known that bear capitula containing an outer whorl of female (pistillate) ray florets surrounding a central disc of hermaphrodite disc florets. In Britain, the radiate variant *S. vulgaris* var. *hibernicus* originated within the last 200 years as a result of gene flow (introgression) from *S. squalidus* L. into *S. vulgaris* (Abbott et al. 2009; Lowe and Abbott 2015) and forms mixed populations with the non-radiate variant at many locations (Abbott et al. 2003; Stace et al. 2015). Presence of ray florets in capitula of this variant is controlled by two tightly linked genes (Kim et al. 2008) inherited together as a single unit (Trow 1912). Progeny resulting from crosses between *S. vulgaris* var. *hibernicus* and non-radiate *S. vulgaris* are easily identified as they produce capitula with shorter ray florets than var. *hibernicus* (Trow 1912). In populations containing both variants, the radiate type always exhibits a higher outcrossing rate

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(Marshall and Abbott 1982, 1984a), because it is more attractive to pollinators (Abbott and Irwin 1988) and its female ray florets outcross at a higher rate than hermaphrodite disc florets (Marshall and Abbott 1984b).

Pollination in *S. vulgaris* involves the plunger or “bottle-brush” system typical of the Asteraceae (Müller 1883). Thus, in each hermaphrodite disc floret, the five anthers are joined at their margins and form a tube near the apex of the corolla tube above the style. Pollen is released and, as the style elongates, it is pushed through the anther tube and out of the floret by sterile hairs protecting a closed and unreciprocative bifid stigma. In this way, pollen is presented to pollinators before the stigma is receptive, thus promoting opportunities for cross-pollination. On opening, the upper surface of the bifid stigma is receptive to both cross- and self-pollen. Interestingly, Trow (1912) reported that some non-radiate *S. vulgaris* plants bear capitula with florets containing long styles with stigmas positioned well above the apex of the corolla tube, while capitula of other plants possess florets having shorter styles with stigmas located just above the anthers (Figure 1). Trow (1912) noted that capitula having long styles “. . . are very sterile, if selfed, but fertile to their own pollen, yielding seed quite freely when pollinated with their own pollen or that of neighbouring long- or short-styled capitula”. Trow probably meant here that capitula having long styles are very sterile “if left to self” (i.e., to auto-self-pollinate) because they do set seed when “. . . pollinated with their own pollen. . .”, that is when self-pollinated either by a pollinator or by hand.

The occurrence of longer styles with stigmas positioned higher above the ring of anthers might prevent stigmas from coming into contact easily with self-pollen following anthesis, thereby delaying self-pollination. If pollination does not occur immediately after the stigmatic arms open, the arms reflex and on shorter styles stigmas will pick up self-pollen more easily from the capitulum surface. Another possibility is that long styles might reflect a delay in self-

pollination and pollen germination on stigmas due to other causes. Such a delay, however caused, will extend the opportunity for cross-pollination and cross-fertilisation to occur. Thus, plants with capitula exhibiting a high frequency of hermaphrodite, long-styled, disc florets might be expected to show higher outcrossing rates than plants bearing capitula containing fewer such florets.

In the study reported here, we tested whether outcrossing rate is positively associated with frequency of long-styled florets in *S. vulgaris* by determining whether the frequency of such florets is greater in capitula produced by the more outcrossing radiate variant relative to the more selfing non-radiate variant. We further examined whether the occurrence of long-styled florets reflects delayed self-pollination and pollen germination by comparing style length in non-pollinated and self-pollinated emasculated florets.

Materials and methods

Plants of the radiate (var. *hibernicus*) and non-radiate variants of *S. vulgaris* were grown from seed in an illuminated greenhouse. Seed came from inbred lines derived (following ca. 10 generations of selfing) from plants that occurred in populations containing both variants at Cardiff and Rhosllanerchrugog (Wales, UK) (see Marshall and Abbott 1984a). Seed was sown on universal compost in 7 cm diameter pots. Following germination, seedlings at a height of ca. 3 cm were thinned to one per pot, repotted into 11.5 cm diameter pots at a height of ca. 5 cm and grown to the flowering stage with water supplied as necessary. During winter, natural light was supplemented with artificial lighting supplied by 400-watt mercury vapour lamps to provide a 16 h photoperiod. Ambient temperature was maintained at ca. 20°C throughout the growth period.

Frequency of long-styled florets in capitula of radiate and non-radiate plants

Plants were examined of eight inbred lines of each variant derived from the Cardiff population and of 10 inbred lines of each variant derived from the Rhosllanerchrugog (hereafter Rhos) population. Shortly after flowering began, florets in 4–12 capitula per plant were marked with a fine-tipped pen containing indelible ink at the stage when they had opened and stigmas had become receptive to pollen. Marked florets were designated as either “outer florets” (present in the outermost whorl of a capitulum) or “inner florets” (present in the remaining inner whorls of a capitulum). In the radiate variant, ray florets comprised the “outer floret” category. After 24 h, each marked floret was removed and fixed in formaldehyde alcohol acetic acid (FAA acid, 10%, 50%, 5% plus 35% double-distilled H₂O). Subsequently, a record was taken of the number of florets containing long or short styles in each of the “outer” and “inner” floret fractions.

The frequencies of long-styled florets in the “outer” and “inner” floret categories were calculated as

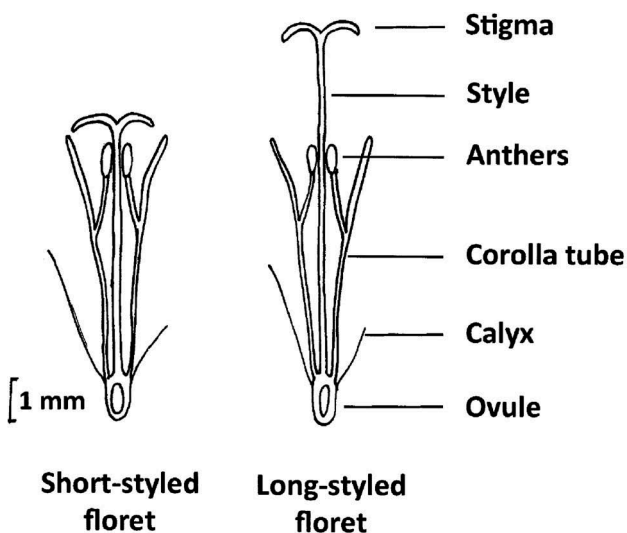


Figure 1. Longitudinal sections of short- and long-styled hermaphrodite disc florets present in capitula of *Senecio vulgaris*.

percentages that were arcsine transformed before conducting analysis of variance (ANOVA) on the total data set using the generalised liner model procedure (Proc GLM) of the SAS statistical software v.9.3, USA (SAS 2011). ANOVA determined whether differences were significant between the means of the three main effects, i.e., populations (Cardiff vs. Rhos), variants (radiate vs. non-radiate) and floret positions (outer vs. inner), and also whether the interactions between these main effects were significant.

Style length in non-pollinated vs. self-pollinated emasculated florets within radiate and non-radiate capitula

A single capitulum was selected on individual plants representing each of five inbred lines of both radiate and non-radiate variants derived from each of the Cardiff and Rhos populations. At the stage shortly before florets began opening and anthers had dehisced, each capitulum was emasculated using a razor to remove the top 2–3 mm of corolla tube containing the anthers. At this stage, the style was positioned below the ring of anthers and remained intact and undamaged. Emasculated capitula were immediately covered by a small bag made from lens tissue and left until stigmas in each floret became receptive to pollen a day or so later. Each capitulum was then divided into two by inserting a small square of thin acetate sheet vertically into the capitulum. The florets in one half of the capitulum were selfed by applying pollen from a newly opened capitulum on the same plant, while those of the other half were left unpollinated. The square of acetate sheet served as a barrier between the two treatments, thus preventing accidental pollination of “non-pollinated” florets. Following treatment, each capitulum was rebagged and left for 24 h before 10 florets were collected from each half and measured for style length using a graticule with 10× magnification. For convenience, measurement was made from the base of the ovary to the point of the style where the two stigmas separated. Thus, “style length” as recorded included the length of the ovary which added ca. 1 mm to the actual style length.

To check that self-pollinated stigmas had germinated pollen on them while non-pollinated stigmas were

pollen free, styles from a further five florets from each half of a capitulum were collected and fixed. The stigmas were checked for pollen grain germination using decolourised aniline blue staining and viewed with a Leica fluorescence microscope fitted with an ultraviolet light filter (method modified from Martin 1959). Whereas all stigmas of non-pollinated styles were found to be pollen-free, 90% of self-pollinated styles had germinated pollen on their stigmas. The lack of germinated pollen on the remaining 10% of self-pollinated stigmas could reflect that they had not been successfully pollinated or that germinated pollen had been accidentally washed off during the examination procedure.

Style length data were subjected to a repeated ANOVA with population (P) and variant (V) considered as fixed effects and pollination treatment (PT) considered as the repeated factor. This analysis tested for an overall significant difference between pollination treatments (self-pollinated vs. non-pollinated) and also significance of differences between population (P), variant (V) and of all interactions among these three factors, i.e., $PT \times P$, $PT \times V$, $P \times V$, and $PT \times P \times V$. Prior to analysis, the homoscedasticity of residuals of the model was visually checked. Analysis was conducted using Proc GLM of the SAS statistical software v.9.3, USA (SAS 2011).

Results

Frequency of long-styled florets in capitula of radiate and non-radiate plants

The radiate variant produced capitula containing a higher frequency of long-styled florets (Table 1). This was apparent in both the “outer” and “inner” floret fractions. It was also evident that in both the radiate and non-radiate variants there was a higher frequency of long-styled florets in the outer whorl of the capitulum than in the inner whorls. These differences were highly significant ($P < 0.0001$, Table 2). However, there was no significant difference in mean frequency of long-styled florets between the two populations examined (Cardiff vs. Rhos), and none of the interactions between the three main effects were significant (Table 2).

Table 1. Mean frequency (percentage) of long-styled florets per capitulum in the outer whorl and inner whorls for the radiate and non-radiate variants of *Senecio vulgaris*. Standard deviation (sd) is in brackets along with the mean number of florets examined per capitulum (n).

		Floret position	
		Outer whorl	Inner whorls
Variant	Radiate	75.92 (sd = 0.12, n = 10.74)	57.56 (sd = 0.17, n = 35.45)
	Non-radiate	57.98 (sd = 0.09, n = 9.60)	38.76 (sd = 0.10, n = 38.87)

Table 2. Results of ANOVA of frequency (percentage) of long-styled florets per capitulum. The two variants of *Senecio vulgaris*, radiate and non-radiate, were derived from each of two populations at Cardiff and Rhosllanerchrugog (Rhos) in Wales.

Source	df	Sum of squares	Mean square	F	P
Population (P)	1	0.0032	0.0032	0.20	0.6535
Variant (V)	1	0.5732	0.5732	36.17	<0.0001
P × V	1	0.0239	0.0239	1.51	0.2236
Floret position (FP)	1	0.6125	0.6125	38.66	<0.0001
P × FP	1	0.0074	0.0074	0.47	0.4967
V × FP	1	0.0005	0.0005	0.03	0.8605
P × V × FP	1	0.0013	0.0013	0.08	0.7735
Error	64	1.0141	0.0158		
Corrected total	71	2.2927			

Style length of non-pollinated vs. self-pollinated emasculated florets in radiate and non-radiate capitula

Following emasculation, non-pollinated styles were significantly ($P < 0.0001$) longer than styles that were manually self-pollinated and left for 24 h (Tables 3 and 4). This was the case for both the radiate and non-radiate variants irrespective of the population from which they were derived. There was no significant

difference between the two variants or the two populations in mean style length within either the non-pollinated or self-pollinated treatment (Tables 3 and 4) and none of the interactions between pollination treatment, populations and variants were significant (Table 4). Thus, the styles of both variants grew to the same length if not pollinated, and either ceased growing or shrank to the same length when self-pollinated.

Table 3. Mean style length (mm) of non-pollinated and self-pollinated florets of the radiate and non-radiate variants of *Senecio vulgaris* 24 h after treatment. Both variants came from populations at Cardiff and Rhosllanerchrugog (Rhos) in Wales. sds are in brackets.

		Pollination treatment (PT)	
		Non-pollinated	Self-pollinated
Population (P)	Variant (V)		
Cardiff	Radiate	10.34 (sd = 0.82)	6.28 (sd = 0.38)
	Non-radiate	10.29 (sd = 0.77)	6.03 (sd = 1.27)
Rhos	Radiate	10.55 (sd = 0.23)	6.40 (sd = 0.81)
	Non-radiate	10.23 (sd = 0.27)	6.94 (sd = 0.53)

Table 4. Results of repeated measures ANOVA of style length for (a) differences between pollination treatments (PT) and the interactions between pollination treatments (PT), populations (P) and variants (V); and (b) differences between populations and variants and their interaction. Variants are the radiate and non-radiate variants of *Senecio vulgaris* derived from populations at Cardiff and Rhosllanerchrugog (Rhos) in Wales.

Source	df	Sum of squares	Mean square	F	P
(a)					
Pollination treatment (PT)	1	1.5504	1.5504	275.87	<0.0001
PT × P	1	0.0048	0.0048	0.86	0.3682
PT × V	1	0.0027	0.0027	0.49	0.4951
PT × P × V	1	0.0071	0.0071	1.26	0.2775
Error	16	0.0899			
(b)					
Population (P)	1	0.0088	0.0088	1.90	0.1866
Variant (V)	1	0.00003	0.00003	0.01	0.9361
P × V	1	0.0017	0.0017	0.36	0.5552
Error	16	0.0739	0.0046		

Discussion

The results reported here show that the radiate variant of *S. vulgaris* (var. *hibernicus*) produces capitula containing a higher frequency of long-styled florets compared with the non-radiate variant. In both variants, long-styled florets were more frequent in the outer whorl than in the inner whorls of a capitulum. However, the radiate variant produced more long-styled florets relative to the non-radiate variant in both outer and inner whorls. Trow (1912) pointed out that long-styled florets in *S. vulgaris* tend not to set seed if left to auto-self-pollinate, although they do set seed if self- or cross-pollinated by pollinators or by hand. Thus, plants with capitula containing a higher proportion of long-styled florets might be expected to exhibit greater potential to outcross, and the fact that in mixed populations, the radiate variant does consistently outcross at a higher frequency than the non-radiate variant (Marshall and Abbott 1982, 1984a) lends support to this hypothesis.

In long-styled florets, receptive stigmas are positioned at a greater height above the apex of the corolla tube, and therefore, the apical surface of the capitulum on which self-pollen is deposited after it is pushed out of florets by elongating styles. Consequently, even when the two arms of a receptive stigma on a long style reflex towards the capitulum surface, they may not easily pick up self-pollen. In contrast, the reflexed arms of a stigma on a shorter style will be expected to pick up self-pollen more easily and become self-pollinated. This could be one reason why increased outcrossing is associated with long style length in *S. vulgaris*, i.e., the development of a long style constrains self-pollination. However, the results of the experiment that compared the mean style lengths of non-pollinated vs. self-pollinated styles in emasculated florets suggest an alternative reason for this association. This experiment showed that 24 h after styles had been either self-pollinated or left non-pollinated, non-pollinated styles of both the radiate and non-radiate variants were long and of similar length, whereas self-pollinated styles of both variants were of a similar shorter length. Most pollinated styles (90%) were found to have germinated pollen on their stigmas, whereas germinated pollen was absent, as expected, from stigmas of non-pollinated styles. Thus, there was no indication from the results of this experiment that non-pollinated styles of the non-radiate variant are inherently shorter than those of the radiate variant or that self-pollination causes the production of shorter styles in the non-radiate variant relative to the radiate variant.

The difference in length between non-pollinated and self-pollinated styles in both variants could have arisen if non-pollinated styles had continued to elongate during the 24 h period, whereas self-pollinated styles stopped elongating over the same period. Alternatively, the difference may have arisen if both non-pollinated and self-pollinated styles were similarly elongated at the start of the 24-h treatment period, but self-pollinated styles shrank in length

during the 24 h following self-pollination whereas non-pollinated styles remained elongated. We did not measure style length at the start of the 24-h treatment period and so cannot distinguish between these two possible explanations from our results. However, evidence from a study by Wagenius (2004) on self-incompatible *Echinacea angustifolia* (Asteraceae) indicates that the second explanation is more likely. This study showed that receptive styles of *E. angustifolia* are exerted from florets prior to pollination and remain exerted for up to 10 days if self-pollinated, but shrink in length within 24 h when cross-pollinated. Because *E. angustifolia* is self-incompatible, the effect of self-pollination in this species can be considered equivalent to non-pollination in self-compatible *S. vulgaris*, in that, it results in failure of pollen germination on stigmas. Thus, if the effect of pollen germination on style length reported in *E. angustifolia* is general for the Asteraceae, we may conclude that in *S. vulgaris* short styles result from shrinkage of long styles within 24 h of pollen deposition and germination on stigmas.

Based on the results of our second experiment, we propose that the development of long-styled florets in capitula of *S. vulgaris* is an effect of delayed self-pollen deposition and germination on stigmas. Thus, florets in which self-pollen deposition and germination is delayed contain long styles, whereas florets in which self-pollen deposition and germination proceed quickly contain short styles.

Two questions remain regarding the relative frequencies of long-styled and short-styled florets in capitula. First, why are long-styled florets more frequent in the outer whorl of the capitulum than in the inner whorls? The answer seems straightforward for the radiate variant, in that, the outer whorl comprises pistillate ray florets in which anthers are absent, whereas the inner whorls comprise hermaphrodite disc florets containing both functional anthers and pistils. Thus, given the centripetal development of florets in the capitulum resulting in florets opening sequentially first in the outer whorl and later in the inner whorls, styles of ray florets are likely to elongate to their full length before self-pollen is available from hermaphrodite florets located within the same capitulum. Thus, with their styles elongated, there is likely to be a significant delay in self-pollen deposition on stigmas of these florets, which will increase their opportunity to outcross (Marshall and Abbott 1984b). It is more difficult to explain why long-styled florets are also more common in the outer than the inner whorls of non-radiate capitula. The number of pollen grains produced by anthers in the outer florets of such capitula (estimated to range from 93 to 153) is known to be significantly lower than is produced by florets from the inner whorls (Ross and Abbott 1987); nonetheless, the number should be more than sufficient to ensure self-pollination. It is unlikely, therefore, that reduced pollen number is a cause of these florets being more frequently long-styled.

Second, why are long-styled florets more frequent in radiate than non-radiate capitula? Clearly, the fact that

radiate capitula bear pistillate ray florets will contribute to the increased frequency of long-styled florets in radiate capitula for reasons given above, but this cannot be the only cause of the difference as long-styled florets are also more frequent in the inner whorls of radiate capitula comprising hermaphrodite florets. Currently, we cannot explain precisely why delayed self-pollen deposition and germination resulting in the occurrence of long-styled florets are more prevalent among hermaphrodite disc florets produced in the inner whorls of radiate capitula than those of non-radiate capitula. Ross and Abbott (1987) reported that average pollen grain number of hermaphrodite florets exceeded 240 in the radiate variant, which is more than enough to ensure self-pollination. It is feasible that self-pollen germination following self-pollination could be delayed due to a weakened, but residual form of self-incompatibility in the radiate variant (which might have been inherited from self-incompatible *S. squalidus* during the introgressive origin of the radiate variant). However, this explanation seems unlikely given that the radiate variant readily sets seed when self-pollinated (e.g., Abbott et al. 1990) and that we recorded germinated pollen on 90% of self-pollinated styles 24 h after pollination in the experiment which compared style lengths of non-pollinated and self-pollinated florets. Another possibility is that styles might elongate more rapidly in the radiate variant relative to the non-radiate variant, which in turn might enable them to avoid self-pollination more effectively, thus causing a greater number of long-styled florets to occur in radiate capitula. A test of this possibility should be carried out in the future. Clearly, further research is required to determine why long-styled florets are more frequent in the inner whorls of radiate relative to those of non-radiate capitula.

Conclusions

Although we cannot provide a cause of why delayed self-pollen deposition and germination is more frequent in the disc florets of the radiate variant relative to the non-radiate variant, we do conclude that such delay results in the occurrence of a long style length in such florets and increases the opportunity for cross-pollination and outcrossing. We found that the radiate variant contains a higher frequency of long-styled disc florets relative to the non-radiate variant and infer, therefore, that self-pollen deposition and germination is delayed longer in the disc florets of this variant, which in addition to the presence of pistillate ray florets should be a factor that contributes to the greater outcrossing rate of the radiate variant. Interestingly, Trow (1912) reported that non-radiate plants occasionally produce capitula exhibiting a high frequency of long-styled florets indicating that certain genotypes of this variant might exhibit higher outcrossing rates than others as reported by Campbell and Abbott (1976).

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Disclosure statement

No potential conflict of interest was reported by the authors.

Notes on contributors

Judith Irwin is a Senior Scientist; she conducts research on the genetics of vernalisation response in *Brassica*. She completed the experimental work reported in this paper as part of her Honours BSc. research project for the degree of Biology (Genetics) at St Andrews University.

Paul Ashton is a Reader; he conducts research on the ecological genetics of *Tilia* spp. and *Carex* spp. He completed important preliminary work that led to the experimental work reported in this paper. This was conducted as part of his Honours BSc research project for the degree of Botany and Ecology at St Andrews University.

Francois Bretagnolle is a Professor; he conducts research into diverse aspects of ecology and evolution. He completed the analyses of data reported in this paper while employed as a Postdoctoral Research Assistant at St Andrews University.

Richard Abbott is Emeritus Professor of Biology; he conducts research on diverse aspects of plant evolution, especially plant speciation and the evolutionary consequences of hybridisation. He supervised the research reported, helped with the analysis of data and wrote the paper.

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