APPLICATION FOR FEDERAL ASSISTANCE

SF 424 (R&R)

2. DATE SUBMITTED	Applicant Identifier
	A53507
3. DATE RECEIVED BY STATE	**State Application Identifier**

1. * TYPE OF SUBMISSION

☐ Pre-application ☒ Application ☐ Changed/Corrected Application

4. Federal Identifier R01GM088805

5. APPLICANT INFORMATION

*** Organizational DUNS:** 605799469

* Legal Name: University of Washington

Department: Office of Sponsored Programs Division: Office of Research

* Street1: 4333 Brooklyn Ave NE

Street2: Box 359472

* City: Seattle County: King

* State: WA: Washington Province:

* Country: USA: UNITED STATES * ZIP / Postal Code: 98195-9472

Person to be contacted on matters involving this application

Prefix: * First Name: Lynne Middle Name:

* Last Name: Chronister Suffix:

* Phone Number: 206-543-4043 Fax Number: 206-685-1732

Email: soderljm@u.washington.edu

6. * EMPLOYER IDENTIFICATION *(EIN) or (TIN):* 91-6001537

7. * TYPE OF APPLICANT: H: Public/State Controlled Institution of Higher Education

Other (Specify):

Small Business Organization Type ☐ Women Owned ☐ Socially and Economically Disadvantaged

8. * TYPE OF APPLICATION:

☐ New ☒ Resubmission

☐ Renewal ☐ Continuation ☐ Revision

If Revision, mark appropriate box(es).

☐ A. Increase Award ☐ B. Decrease Award ☐ C. Increase Duration ☐ D. Decrease Duration

☐ E. Other (specify):

* Is this application being submitted to other agencies? Yes ☐ No ☒ What other Agencies?

9. * NAME OF FEDERAL AGENCY: National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER:

TITLE: Research Project Grant (Parent R01)

11. * DESCRIPTIVE TITLE OF APPLICANT'S PROJECT:

Genetics of prezygotic reproductive isolation in natural populations of monkeyflowers (Mimulus)

12. * AREAS AFFECTED BY PROJECT *(cities, counties, states, etc.)*

Seattle, King, WA, USA

13. PROPOSED PROJECT:

* Start Date: 08/01/2010 * Ending Date: 07/31/2015

14. CONGRESSIONAL DISTRICTS OF:

a. * Applicant: WA-007 b. * Project: WA-007

15. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: * First Name: Harvey Middle Name: D

* Last Name: Bradshaw Suffix: Jr.

Position/Title: Professor

* Organization Name: University of Washington

Department: BIOLOGY Division: BIOLOGY

* Street1: University of Washington

Street2: Box 355325

* City: Seattle County: King

* State: WA: Washington Province:

* Country: USA: UNITED STATES * ZIP / Postal Code: 98195

* Phone Number: +1 206 616-1796 Fax Number:

* Email: toby@u.washington.edu

OMB Number: 4040-0001
Expiration Date: 04/30/2008

16. ESTIMATED PROJECT FUNDING

a. * Total Estimated Project Funding	1,798,018.12
b. * Total Federal & Non-Federal Funds	1,798,018.12
c. * Estimated Program Income	0.00

17. * IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?

a. YES ☐ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:

DATE:

b. NO ☒ PROGRAM IS NOT COVERED BY E.O. 12372; OR

☐ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

18. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

☒ *** I agree**

**** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.***

19. Authorized Representative

Prefix: * First Name: Lynne Middle Name:

* Last Name: Chronister Suffix:

* Position/Title: Asst Vice Provost for Research

* Organization: University of Washington

Department: Office of Sponsored Programs Division: Office of Research

* Street1: 4333 Brooklyn Ave NE

Street2: Box 359472

* City: Seattle County: King

* State: WA: Washington Province:

* Country: USA: UNITED STATES * ZIP / Postal Code: 98195-9472

* Phone Number: 206-543-4043 Fax Number: 206-685-1732

* Email: soderljm@u.washington.edu

*** Signature of Authorized Representative**: Lynne Chronister

*** Date Signed**: 11/19/2009

20. Pre-application Add Attachment | Delete Attachment | View Attachment

21. Attach an additional list of Project Congressional Districts if needed.

Add Attachment | Delete Attachment | View Attachment

OMB Number: 4040-0001
Expiration Date: 04/30/2008

424 R&R and PHS-398 Specific Table Of Contents

RESEARCH & RELATED Project/Performance Site Location(s)

Project/Performance Site Primary Location

Organization Name: Office of Sponsored Programs, University of Washington
* Street1: 4333 Brooklyn Ave NE
Street2: Box 359472
* City: Seattle County: King
* State: WA: Washington Province:
* Country: USA: UNITED STATES * ZIP / Postal Code: 98195-9472

Project/Performance Site Location 1

Organization Name: University of Washington
* Street1: Department of Biology
Street2: Box 355325
* City: Seattle County:
* State: WA: Washington Province:
* Country: USA: UNITED STATES * ZIP / Postal Code: 98195

Project/Performance Site Location 2

Organization Name: Michigan State University
* Street1: Department of Plant Biology
Street2: 166 Plant Biology
* City: East Lansing County:
* State: MI: Michigan Province:
* Country: USA: UNITED STATES * ZIP / Postal Code: 48824

Additional Location(s) Add Attachment Delete Attachment View Attachment

OMB Number: 4040-0001
Expiration Date: 04/30/2008

Close Form | Print Page | About

RESEARCH & RELATED Other Project Information

1. * Are Human Subjects Involved? ☐ Yes ☒ No

1.a If YES to Human Subjects

Is the IRB review Pending? ☐ Yes ☐ No

IRB Approval Date:

Exemption Number: ☐1 ☐2 ☐3 ☐4 ☐5 ☐6

Human Subject Assurance Number:

2. * Are Vertebrate Animals Used? ☐ Yes ☒ No

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ☐ Yes ☐ No

IACUC Approval Date:

Animal Welfare Assurance Number

3. * Is proprietary/privileged information included in the application? ☐ Yes ☒ No

4.a. * Does this project have an actual or potential impact on the environment? ☐ Yes ☒ No

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ☐ Yes ☐ No

4.d. If yes, please explain:

5.a. * Does this project involve activities outside the U.S. or partnership with International Collaborators? ☐ Yes ☒ No

5.b. If yes, identify countries:

5.c. Optional Explanation:

6. * Project Summary/Abstract ProjSumm.pdf | Add Attachment | Delete Attachment | View Attachment

7. * Project Narrative ProjNarr.pdf | Add Attachment | Delete Attachment | View Attachment

8. Bibliography & References Cited LitCited.pdf | Add Attachment | Delete Attachment | View Attachment

9. Facilities & Other Resources Facilities.pdf | Add Attachment | Delete Attachment | View Attachment

10. Equipment MajorEqpt.pdf | Add Attachment | Delete Attachment | View Attachment

11. Other Attachments Add Attachments | Delete Attachments | View Attachments ☐

OMB Number: 4040-0001
Expiration Date: 04/30/2008

Project Summary/Abstract

The long-term goal of our research is to understand the genetics and evolution of reproductive isolation in natural populations of sexually-reproducing organisms: *i.e.*, the origin of species.

This proposal focuses on the genetics of prezygotic reproductive isolation in wild populations of two sister species of monkeyflower, *Mimulus lewisii* and *M. cardinalis*. Previous studies have demonstrated that reproductive isolation between sympatric populations of these species is mediated almost exclusively by the fidelity of their different pollinator guilds. *M. lewisii* is pollinated by bumblebees, *M. cardinalis* is pollinated by hummingbirds, and pollinator preference depends strongly on flower color differences between them.

The Specific Aims of this project are to:

Aim 1. Produce high-resolution genetic and physical maps of the 4 major quantitative trait loci (QTL) controlling differences in flower color between bumblebee-pollinated *M. lewisii* and hummingbird-pollinated *M. cardinalis*. Physical regions containing these QTLs will be sequenced an annotated.

Aim 2. Construct and characterize high-resolution near-isogenic lines (hrNILs) for each of the 4 major loci controlling differences in flower color between *M. lewisii* and *M. cardinalis*. hrNILs will approximate single-gene substitutions, and will be suitable for field experiments.

Aim 3. Determine the proportion of the total prezygotic reproductive isolation between *M. lewisii* and *M. cardinalis* attributable to each of the 4 flower color loci, and to their combinatorial effects, by direct observation of hrNILs in common garden field experiments in areas of natural sympatry. The effect of each of the 4 flower color loci (in all 15 possible combinations) on pollinator preference will be assessed in field experiments with arrays of hrNILs and the “ancestral” *M. lewisii*. A final experiment will directly compare the preference of bumblebees and hummingbirds for the “ancestral” *M. lewisii* phenotype, a 4-locus hrNIL in a *M. lewisii* background, and the derived *M. cardinalis*. The pollinator visitation phenotypes of all 15 hrNIL combinations, combined with data from parental *M. lewisii* and *M. cardinalis*, will allow us to estimate the individual and aggregate effect of all 4 loci on reproductive isolation. Our expectation is that the vast majority (>75%) of reproductive isolation in sympatry will be attributable to just these 4 flower color loci. These experiments will provide our first glimpse into the multi-locus genetics of prezygotic reproductive isolation in any natural system.

Project Narrative

The long-term goal of our research is to understand the genetics and evolution of reproductive isolation in natural populations of sexually-reproducing organisms: *i.e.*, the origin of species. All of the biodiversity on Earth, including the origin of humans, is a result of the process of speciation.

Facilities

Lab: A molecular genetics lab of 30m^2, used primarily for DNA extraction and genotyping, is equipped with 4 Bio-Rad i-Cyclers (2 96-well and 2 384-well), a Bio101 FastPrep plant DNA extraction machine, agarose gel electrophoresis rigs, and manual and electronic single- and multi-channel pipets.

The lab has an attached greenhouse (120m^2), and is a short walk (10m) from the main Biology greenhouse, which has 800m^2 of research space for propagating large mapping populations. We can propagate 20K *Mimulus* seedlings simultaneously.

Office: My office and two multi-desk offices for grad students and technicians are all adjacent to the lab. There is also a central common area/meeting space for lab meetings, lunch, etc.

Clinical: N/A

Animal: N/A

Computers: Every desk in the offices is equipped with a desktop or laptop no more than 4 years old, with the choice of OS (Mac, Windows, Linux) depending on the user.

Other: The UW Department of Biology has considerable strength in ecology and evolutionary biology, with weekly informal seminars in both areas that draw grad students, postdocs, and faculty from around the Seattle area. My most frequent scientific interactions are with the labs of Katie Peichel, Josh Tewksbury, Veronica Di Stilio, Dick Olmstead, and Joe Felsenstein.

The Department of Biology Comparative Genomics Center has a variety of shared equipment that we use for Sanger sequencing (ABI 3730, ABI 3130), plate reading (P-E Victor), robotic liquid handling (Apricot pipettor), robotic tissue grinding and DNA extraction in 96-well format (Qiagen Biorobot), and computational analysis. Use of this equipment is on a cost recovery basis.

The UW Microbiology core facility has a 454 GS FLX, which we will use on a cost recovery basis (see letter of support from Dr. Roger Bumgarner, who manages the core facility).

Major Equipment

4 Bio-Rad i-Cyclers (2 96-well and 2 384-well)
Bio101 FastPrep plant DNA extraction machine
agarose gel electrophoresis rigs
microfuge
manual and electronic single- and multi-channel pipets
-20 and -80 freezers

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: | * First Name: Harvey | Middle Name: D

* Last Name: Bradshaw | Suffix: Jr.

Position/Title: Professor | Department: BIOLOGY

Organization Name: University of Washington | Division: BIOLOGY

* Street1: University of Washington

Street2: Box 355325

* City: Seattle | County: King

* State: WA: Washington | Province:

* Country: USA: UNITED STATES | * Zip / Postal Code: 98195

* Phone Number: +1 206 616-1796 | Fax Number:

* E-Mail: toby@u.washington.edu

Credential, e.g., agency login: tobybradshaw

*** Project Role:** PD/PI | **Other Project Role Category:**

***Attach Biographical Sketch** biosketch_Bradshaw.pdf | Add Attachment | Delete Attachment | View Attachment

Attach Current & Pending Support | Add Attachment | Delete Attachment | View Attachment

PROFILE - Senior/Key Person 1

Prefix: | * First Name: Douglas | Middle Name:

* Last Name: Schemske | Suffix:

Position/Title: Professor | Department:

Organization Name: Michigan State University | Division:

* Street1: 166 Plant Biology

Street2: Michigan State University

* City: East Lansing | County: Ingham

* State: MI: Michigan | Province:

* Country: USA: UNITED STATES | * Zip / Postal Code: 48824

* Phone Number: 269-671-2264 | Fax Number:

* E-Mail: schem@u.washington.edu

Credential, e.g., agency login: msuschemske

*** Project Role:** PD/PI | **Other Project Role Category:**

***Attach Biographical Sketch** biosketch_Schemske.pdf | Add Attachment | Delete Attachment | View Attachment

Attach Current & Pending Support | Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Senior/Key Person 2

Prefix:		* First Name: Kelsey	Middle Name: Jrp
* Last Name: Byers		Suffix:	
Position/Title:		Department:	
Organization Name: University of Washington		Division:	
* Street1: University of Washington Box 351800			
Street2:			
* City: Seattle		County: King	
* State: WA: Washington		Province:	
* Country: USA: UNITED STATES		* Zip / Postal Code: 98195	
* Phone Number: +1 978 460-3581		Fax Number:	
* E-Mail: kjbyers@u.washington.edu			

Credential, e.g., agency login:

*** Project Role:** Graduate Student **Other Project Role Category:**

***Attach Biographical Sketch**	biosketch_Byers.pdf	Add Attachment	Delete Attachment	View Attachment
Attach Current & Pending Support		Add Attachment	Delete Attachment	View Attachment

ADDITIONAL SENIOR/KEY PERSON PROFILE(S)		Add Attachment	Delete Attachment	View Attachment
Additional Biographical Sketch(es) (Senior/Key Person)		Add Attachment	Delete Attachment	View Attachment
Additional Current and Pending Support(s)		Add Attachment	Delete Attachment	View Attachment

OMB Number: 4040-0001
Expiration Date: 04/30/2008

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Bradshaw, H.D., Jr.	Professor of Biology
eRA COMMONS USER NAME tobybradshaw	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
East Carolina University	B.S.	1979	Biology
Louisiana State University Medical Center	Ph.D.	1984	Biochemistry
University of Washington	postdoc	1984-89	Plant molecular biology

A. Positions and Honors.

Positions and Employment

1980-1983 NSF Predoctoral Fellow (William C. Claycomb lab, LSU Med. Ctr.)
1980-1981 American Heart Association Graduate Fellow (William C. Claycomb lab, LSU Med. Ctr.)
1984-1987 Helen Hay Whitney Postdoctoral Fellow (Milton P. Gordon lab, Univ. Washington)
1989-1994 Research Assistant Professor, Department of Biochemistry, Univ. Washington
1994-1995 Research Assistant Professor, College of Forest Resources, Univ. Washington
1995-2003 Research Associate Professor, College of Forest Resources, Univ. Washington
2003-present Professor, Department of Biology, Univ. Washington

Other Experience and Professional Memberships

2003-present Associate Chair and Graduate Program Coordinator, Department of Biology
2004-present NIH Genetic Variation and Evolution Study Section member
1998, 2002 NSF Population Biology Review Panel member
1997-2002 Co-organizer (with David Neale), Forest Tree Genome Workshop, San Diego
2002 National Academy of Sciences Workshop on the Plant Genome Initiative participant
2001 Co-organizer (with Steve Strauss), Tree Biotechnology in the Next Millennium conference

Honors

2005-2008 Washington Research Foundation Professor of Basic Biological Sciences
2007 American Association for the Advancement of Science Fellow

B. Selected peer-reviewed publications (in descending chronological order).

(Publications selected from 49 peer-reviewed publications)

Angert, A.L., Bradshaw, H.D., Jr., & Schemske, D.W. (2008) Using experimental evolution to investigate geographic range limits in monkeyflowers. *Evolution* 62: 2660-2675.

Bradshaw, H.D., Jr. (2005) Mutations in *CAX1* produce phenotypes characteristic of plants tolerant to serpentine soils. *New Phytologist* 167: 81-88.

Bradshaw, H.D., Jr. & Schemske, D.W. (2003) Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature* 426: 176-178.

Ramsey, J. R., Bradshaw, H. D., Jr., & Schemske, D. W. (2003) Components of reproductive isolation in the monkeyflowers *Mimulus lewisii and M. cardinalis* (Phrymaceae). *Evolution* 57(7): 1520-1534.

Bradshaw, H.D., Jr. & Schemske, D.W. (2001) The birds and the bees of plant evolution. *Northwest Science and Technology* Spring 2001: 44-49.

Frewen, B.E., Chen, T.H.H., Howe, G., Davis, J., Rohde, A., Boerjan, W., & Bradshaw, H.D., Jr. (2000) QTL and candidate gene mapping of bud set and bud flush in *Populus*. *Genetics* 154: 837-845.

Schemske, D.W. & Bradshaw, H.D., Jr. (1999) Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proceedings of the National Academy of Sciences USA* 96(21): 11910-11915.

Bradshaw, H.D., Jr., Otto, K.G., Frewen, B.E., McKay, J.K. & Schemske, D.W. (1998) Quantitative trait loci affecting differences in floral morphology between two species of monkeyflower (*Mimulus*). *Genetics* 149: 367-382.

Wilbert, S.M., Schemske, D.W., & Bradshaw, H.D., Jr. (1997) Floral anthocyanins from two monkeyflower species with different pollinators. *Biochemical Systematics and Ecology* 25(5): 437-443.

Villar, M., Lefevre, F., Bradshaw, H.D., & Teissier du Cros, E. (1996) Molecular genetics of rust resistance in poplars (*Melampsora larici-populina* Kleb./*Populus* sp.) by bulked segregant analysis in a 2 x 2 factorial mating design. *Genetics* 143: 531-536.

Bradshaw, H.D., Jr., Wilbert, S.M., Otto, K.G., & Schemske, D.W. (1995) Genetic mapping of floral traits associated with reproductive isolation in monkeyflowers (*Mimulus*). *Nature* 376: 762-765.

Bradshaw, H.D., Jr. & Stettler, R.F. (1995) Molecular genetics of growth and development in *Populus*. IV. Mapping QTLs with large effects on growth, form, and phenology traits in a forest tree. *Genetics* 139: 963-973.

C. Research Support

Ongoing Research Support

NSF FIBR 0328636 Willis (PI) 9/01/03-8/31/08 (no-cost extension to 8/31/09)
Integrated Ecological and Genomic Analysis of Speciation in *Mimulus*.
This project is to develop genetics and genomics resources for *Mimulus*, to make possible the positional cloning of genes involved in speciation.
Role: co-PI

Completed Research Support (relevant to the current proposal)

NSF DEB 0075660 Bradshaw (PI) 2000-2004
The Genetic Basis of Adaptation: A Combined Molecular and Ecological Approach.
The goal of this study was to examine the genetic basis of floral and physiological differentiation in two closely-related plant species.
Role: PI

NSF DEB 9616522 Bradshaw (PI) 1997-2000
Molecular Genetics of Reproductive Isolation in *Mimulus*.
The objective of this study was to identify the genetic basis of floral traits that contribute to speciation.
Role: PI

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Schemske, Douglas W.	Professor of Plant Biology
eRA COMMONS USER NAME msuschemske	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Illinois	B. S.	1970	Biology
University of Illinois	Ph.D	1977	Evolution, Ecology
Smithsonian Tropical Research Institute	Postdoctoral	1978	Evolution, Ecology

A. Positions and Honors.

Positions and Employment

1978-1979 Assistant Professor, Amherst College, Amherst, MA
1979-1985 Assistant Professor, University of Chicago, Chicago, IL
1985-1989 Associate Professor, University of Chicago, Chicago, IL
1989-1992 Associate Professor, University of Washington, Seattle, WA
1992-2001 Professor, University of Washington, Seattle, WA
2001- Professor and Hannah Chair of Biology, Michigan State University, E. Lansing, MI

Other Experience and Professional Memberships

1984-1987 Associate Editor, Evolution
1987-1989 Member of the Council, Society for the Study of Evolution
1989-1992 Associate Editor, Ecology
1996-1999 Associate Editor, American Naturalist
2002-2003 Vice President, Society for the Study of Evolution
2002-2005 Associate Editor, Biotropica
2002-2007 Associate Editor, Annual Review of Ecology, Evolution and Systematics
2007-2009 Vice President, American Society of Naturalists
various- Member, American Association for the Advancement of Science
various- Member, American Society of Naturalists
various- Member, Association for Tropical Biology
various- Member, Botanical Society of America
various- Member, Ecological Society of America
various- Member, Genetics Society of America
various- Member, Society for the Study of Evolution

Honors

1986 Mercer Award (best paper in the field of ecology in a given year), Ecological Society of America
2002 E. O. Wilson Naturalist Award, American Society of Naturalists
2003 American Academy of Arts and Sciences, Elected fellow
ISI Highly Cited Researcher

B. Selected peer-reviewed publications (in chronological order).

(Selected from 98 peer-reviewed publications. An additional 49 papers have been published by my graduate students)

1. Schemske DW, Horvitz CC. Variation among floral visitors in pollination ability: A precondition for mutualism specialization. Science 1984;225:519-521.
2. Schemske DW. Population structure and local selection in *Impatiens pallida* (Balsaminaceae), a selfing annual. Evolution 1984;38:817-832.
3. Lande R, Schemske DW. The evolution of self-fertilization and inbreeding depression in plants. I. Genetic models. Evolution 1985;39:24-40.
4. Schemske, D. W. and C. C. Horvitz. Temporal variation in selection on a floral character. Evolution 1989;43:461-465.
5. Schemske, D. W. and J. Ågren. Deceit pollination and selection on female flower size in *Begonia involucrata*: An experimental approach. Evolution 1995;49: 207-214.
6. Bradshaw, H. D. Jr., S. M. Wilbert, K. G. Otto and D. W. Schemske. Genetic mapping of floral traits associated with reproductive isolation in monkeyflowers. Nature 1995;376: 762-765.
7. Husband, B. C. and D. W. Schemske. Evolution of the magnitude and timing of inbreeding depression in plants. Evolution 1996;50:54-70.
8. Bradshaw, H. D., Jr., K. G. Otto, B. E. Frewen, J. K. McKay and D. W. Schemske. Quantitative trait loci affecting differences in floral morphology between two species of monkeyflower (*Mimulus)*. Genetics 1998;149:367-382.
9. Ramsey, J. and D. W. Schemske. Pathways, mechanisms and rates of polyploid formation in flowering plants. Annual Review of Ecology and Systematics 1998;29:477-501.
10. Schemske, D. W. and H. D. Bradshaw, Jr. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). Proceedings of the National Academy of Sciences 1999;96:11910-11915.
11. Schemske, D. W. and P. Bierzychudek. Evolution of flower color in the desert annual *Linanthus parryae*: Wright revisited. Evolution 2001;55:1269-1282.
12. Turelli, M., D. W. Schemske and P. Bierzychudek. Stable two-allele polymorphisms maintained by fluctuating fitnesses and seed banks: Protecting the blues in *Linanthus parryae*. Evolution 2001; 55:1283-1298.
13. Ramsey, J. R., H. D. Bradshaw, Jr. and D. W. Schemske. Components of reproductive isolation between the monkeyflowers *Mimulus lewisii and M. cardinalis* (Phrymaceae). Evolution 2003;57:1520-1534.
14. Bradshaw, H. D., Jr. and D. W. Schemske. Allele substitution at a flower color locus produces a pollinator shift in two monkeyflower species (*Mimulus*). Nature 2003;426:176-178.
15. Angert, A. L. and D. W. Schemske. The evolution of species' distributions: reciprocal transplants across the elevation ranges of *Mimulus cardinalis* and *M. lewisii*. Evolution 2005;59:1671-1684.
16. Schemske, D. W. and P. Bierzychudek. Spatial differentiation for flower color in the desert annual *Linanthus parryae*: Was Wright right? Evolution 2007;61:2528-2543.
17. M. A. Grillo, C. Li, A. M. Fowlkes, A. Zhou, T. M. Briggeman, D. W. Schemske, and T. Sang. Genetic architecture for the adaptive origin of annual wild rice, *Oryza nivara*. (in press, Evolution).
18. Angert, A. L., H. D. Bradshaw and D. W. Schemske. Using experimental evolution to investigate geographic range limits in monkeyflowers (in press, Evolution).

C. Research Support

<u>Ongoing Research Support</u>

NSF 2003-2009
(Frontiers in Integrative Biological Research; FIBR)
Integrated Ecological and Genomic Analysis of Speciation in *Mimulus*.
This project is developing genomic tools for studies of evolutionary genetics in the model system *Mimulus*.
Role: Co-Investigator

USDA 2004-2008
Using demographic models to assess biocontrol of an invasive plant
The goal of this project is to evaluate rates of spread and the potential for biocontrol of an invasive plant.
Role: Co-Investigator

NSF 2005-2008
Genetics of adaptation of wild *Oryza* species.
This study uses molecular genetic techniques to identify QTL for adaptive divergence of two wild rice species.
Role: Co-Investigator

Michigan State University 2007-2010
Integrating Ecological and Genomic Approaches towards Sustainable Biomass Production in Switchgrass.
This study examines the role of polyploidy in gene expression and biomass production in switchgrass.
Role: PI

Department of Energy 2007-2012.
Development of a Sustainable Bioenergy Economy; Biodiversity Responses.
The goal of this study is to examine the consequences of different biofuel production systems on biodiversity and ecosystem services.
Role: Co-Investigator

NSF 2005-2008
National Center for Ecological Analysis and Synthesis (NCEAS).
Gradients in Biodiversity and Speciation
The goal of this working group is to review evolutionary explanations for the latitudinal biodiversity gradient.
Role: Co-Investigator

Completed Research Support

NSF 1981-1983.
The Evolutionary Significance of Selfing and Outcrossing in *Impatiens pallida* (Balsaminaceae): An Experimental Approach.
The goal of this study was to evaluate the evolutionary dynamics of mixed mating systems.
Role: PI

NSF 1982-1984
Demographic Consequences of Stage-Specific Plant-Animal Interactions in a Tropical Herb, *Calathea ovandensis* (Marantaceae).
The goal of the study was to apply stage-structured matrix models to the study of plant-animal interactions.
Role: Co-Investigator

NSF 1985-1988
Temporal and Spatial Variation in Plant- Animal Interactions: Demographic Consequences for a Neotropical Herb.
This study utilized matrix models to estimate the demographic effects of plant- animal interactions.
Role: Co-Investigator

NSF 1990-1993
Evolutionary Dynamics of a Flower- Color Polymorphism in the Desert Annual *Linanthus parryae*.
The goal of this study was to determine the relative importance of genetic drift and natural selection to the maintenance of a conspicuous polymorphism.
Role: PI

Mellon Foundation 1992-1994
Evolution of the Mating System in Neotropical *Begonia*.

This study examined the evolution of floral traits and deceptive pollination in *Begonia* spp.
Role: PI

NSF 1997-2000
Molecular Genetics of Reproductive Isolation in *Mimulus*.
The objective of this study was to identify the genetic basis of floral traits that contribute to speciation.
Role: Co-Investigator

Mellon Foundation 1997-2001
The evolution of floral characters and reproductive isolation in Neotropical *Costus* (Costaceae).
This study examined the role of pollinator switches in the speciation of a diverse group of Neotropical plants.
Role: PI

NSF 2000-2004
The Genetic Basis of Adaptation: A Combined Molecular and Ecological Approach.
The goal of this study was to examine the genetic basis of floral and physiological differentiation in two closely-related plant species.
Role: Co-Investigator

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Byers, Kelsey	Graduate Student
eRA COMMONS USER NAME kbyers	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Massachusetts Institute of Technology, Cambridge, MA, USA	S.B.	2003-2007	Biology
University ofWashington, Seattle, WA, USA	Ph.D. (in progress)	2008-	Biology

A. Positions and Honors

Positions and Employment

2007-2008 Technical Research Assistant, Harvard Medical School/Brigham and Women's Hospital

Honors

2008-2011 Achievement Rewards for College Scientists (ARCS) Foundation Fellowship
2008-2010 GenOM Project Graduate Fellowship
2008-2010 Graduate Opportunity Program Research Assistantship
2009-2010 Plant Biology Fellowship

B. Selected peer-reviewed publications (in chronological order).

C. Zhu*, **K. Byers***, *et al.* High-Resolution DNA Binding Specificity Survey of Yeast Transcription Factors. *Cell*, in review. (*these authors contributed equally)

C. Research Support

Achievement Rewards for College Scientists (ARCS) Foundation Fellowship
GenOM Project Graduate Fellowship
Plant Biology Fellowship

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OMB Number: 0925-0001

1. Project Director / Principal Investigator (PD/PI)

Prefix:
* First Name: Harvey
Middle Name: D
* Last Name: Bradshaw
Suffix: Jr.

* New Investigator? ☐ No ☒ Yes

Degrees: PhD

2. Human Subjects

Clinical Trial? ☒ No ☐ Yes

* Agency-Defined Phase III Clinical Trial? ☐ No ☐ Yes

3. Applicant Organization Contact

Person to be contacted on matters involving this application

Prefix:
* First Name: Lynne
Middle Name:
* Last Name: Chronister
Suffix:

* Phone Number: 206-543-4043 Fax Number: 206-685-1732
Email: soderljm@u.washington.edu

* Title: Asst Vice Provost for Research

* Street1: 4333 Brooklyn Ave NE
Street2: Box 359472
* City: Seattle
County: King
* State: WA: Washington
Province:
* Country: USA: UNITED STATES * Zip / Postal Code: 98195-9472

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4. Human Embryonic Stem Cells

* Does the proposed project involve human embryonic stem cells? ☒ No ☐ Yes

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://stemcells.nih.gov/registry/index.asp. Or, if a specific stem cell line cannot be referenced at this time, please check the box indicating that one from the registry will be used:

Cell Line(s): ☐ Specific stem cell line cannot be referenced at this time. One from the registry will be used.

PHS 398 Modular Budget, Periods 1 and 2

OMB Number: 0925-0001

Budget Period: 1

Start Date: 08/01/2010 End Date: 07/31/2011

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	250000.00
Consortium F&A	0.00
* Total Direct Costs	250,000.00

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	MTDC	56	237,958.00	133,256.48
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Patrick Smith, (415) 437-7820

Indirect Cost Rate Agreement Date: 11/05/2008

Total Indirect Costs: 133,256.48

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($): 383,256.48

Budget Period: 2

Start Date: 08/01/2011 End Date: 07/31/2012

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	250000.00
Consortium F&A	24,071.00
* Total Direct Costs	274,071.00

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	MTDC	56	215,464.00	120,659.84
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Patrick Smith, (415) 437-7820

Indirect Cost Rate Agreement Date: 11/05/2008

Total Indirect Costs: 120,659.84

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($): 394,730.84

PHS 398 Modular Budget, Periods 3 and 4

Budget Period: 3

Start Date: 08/01/2012 End Date: 07/31/2013

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	225000.00
Consortium F&A	24,736.00
* Total Direct Costs	249,736.00

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	MTDC	56	162,859.00	91,201.04
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Patrick Smith, (415) 437-7820

Indirect Cost Rate Agreement Date: 11/05/2008

Total Indirect Costs: 91,201.04

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($): 340,937.04

Budget Period: 4

Start Date: 08/01/2013 End Date: 07/31/2014

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	225000.00
Consortium F&A	25,364.00
* Total Direct Costs	250,364.00

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	MTDC	56	160,195.00	89,709.20
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Patrick Smith, (415) 437-7820

Indirect Cost Rate Agreement Date: 11/05/2008

Total Indirect Costs: 89,709.20

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($): 340,073.20

PHS 398 Modular Budget, Periods 5 and Cumulative

Budget Period: 5

Start Date: 08/01/2014 End Date: 07/31/2015

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	225000.00
Consortium F&A	27,374.00
* Total Direct Costs	252,374.00

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	MTDC	56	154,726.00	86,646.56
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Patrick Smith, (415) 437-7820

Indirect Cost Rate Agreement Date: 11/05/2008

Total Indirect Costs: 86,646.56

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($): 339,020.56

Cumulative Budget Information

1. Total Costs, Entire Project Period

*Section A, Total Direct Cost less Consortium F&A for Entire Project Period	$	1,175,000.00
Section A, Total Consortium F&A for Entire Project Period	$	101,545.00
*Section A, Total Direct Costs for Entire Project Period	$	1,276,545.00
*Section B, Total Indirect Costs for Entire Project Period	$	521,473.12
*Section C, Total Direct and Indirect Costs (A+B) for Entire Project Period	$	1,798,018.12

2. Budget Justifications

Personnel Justification	BudgetJust.pdf	Add Attachment	Delete Attachment	View Attachment
Consortium Justification	ConsortiumJust.pdf	Add Attachment	Delete Attachment	View Attachment
Additional Narrative Justification	BudgetJustAddn.pdf	Add Attachment	Delete Attachment	View Attachment

Budget Justification, Personnel

H.D. Bradshaw, Jr., Principal Investigator (UW), 2.0 months summer, responsible for overall project coordination, data analysis and interpretation, preparation of manuscripts, supervision of graduate student, technician, and undergraduate research assistant.

Douglas W. Schemske, co-PI (MSU), no salary requested (since he is supported by an endowed professorship), will join Bradshaw in coordinating and supervising field experiments, performing statistical analysis of field experiments, contributing to project publications. Schemske will supervise the postdoctoral research associate and undergraduate research assistant who are employed on the subaward to MSU.

Postdoctoral Research Associate (to be named) (MSU), 6.0 months in Years 2-5, responsible for conducting greenhouse experiments to evaluate the phenotypes of hrNILs, executing the field experiments, supervising the undergraduate research assistant and the grad student in the field, and assisting in data analysis, interpretation, and publication.

Kelsey Byers, Graduate Student (UW), 12.0 months, responsible for fine-scale mapping and hrNIL construction, supervising undergraduate research assistant, assisting postdoc with summer field experiments, assisting in data analysis, interpretation, and publication. [*NB*: Ms. Byers is a new graduate student who was heavily recruited by several top programs around the country. She has a 3.7GPA from MIT, GRE scores in the >95th percentile, and is Hispanic.]

Technician (UW), 12.0 months, responsible for planting and caring for large mapping populations, DNA extractions, and routine lab maintenance (ordering, etc.).

Jason Phillips, Consultant, 2.0 months, responsible for annotation (and perhaps some assembly) of BAC DNA sequences from 454 reads.

Undergraduate Research Assistant (UW), 12.0 months, responsible for helping the grad student and technician carry out the fine-scale mapping and hrNIL construction, and for helping with field experiments.

Undergraduate Research Assistant (MSU), 5.0 months in Years 2-5, responsible for helping the postdoc carry out the hrNIL phenotype characterization and field experiments.

Consortium Justification

Co-PIs Bradshaw and Schemske have collaborated on the evolutionary genetics and ecology of *Mimulus lewisii* and *M. cardinalis* for 15 years. The collaboration continued uninterrupted when Prof. Schemske moved to Michigan State University in 2001. The general division of labor has Prof. Bradshaw responsible for the wet lab work, especially the positional cloning (*e.g*., Bradshaw 2005), and Prof. Schemske responsible for the field work. But there is a good deal of overlap (*e.g*., when phenotyping plants in the greenhouse), with plenty of opportunity to discuss and plan all aspects of the project.

Douglas W. Schemske, PI (MSU), no salary requested for 10% effort (1.2 calendar months), responsible for coordinating and supervising field experiments, performing statistical analysis of field experiments, contributing to project publications, and supervising the postdoctoral research associate and undergraduate research assistant who are employed on the subaward to MSU.

Postdoctoral Research Associate (to be named) (MSU), 6.0 months in Years 2-5, responsible for conducting greenhouse experiments to evaluate the phenotypes of hrNILs, executing the field experiments, supervising the undergraduate research assistant and the grad student in the field, and assisting in data analysis, interpretation, and publication.

Undergraduate Research Assistant (MSU), 5.0 months in Years 2-5, responsible for helping the postdoc carry out the hrNIL phenotype characterization and field experiments.

		Direct + F&A
Michigan State University (domestic)	Year 1	$ 0
	Year 2	$70,000
	Year 3	$72,000
	Year 4	$74,000
	Year 5	$80,000

Budget Justification, Additional Narrative

The additional budget module requested in Years 1 and 2 is because the 10x *M. lewisii* BAC library and filters (~$14K), BAC contig 454 sequencing (8 runs x $7K/run), and the majority of genotyping for hrNIL construction fall in Years 1 and 2.

Close Form | Print Page | About

OMB Number: 0925-0001

PHS 398 Research Plan

1. Application Type:

From SF 424 (R&R) Cover Page and PHS398 Checklist. The responses provided on these pages, regarding the type of application being submitted, are repeated for your reference, as you attach the appropriate sections of the research plan.

*Type of Application:

☐ New ☒ Resubmission ☐ Renewal ☐ Continuation ☐ Revision

2. Research Plan Attachments:

Please attach applicable sections of the research plan, below.

1. Introduction to Application (for RESUBMISSION or REVISION only)	Intro.pdf	Add Attachment	Delete Attachment	View Attachment
2. Specific Aims	SpecificAims.pdf	Add Attachment	Delete Attachment	View Attachment
3. Background and Significance	Background.pdf	Add Attachment	Delete Attachment	View Attachment
4. Preliminary Studies / Progress Report	PrelimStudies.pdf	Add Attachment	Delete Attachment	View Attachment
5. Research Design and Methods	ExpDesign.pdf	Add Attachment	Delete Attachment	View Attachment
6. Inclusion Enrollment Report		Add Attachment	Delete Attachment	View Attachment
7. Progress Report Publication List		Add Attachment	Delete Attachment	View Attachment

Human Subjects Sections

Attachments 8-11 apply only when you have answered "yes" to the question "are human subjects involved" on the R&R Other Project Information Form. In this case, attachments 8-11 may be required, and you are encouraged to consult the Application guide instructions and/or the specific Funding Opportunity Announcement to determine which sections must be submitted with this application.

8. Protection of Human Subjects		Add Attachment	Delete Attachment	View Attachment
9. Inclusion of Women and Minorities		Add Attachment	Delete Attachment	View Attachment
10. Targeted/Planned Enrollment		Add Attachment	Delete Attachment	View Attachment
11. Inclusion of Children		Add Attachment	Delete Attachment	View Attachment

Other Research Plan Sections

12. Vertebrate Animals		Add Attachment	Delete Attachment	View Attachment
13. Select Agent Research		Add Attachment	Delete Attachment	View Attachment
14. Multiple PI Leadership Plan		Add Attachment	Delete Attachment	View Attachment
15. Consortium/Contractual Arrangements		Add Attachment	Delete Attachment	View Attachment
16. Letters of Support		Add Attachment	Delete Attachment	View Attachment
17. Resource Sharing Plan(s)		Add Attachment	Delete Attachment	View Attachment

18. Appendix: Add Attachments | Remove Attachments | View Attachments

Introduction to Revised Application

The reviewers expressed considerable enthusiasm for the significance and aims of the proposed research, and for the suitability of the *Mimulus* system for addressing unanswered questions about the evolutionary genetics of reproductive isolation. Below we respond to the three reviewers' criticisms grouped by shared concerns. We have also added a section **D.3.4. Potential pitfalls and alternative strategies** to the proposal in order to address these concerns more fully.

R1. Because it is not possible to reconstruct either the ecological milieu in which the species diverged or the order of gene substitution, the inferences will bear much more tenuously on the process of speciation. … A further concern is that it is presumably unknown and unknowable whether the species diverged in sympatry, which is the context in which the field assays will be (and have been) done. As a result, though the work will bear on the maintenance of species boundaries, its connection to speciation is tenuous.

Response: We agree that the (genetic, ecological, spatial) history of divergence leading to speciation is not known and not knowable in *Mimulus* (nor in any of the other classical – almost exclusively allopatric – systems for studying the genetics of speciation). But (biological) *speciation is defined by reproductive isolation*, and *M. lewisii* and *M. cardinalis* are reproductively isolated in sympatry primarily (>99%) by pollinator preference – precisely the trait we propose to measure in the native range and natural habitat of both plant species and their pollinators. Even if *M. lewisii* and *M. cardinalis* speciated in allopatry (as most pairs of sister taxa have), they are sympatric *now*, and remain distinct species primarily because of evolved pollinator preference. *The behavior of diverged populations when brought into secondary contact by natural mechanisms is the best possible scenario for assessing whether (and how) speciation has occurred.* Most importantly, we propose to investigate (at a detailed genetic level) prezygotic reproductive isolation, which, despite its well-known role in speciation, has been very little studied in comparison to postzygotic isolation. Arguably, the relative roles of various prezygotic and postzygotic reproductive isolating mechanisms in nature are better known in the *Mimulus* system than in any other pair of taxa (Ramsey et al. 2003).

R1. The mapping work has evidently assumed that few loci underlie the phenotypic differences and that each of the major QTLs represents a single genetic locus.
R2. An assumption that a between species QTL of large effect is due to a single genetic substitution. Thus QTL/locus/alleles are used interchangeably. What if the differences are due to several fixation events that have occurred at the same locus (much like the "shaven baby" story of D. Stern et al.?) … It is unclear to me that the path of evolution is reconstructed if in fact QTLs represent several substitutions. … It will ultimately be important to determine how many substitutions define each QTL.

Response: In this revised proposal we have tried to be more explicit about the relationship among QTLs, the underlying genes, and the mutations that define the allelic differences between *M. lewisii* and *M. cardinalis*. In brief:

We expect that the 4 flower color loci are single genes, and our sub-cM fine-mapping data for *YELLOW UPPER* and *ROSE INTENSITY*, with short lists of candidate genes inferred from orthology with the sequenced *M. guttatus* genome, point in this direction. Regardless, our fine-mapping will determine precisely how many genes are in each QTL.

Until the gene(s) underlying each QTL are cloned and sequenced, we will not know how many mutations have been fixed during the divergence of *M. lewisii* and *M. cardinalis*. We have added to the **Background** section that these two species are 97% identical in noncoding regions of the genome, and >99% identical in coding regions, so there are likely to be very few functional mutations at the loci differentiating their floral forms.

Nevertheless, we are not proposing to reconstruct a mutation-by-mutation account of speciation, but rather to assess the effect of substituting 4 very small (gene-sized, ~10kb) segments of the *M. cardinalis* genome into a *M. lewisii* genetic background, and determining their individual and combinatorial effects on reproductive isolation in nature – effects that we expect to be impressively large and directly relevant to the evolutionary genetics of speciation. The extent to which we are able to pinpoint the allelic differences at each locus depends on the resolution of our fine-mapping. Preliminary data suggest that this fine-mapping will be below single-gene resolution (*i.e.*, we expect intragenic recombinants). Whether this level of resolution results in a single QTN (or other polymorphism, such as a gene duplication) remains to be seen. **It is worth highlighting that for 3 of the 4 flower color loci the derived *M. cardinalis* allele is completely recessive (suggesting a loss of function), so that for all practical purposes *any* mutation that eliminates the function of the cognate *M. lewisii* allele is equivalent to a (knockout) mutational step towards hummingbird pollination and away from bumblebee pollination.**

R1. To what extent are these genotypes representative of the species from which they were drawn?

Response: The reviewer recognizes the practical reason for our use of single inbred lines to represent each species. But even at the conceptual level the floral traits under study are diagnostic (think "field guide"), fixed differences between the two species (Figs. 1-2) that dwarf the variation within each species, making the choice of a single inbred from each species a logical (as well as a practical) one.

R1. It should also be acknowledged that a) they are (apparently) evaluating only pollinator attractiveness and thus may miss losses of fitness with respect to other components and b) fitness loss to some extent may not definitively rule out a particular path, unless the plants are absolutely dependent on pollination, which seems unlikely in view of the claimed absence of inbreeding depression.

Response: Certainly we are aware of (and have accounted in great detail for) the many components affecting reproductive isolation between *M. lewisii* and *M. cardinalis*, by previously

estimating (for the first time in any system) the contributions of ecology, pollinator visitation ("mating"), postmating gamete competition, hybrid pollen viability, hybrid seed set, and hybrid vegetative growth to overall reproductive isolation (Ramsey et al. 2003). We choose now to focus on pollinator preference because it is the most "upstream" component of reproductive isolation in sympatry (sympatry being the most rigorous test of reproductive isolation), and as the "first line of defense" pollinator preference accounts for >99% of *total* reproductive isolation in sympatry. [This says nothing about the order in which the various isolating mechanisms evolved, but it does bear on the unresolved issue of the minimum number of genes required to produce substantial reproductive isolation, just as those studying one of many Dobzhansky-Müller incompatibility gene pairs in sister taxa have argued.]

Regarding the lack of inbreeding depression, we have changed our description of the mating system from "outcrossing" to "*obligately* outcrossing" in an attempt to be even more clear that our observations of hundreds of thousands of flowers in the greenhouse, as well as >50 years of fieldwork by us and others, make it apparent that *pollinator service is absolutely required for seed set*. The lack of inbreeding depression could be explained by other factors, such as recent bottlenecks, which also would explain the low nucleotide diversity we have observed within each species.

R2. The ability to fine map QTL via pure genetic approaches may be overstated.

Response: Given our success in fine-mapping *YUP* (**C.3.1.**) and *ROI* (**C.3.3**.) to sub-cM regions of the genome with short lists of candidate genes, the large number of meioses we plan to sample (8000/locus), and the very favorable physical:genetic distance ratios discovered so far (20-250kb/cM), we think the rationale of using recombination as a genetic tool for fine-mapping allele replacement is well justified. Such recombinational mapping was used in the first QTL positional cloning experiments (which were done in tomato) to confine a causative mutation to a 484bp region (Fridman et al. 2000). The additional, critical, value of this non-transgenic approach for permitting field studies in a National Park is described in the proposal.

R3. Lack of a succinct statistical model for analyzing the data collected from the field.

Response: This comment is a bit of a mystery to us. Section **D.3.2.** of the Experimental Design spells out the orthodox ANOVA approach we propose to detect the effects of single and multiple allele substitutions. The other two reviewers recognized that we had expanded and clarified the form of the data analysis following the first round of reviews. We have now included an explicit model for the ANOVA.

R1. Resource sharing is not discussed.
R2. A resource sharing plan was not in the application.

Response: We have always made seed stocks, markers, etc. available to anyone who asked, but we have included a (brief) resource sharing plan in this version of the proposal.

A. Specific Aims

The long-term goal of our research is to understand the genetics and evolution of reproductive isolation in natural populations of sexually-reproducing organisms: *i.e.*, the origin of species. This proposal focuses on the genetics of prezygotic reproductive isolation in wild populations of two sister species of monkeyflower, *Mimulus lewisii* and *M. cardinalis*. Previous studies have demonstrated that reproductive isolation between sympatric populations of these species is mediated almost exclusively by the fidelity of their different pollinator guilds (Hiesey *et al.* 1971; Ramsey *et al.* 2003). *M. lewisii* is pollinated by bumblebees, *M. cardinalis* is pollinated by hummingbirds, and pollinator preference depends strongly on flower color differences between them (Hiesey *et al.* 1971; Schemske & Bradshaw 1999; Bradshaw & Schemske 2003).

Aim 1. Produce high-resolution genetic and physical maps of the 4 major quantitative trait loci (QTLs) controlling differences in flower color between bumblebee-pollinated *M. lewisii* and hummingbird-pollinated *M. cardinalis*. All 4 major flower color QTLs have been isolated in low-resolution near-isogenic lines (lrNILs). For each QTL a moderately dense (~3cM spacing) genetic map made with $\geq$500 meioses will be used to flank each QTL position at 1-5cM resolution and identify a BAC contig containing the target flower color locus. The BAC contig will be sequenced and annotated to guide the development of new SNP markers for every gene in candidate region. High-resolution genetic mapping with 8000 additional meioses will refine the target QTL position with 0.012cM (~2.5kb) precision, less than the 9kb average spacing between single genes in *Mimulus*.

Aim 2. Construct and characterize high-resolution near-isogenic lines (hrNILs) for each of the 4 major loci controlling differences in flower color between *M. lewisii* and *M. cardinalis*. From the high-resolution QTL mapping population in **Aim 1**, hrNILs will be constructed for each of the 4 flower color loci. The average size of the introgressed segment will be ~10kb, resulting in the approximate substitution of a single *M. cardinalis* flower color allele in a *M. lewisii* genetic background. The phenotype of each hrNIL will be characterized.

Aim 3. Determine the proportion of the total prezygotic reproductive isolation between *M. lewisii* and *M. cardinalis* attributable to each of the 4 flower color loci, and to their combinatorial effects, by direct observation of hrNILs in common garden field experiments in areas of natural sympatry. The effect of each of the 4 flower color QTLs on pollinator preference will be assessed in field experiments with arrays of hrNILs. Each of the 4 ~single-locus hrNILs will be tested for its contribution to prezygotic reproductive isolation from the "ancestral" *M. lewisii* in the first field season, all 6 two-locus combinations ("doubles") in the second field season, all 4 triples and the quadruple in the third field season, and the quadruple NIL *vs*. *M. lewisii vs*. *M. cardinalis* in the fourth field season.

Our working hypotheses are that: 1) each *M. cardinalis* flower color allele introgressed into *M. lewisii* will increase hummingbird visitation and decrease bumblebee visitation; 2) each locus will have an additive effect on reproductive isolation; and, 3) the cumulative effect of all 4 loci on reproductive isolation will account for at least 75% of the reproductive isolation between *M. lewisii* and *M. cardinalis* in sympatry.

B. Background and Significance

B.1. Reproductive isolation and the origin of species. Perhaps Darwin's most profound insight is that all of the Earth's biota are descended from a single common ancestor. The "endless forms" (Darwin 1859) derived from that common ancestor arose by the process of speciation – a fundamental evolutionary process whose genetics we seek to understand.

There is no shortage of species concepts and definitions, but for purposes of studying the evolutionary *process* of speciation, the Biological Species Concept is most appropriate (Coyne & Orr 2004, pp. 26-33). Although owing much to several of the architects of the Modern Synthesis, the BSC is usually attributed to Ernst Mayr (1942):

> *"Species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups."*

The stable coexistence of sister species with overlapping ranges in nature (*i.e.*, in sympatry) thus depends upon reproductive isolation – barriers to gene exchange that maintain the independent evolutionary trajectories of the two distinct species. In the absence of reproductive isolation the two species will merge into one by hybridization or introgression.

Coyne (1992) has summarized the situation succinctly:

> *"When we understand the origin of reproductive isolation, we understand the origin of species."*

It is worth noting that the reproductive isolation factor being addressed in our proposal is pollinator-mediated selection on flower traits. In their analysis of macroevolutionary patterns, Coyne and Orr (2004, p. 441) say:

> *"Our major conclusion is that two sets of key factors – traits increasing sexual selection in animals, and traits promoting animal pollination in plants – appear to increase the rate of speciation."*

Thus, a detailed genetic understanding of floral traits affecting pollinator choice is of particular relevance not just to the process of speciation, but to increased rates of speciation that characterize the major radiations of extant taxa. Darwin (1879) called the origin and diversification of >250,000 species of flowering plants "an abominable mystery." We expect to solve at least part of that mystery with the work proposed here.

B.2. The role of prezygotic factors in reproductive isolation. Reproductive isolation has been partitioned into prezygotic factors (*e.g.*, mate choice behavior, habitat preference, timing of reproduction, mechanical fit between copulatory organs, gamete compatibility) and postzygotic factors (*e.g.*, hybrid sterility, hybrid inviability). Historically, postzygotic barriers to gene flow have received vastly more attention from geneticists than prezygotic barriers because hybrid sterility or inviability are easily quantified and studied in the laboratory (Coyne & Orr 2004, p. 179), whereas the behavioral and ecological components of prezygotic reproductive isolation generally must be studied in the field (Schemske 2000).

Prezygotic barriers play a major role in the evolution of reproductive isolation (*e.g.*, Dobzhansky 1937; Mayr 1947, 1963; Stebbins 1950; Schluter 2001; Price & Bouvier 2002; Coyne & Orr 2004; Rundle & Nosil 2005; Rieseberg & Willis 2007). Because they act early in the interaction between potential mates, prezygotic factors have a disproportionately large effect on reproductive isolation. Postzygotic barriers can only be responsible for the fraction of reproductive isolation that remains after prezygotic factors have had their effect (Coyne & Orr 1989; Ramsey *et al.* 2003).

It has been argued that prezygotic barriers are likely to evolve before postzygotic barriers (*e.g.*, Jiggins *et al.* 2001; Kirkpatrick & Ravigné 2002), but there is some question as to whether there are enough data to support this view, at least for animals (Coyne & Orr 2004, pp. 65-69). In flowering plants, there is persuasive (if indirect) evidence that prezygotic barriers commonly evolve before postzygotic barriers, since more than 80% of crosses among closely related species produce viable, fertile progeny; *i.e.*, there is no intrinsic postzygotic isolation (Rieseberg *et al.* 2006). Prezygotic barriers to gene flow in flowering plants are primarily ecological: habitat preference due to local adaptation, and pollinator preference due to selection on floral traits (reviewed in Rieseberg & Willis 2007; Lowry *et al.* 2008).

Phenotypic variation in floral traits that produce prezygotic reproductive isolation by pollinator discrimination is potentially very amenable to detailed genetic analysis. There are substantial research efforts underway to determine the genetic basis of pollinator choice in several plant genera: *Antirrhinum* (snapdragon; Glover & Martin 1998; Comba *et al.* 2000; Jones & Reithel 2001; Whibley *et al.* 2006), *Ipomoea* (morning glory; Zufall & Rausher 2003, 2004); *Aquilegia* (columbine; Whittall *et al.* 2006; Whittall & Hodges 2007), *Nicotiana* (tobacco; Kessler & Baldwin 2006; Kessler *et al.* 2008), *Petunia* (Hoballah *et al.* 2007), and *Mimulus* (monkeyflower; Bradshaw *et al.* 1995, 1998; Schemske & Bradshaw 1999; Bradshaw & Schemske 2003; Streisfeld & Kohn 2005, 2007).

Despite these ongoing efforts devoted to studying the genetics of pollinator-mediated reproductive isolation, in none of the available systems have the following criteria – all of which are necessary to claim a detailed genetic understanding of pollinator preference – been met.

1. Sympatric sister taxa with different pollinator guilds (*e.g.*, bee, hummingbird, hawkmoth)
2. Genetic mapping of the major loci contributing to pollinator preference
3. Discovery of the identity of the gene(s) within the mapped QTLs (and their alleles)
4. Assessment of the effect of single-allele substitutions and combinatorial substitutions on pollinator preference in the natural habitat and native range of both plants and pollinators

B.3. Basic unanswered questions on the genetics and evolution of prezygotic reproductive isolation. Considerable progress has been made in identifying genes responsible for postzygotic barriers to hybridization, including hybrid sterility and hybrid inviability. Dobzhanksy (1936) was the first to map a chromosomal region contributing to hybrid sterility, and many chromosomal regions contributing to hybrid sterility or inviability have since been dissected with high precision in *Drosophila* (*e.g.*, Perez & Wu 1995; Tao *et al.* 2003; Sawamura *et al.* 2004). Several of the underlying genes have been pinpointed, and their selection history revealed by molecular evolutionary analysis (Ting *et al.* 1998; Barbash *et al.* 2003; Presgraves 2003; Presgraves *et al.* 2003; Brideau *et al.* 2006; Masly *et al.* 2006; Orr *et al.* 2007; Phadnis & Orr 2009; Tang & Presgraves 2009).

In contrast, there has been little comparable progress in identifying the genes responsible for prezygotic barriers, except in sperm-egg interactions (*e.g.*, Vacquier 1998). Schluter (2009) puts it this way:

> *"The most obvious shortcoming of our current understanding of speciation is that the threads connecting genes and selection are still few. We have many cases of ecological selection generating reproductive isolation with little knowledge of the genes that allow it."*

In the specific case of pollinator-mediated selection on floral morphology (reviewed in Fenster *et al.* 2004), we do not know whether alleles at the relevant loci have large phenotypic effects (although QTL mapping suggests that some of them do). Are the QTLs composed of single genes, or multiple linked genes? Are the genes structural (*i.e.*, coding for enzymes in floral pigment biosynthesis), or regulatory (*i.e.*, encoding transcription factors)? Are the derived alleles due to mutations in coding regions, or noncoding regions (a controversy that has boiled over again recently; Hoekstra & Coyne 2007; Pennisi 2008)? Are the derived alleles the result of single mutations, or multiple mutations in the same gene? Is the cumulative effect of individual QTLs additive, or multiplicative? What role does epistasis play, and can we use this information to infer the order of mutations that are possible in an adaptive walk (Weinreich *et al*. 2006) from one pollination syndrome to another? In multiple independent but parallel evolutionary trajectories (*e.g.*, from bee-pollinated to hummingbird-pollinated flowers; Grant 1994; Beardsley *et al.* 2003; Kay *et al.* 2005), how often are the same genes and pathways/networks the targets of selection (*i.e.*, how repeatable or predictable is phenotypic evolution)? Is the adaptive landscape for pollinator preference smooth, rugged, or ridged (Whibley *et al.* 2006)? These questions can only be addressed in model systems where detailed genetic studies can be conducted in tandem with ecological experiments.

B.4. *Mimulus* as a model system. The Plant Biology group at the Carnegie Institution of Washington (Stanford University) carried out the most extensive and visionary research program in ecological and physiological genetics ever conceived (Clausen, Keck, & Hiesey 1940, 1948; Clausen & Hiesey 1958; Hiesey, Nobs, & Björkman 1971). They began working with *Mimulus lewisii* and *M. cardinalis* in the 1929 field season and devoted their fifth and final monograph of the series *Experimental Studies on the Nature of Species* to the *Mimulus* system 42 years later (Hiesey *et al.* 1971). David Keck, the team's plant taxonomist, was instrumental in guiding the choice of experimental systems for the Carnegie's work – no small task considering the richness of the California floristic province. The rationale for choosing *M. lewisii*, *M. cardinalis*, and their relatives in the *Erythranthe* section of the genus *Mimulus* is as valid today as it was in 1929:

1. The two species have dramatically different adaptations, including floral characters that lead to the preference of bumblebees for *M. lewisii* and hummingbirds for *M. cardinalis* (Fig.1).
2. Despite their dissimilar phenotypes, *M. lewisii* and *M. cardinalis* are very closely related, readily producing vigorous, fertile hybrids when hand-pollinated. Recently, molecular phylogenetic analysis has shown that these two species are sister taxa (Beardsley *et al.*

Figure 1. *Mimulus lewisii* and *M. cardinalis*.

Bumblebee-pollinated	Hummingbird-pollinated
Pink	Red
Wide corolla opening	Narrow, tubular corolla
Inserted stigma/anther	Exserted stigma/anther
1-2μl nectar	40-100μl nectar

2003), 97% identical in noncoding regions of the genome, and >99% identical in coding regions.
3. F_2 and F_3 populations derived from the F_1 hybrids segregate for the adaptive traits that differ between *M. lewisii* and *M. cardinalis*.
4. The segregating populations can be cloned by vegetative propagation, allowing recombinant genotypes to be grown in different environments; *e.g.*, the classical Carnegie transplant stations at Stanford (30m elevation), Mather (1400m), and Timberline (3050m).
5. Wild sympatric populations of the two species are found at several locations.

Entering the genomics era, something that the Carnegie team could scarcely have anticipated in 1971, *Mimulus* has proven to have additional strengths:

1. The genome size is approximately 480Mbp, similar to the sequenced genomes of rice and poplar, and only about 3 times larger than *Arabidopsis thaliana*. The Joint Genome Institute has sequenced and assembled (though not yet published) the genome of *M. guttatus*, which shares 94% DNA sequence identity in coding regions with *M. lewisii* and *M. cardinalis*.

2. There is essentially no inbreeding depression (unusual for obligately outcrossing species), making it possible to create vigorous and fertile inbred lines, ideal for replicating genotypes across environments in the field.

3. The generation interval is just 12 weeks seed-to-seed, allowing up to 4 generations per year. Seed set is 1000-2000 seeds per pollination, seeds are viable for decades at room temperature, and there is no seed dormancy.

4. Plants can be grown to flowering age (8 weeks) at very high density in the greenhouse. We can grow as many as 20,000 at a time in the UW greenhouse.

5. The haploid chromosome number is 8, but the total map length of the genome estimated from recombination in *M. lewisii* x *M. cardinalis* hybrids is only 450cM (Bradshaw *et al.* 1995). This suggests an average physical:genetic distance ratio of ~1Mb/cM. However, our recent comparative linkage mapping in intraspecific *M. lewisii* x *M. lewisii* crosses shows that the short map length in the interspecific hybrids is due primarily to a lack of recombination between 2 pairs of chromosomes in the hybrids (C. Owen, B. Christensen, H.D. Bradshaw, Jr., unpubl.). On the freely recombining linkage groups (including all those containing the QTLs targeted in this proposal) the physical:genetic distance ratio, as estimated from genetic maps, physical maps, and DNA sequencing, is on the order of 20-250kb/cM (C.3., below), making fine-scale physical mapping and positional cloning quite approachable with realistic numbers of meioses.

6. An NSF FIBR grant has funded the development of new genetic markers, physical maps, and mapping populations for *Mimulus*, and stimulated the whole-genome sequencing of *M. guttatus* by the JGI (more details in C.1., below).

One of the greatest strengths of the *Mimulus* system is its amenability to large-scale field experiments in its natural habitat and native range. The three historical Carnegie altitudinal transplant stations (Stanford, Mather, Timberline) are still available, and we have used Mather

and Timberline, as well as several other field sites in and around Yosemite National Park, in our own work (Schemske & Bradshaw 1999; Bradshaw & Schemske 2003; Ramsey *et al.* 2003; Angert *et al.* 2008). Areas of sympatry between *M. lewisii* and *M. cardinalis* were reported along the South Forks of the Merced and Tuolumne Rivers (Hiesey *et al.* 1971); these contact zones still exist, and will be used for our proposed field experiments (D.3., below). We have (and have had for more than a decade) permission from the Park Service, US Forest Service, and Carnegie Institution to use any or all of these sites for our field experiments.

Where *M. lewisii* and *M. cardinalis* are sympatric, hybrids are extremely rare (Hiesey *et al.* 1971; Ramsey *et al.* 2003). Ramsey *et al.* (2003) estimate the effective hybridization rate as 0.0009, based upon finding 2 hybrid seeds among 2336 seeds examined from both species in sympatry. Total reproductive isolation between *M. lewisii* and *M. cardinalis* in sympatry thus closely approaches the maximum value of 1 (1 – 0.0009 = 0.9991). **Pollinator preference accounts for >99% of the total reproductive isolation between *M. lewisii* and *M. cardinalis* in sympatry; >99% of pollinator visits to *M. lewisii* are made by bumblebees, and >99% of visits to *M. cardinalis* are by hummingbirds (Bradshaw & Schemske 2003).**

B.5. Long-term objectives. Ultimately, we wish to identify and characterize all of the major genes that contribute to the phenotypic differences between *M. lewisii* and *M. cardinalis*. We will thus discover both the genetic basis of species differences (Orr 2001) and the effects of each allelic substitution on reproductive isolation, as determined in an ecologically relevant context in the field – *natural populations in their native range and habitat*. In addition to QTLs controlling differences in flower color (C.2., below), we have mapped major QTLs for nectar volume, flower size, and the placement of reproductive organs (anthers and stigma), all of which affect pollinator preference (or pollen transfer) in this system (Bradshaw *et al.* 1995; Bradshaw *et al.* 1998; Schemske & Bradshaw 1999). Once we have identifed the genes involved in flower color (this proposal), we will be in a position to dissect QTLs for other floral traits down to individual genes . Ultimately, we expect to generate a comprehensive gene-by-gene account of the components of the prezygotic isolating factors that can lead to the origin of new species. For those genes where there is only one mutational difference between *M. lewisii* and *M. cardinalis*, or for which all observed mutations are equivalent (derived loss-of-function alleles), the mutational trajectory from bee-pollinated to hummingbird-pollinated may be inferred.

B.6. Summary. The significance of our proposed work is:

1. A multi-locus approach to studying the genetics of prezygotic reproductive isolation, which, despite its importance, is poorly known compared to postzygotic reproductive isolation.

2. Prezygotic reproductive isolation is studied in its appropriate context (as far as the Biological Species Concept is concerned) – a zone of sympatry between two species living in their natural habitat and native range and visited by their native, co-evolved pollinators.

3. A test of the hypothesis that a few loci can produce substantial prezygotic reproductive isolation, which bears on the questions surrounding the rapid diversification of flowering plants.

4. We are very likely to discover one or more of the actual genes at the flower color loci, and to identify the mutations that have been fixed in the evolution of hummingbird pollination from a bee-pollinated ancestor, bringing the study of prezygotic reproductive isolation closer to the high genetic standards set by those working on postzygotic reproductive isolation.

C. Preliminary Studies

C.1. Development of gene-based markers and physical maps are enabling detailed characterization of QTL alleles that distinguish *M. lewisii* (bumblebee-pollinated) from *M. cardinalis* (hummingbird-pollinated). We were among the first to apply genome mapping methods to study the evolutionary biology of natural populations, mapping QTLs affecting pollinator-mediated reproductive isolation in *M. lewisii* and *M. cardinalis* (Bradshaw *et al.* 1995; Bradshaw *et al.* 1998; Schemske & Bradshaw 1999; Bradshaw & Schemske 2003). The anonymous RAPD markers (Williams *et al.* 1990) used for these initial QTL mapping experiments led us to discover that "major" QTLs (defined as explaining $\geq$25% of the F_2 phenotypic variance) control most traits associated with pollinator attraction (*e.g.*, flower color), reward (*e.g.*, nectar volume), and pollen transfer efficiency (*e.g.*, stamen length). But because RAPDs are anonymous, dominant, PCR-amplified genetic markers defined only by a short (10nt) primer sequence, they are poorly suited for follow-up experiments to identify the specific genes underlying QTLs.

For the past 5 years we have been part of a large collaborative NSF FIBR project (PI: John Willis, Duke Univ.) to develop the genus *Mimulus* as a model for ecological and evolutionary genetics (Wu *et al.* 2008; http://mimulusevolution.org). The FIBR project, which ends in Aug 2010, has produced genetic and genomic resources for the comprehensive study of fundamental questions in speciation.

C.1.1. Genetic marker development. We now have gene-based markers and physical maps suitable for detailed characterization and positional cloning of QTLs in *Mimulus*. Todd Vision's bioinformatics group at UNC created a software pipeline for converting *Mimulus* expressed sequence tag (EST) sequences into sequence-tagged site (STS) markers. EST sequences (*N* ~ 200,000) were generated by the Joint Genome Institute as part of the *M. guttatus* genome sequencing project, which itself grew out of our FIBR genome mapping project. The STS marker pipeline algorithms have not yet been published, but, in brief:

Mimulus ESTs derived from both *M. lewisii* (markers named MlSTS#### in figures below) and *M. guttatus* (MgSTS###) were aligned with their orthologs in the *Arabidopsis thaliana* genomic sequence. *Mimulus* ESTs which could not be assigned with confidence to a single *A. thaliana* ortholog were rejected. The *Mimulus-Arabidopsis* ortholog alignment was used to infer likely intron positions within the *Mimulus* genes encoding the ESTs. Amplification primers were then designed within adjacent *Mimulus* exons to span a predicted intron, creating exon-primed intron-crossing (EPIC) amplicons in which SNPs or indels can serve as markers (Lessa 1992). Introns are expected to be more polymorphic than coding sequences of the ESTs themselves, making them useful substrates for genetic marker development. Furthermore, the introns can be referenced to specific genes whose orthology relationships can be determined across wide phylogenetic distances (*e.g.*, among flowering plants). A compendium of available *Mimulus* STS markers may be found at http://mimulusevolution.org/viewmap.php.

We sequenced ~1Mbp of amplified introns from inbred lines of *M. lewisii* (line LF10) and *M. cardinalis* (line CE10) to locate SNPs suitable for genetic mapping in interspecific crosses. The frequency of intron SNPs between the two inbred lines is 3%, so essentially every amplified intron (typically 150-500bp in *Mimulus*) has at least one mappable SNP. We have mapped 350 SNPs in *M. lewisii* x *M. cardinalis* hybrids, yielding an average marker spacing of ~1.3cM.

C.1.2. Physical map development. Jeff Tomkins and Anna Blenda at the Clemson University Genomics Institute (CUGI) took the lead on development of a BAC-based physical map for *M. lewisii* (and *M. guttatus*). Genomic DNA from *M. lewisii* inbred line LF10 was used to generate an 8x BAC library (36,864 clones, 125kb inserts). All clones were fingerprinted and end-sequenced. Fingerprints were assembled into contigs with FPC (Soderlund *et al.* 1997). STS-based EPIC genetic markers were anchored to the BAC contigs by overgo hybridization (Ross *et al.* 1999), making it straightforward to align the genetic and physical maps. The current state of the *M. lewisii* physical map, including anchored genetic markers, may be viewed at: http://www.genome.clemson.edu/activities/projects/mimulus/pmap/mlwebfpc.shtml.

C.2. QTL mapping identifies 4 major loci controlling differences in flower color between *M. lewisii* (bumblebee-pollinated) and *M. cardinalis* (hummingbird-pollinated). The pale pink color of *M. lewisii* flowers is produced by a low concentration of magenta anthocyanins (Hiesey *et al.* 1971), primarily pelargonidin glycosides (Wilbert *et al.* 1997). There are no carotenoid (yellow) pigments except in the nectar guides (parallel yellow stripes in the corolla throat; Fig. 2).

The scarlet color of the *M. cardinalis* flower is produced by a combination of intense carotenoid (yellow) pigmentation in the chromoplasts (modified chloroplasts) of the upper and lower epidermis of the petals, and intense pelargonidin/anthocyanin (magenta) pigmentation in the vacuoles, primarily in the upper epidermis of the petals. Neither *M. lewisii* nor *M. cardinalis* flowers reflect in the ultraviolet (DWS & HDB, unpubl.), so only the colors observed in visible light are relevant to pollinator attraction.

Figure 2. Parental inbred lines of *M. lewisii*, *M. cardinalis*, and their F_1 hybrid.

Mimulus lewisii line LF10 | F_1 | *Mimulus cardinalis* line CE10

Our most recent QTL mapping pedigree is a reciprocal backcross (N = 188 BC_1 plants in each direction) rather than an F_2, primarily because the genotyped backcross plants are more useful as starting points for creating near-isogenic lines (NILs). The modest size of the mapping population is all that is required to detect QTLs of large phenotypic effect, and initiate NIL construction for the major QTLs affecting flower color. The founders of the mapping populations (Fig. 3) are inbred lines of *M. lewisii* (LF10) and *M. cardinalis* (CE10) derived from a sympatric population along the South Fork of the Tuolumne River in the central Sierra Nevada of California, just outside Yosemite National Park.

All 376 BC_1 plants were genotyped at a set of 32 framework SNPs spaced at ~20cM intervals. Flower color phenotypes were scored in two ways. First, a digital photograph was taken of each flower, as it would appear to an approaching pollinator, and a section of the image of the ventral petal was analyzed for the intensity of yellow (to estimate carotenoid concentration) and magenta (to estimate anthocyanin concentration) using NIH ImageJ (http://rsbweb.nih.gov/ij/). Second, as originally described by Hiesey, Nobs, and Björkman (1971), visual scores were assigned to the presence or absence of carotenoid pigment in the upper epidermis of the petal (a Mendelian phenotype they called YELLOW UPPER), the intensity (scale of 1-3) of carotenoid pigmentation in the lower epidermis of the petal (*i.e.*, the back of the petal, a quantitative trait called YELLOW LOWER), and the presence/absence of anthocyanin pigmentation adjacent to the yellow nectar guides in the corolla throat (LIGHT AREAS). Annotated images of these phenotypes are found in C.3. (below).

Since *M. cardinalis* carries recessive alleles at most QTLs that distinguish it from *M. lewisii* (Bradshaw *et al.* 1998), the BC_1 to *M. cardinalis* has much more segregating phenotypic variation than the BC_1 to *M. lewisii*. The QTL mapping results shown below are for a backcross to *M. cardinalis*, with inbred line CE10 used as the recurrent parent, in a *M. cardinalis* cytoplasm.

QTLs for each flower color trait (carotenoid concentration, anthocyanin concentration, and YELLOW LOWER visual score) were mapped using MAPMAKER/QTL 3.0 (Lincoln *et al.* 1992) (http://www.broad.mit.edu/genome_software/). *YELLOW UPPER* and *LIGHT AREAS* were scored as Mendelian loci, as originally suggested by Hiesey *et al.* (1971). Results of QTL mapping of floral color phenotypes are summarized in Table 1 below.

Table 1. Major QTLs and Mendelian loci affecting flower color in a backcross between *M. lewisii* and *M. cardinalis*.

Phenotype or locus	Single largest QTL magnitude (% BC_1 variance explained)	LOD score	Linkage group
Carotenoid concentration	90.1%	93.9	7
Anthocyanin concentration	44.0%	20.4	1
YELLOW LOWER visual score	86.0%	40.9	1
YELLOW UPPER	-	-	7
LIGHT AREAS	-	-	1

Based upon the results in Table 1, we can infer that there are 4 major QTLs controlling differences in flower pigment concentration or pattern between *M. lewisii* and *M. cardinalis*. We have verified this inference by construction of NILs for each of the QTLs (C.3.1.-4.). Two of the loci, *YELLOW UPPER* (*YUP*) and *YELLOW LOWER* (*YLO*), control carotenoid (yellow) pigment deposition. *YUP* maps to the QTL on linkage group 7 with the largest effect on carotenoid concentration. The other two loci affect anthocyanin (magenta) pigmentation. The QTL on linkage group 1 that affects anthocyanin concentration is called *ROSE INTENSITY* (*ROI*). The *LIGHT AREAS* (*LAR*) locus controls the pattern of anthocyanin deposition in the corolla.

At all 4 flower color loci it is the *recessive* (or partially recessive, in the case of *ROI*) allele that leads to *increased* pigment deposition, suggesting that the dominant allele (in *M. lewisii*) represses pigment deposition, and that the red flower color associated with hummingbird pollination in *M. cardinalis* has been derived by the successive fixation of loss-of-function alleles at these repressor loci (Bradshaw *et al.* 1998). Dominant (or semidominant) repressors of

anthocyanin pigmentation, such as *Eluta* in *Antirrhinum* (snapdragon, a close relative of *Mimulus*), have been described (Martin *et al.* 1991). It is also possible that dominant loss or reduction of pigmentation results from the production of enzymes that degrade pigments, such as CmCCD4a which oxidizes the yellow carotenoids in *Chrysanthemum* to produce a white flower (Ohmiya *et al.* 2006).

C.3. Genetic mapping, alignment with the *M. lewisii* physical map, and orthology with the whole genome sequence from *M. guttatus* are taking us from QTL to gene for the 4 flower color QTLs (*YUP*, *YLO*, *ROI*, *LAR*). We have set out to characterize these 4 flower color loci at the molecular level, using the increasingly sophisticated tools becoming available for *Mimulus* genetics/genomics. The long-term goal of this characterization, which may not be fully achieved in the requested funding period (and which is not required to carry out the studies proposed here), is to positionally clone each of the QTLs and identify the polymorphism(s) responsible for the allelic differences between *M. lewisii* and *M. cardinalis*. A synopsis of our QTL positional cloning approach for each of the 4 target flower color genes:

1. From the genotyped reciprocal backcross mapping population, develop low-resolution near-isogenic lines (lrNILs) for each of the 4 QTLs. Using these lrNILs as founders of mapping populations forces the phenotype produced by each QTL to segregate in an easily-scored Mendelian fashion. Mendelizing QTLs historically has been a necessary first step in positional cloning (*e.g.*, Frary *et al.* 2000; Fridman *et al.* 2000). **This step has been completed for all 4 QTLs (additional details and images below).**
2. Map each QTL at 1-5cM (~200-1000kb) resolution using $\geq$500 meioses from the lrNIL-derived mapping population and all 350 available SNP markers to flank the QTL. **This step has been completed for 3 of the 4 QTLs (*YUP*, *YLO*, *ROI*; details below).**
3. Identify a single BAC contig from the *M. lewisii* physical map that contains at least one flanking marker on each side of the target QTL. **This step has been completed for 1 of the 4 loci (*ROI*; details below).** Sequence and annotate the BACs representing the minimum tile path known to contain the QTL, develop SNP markers in candidate genes within the target region, and map recombination breakpoints as closely as allowed by the 500 meioses (average distance between breakpoints = 0.2cM ~ 40kb).
4. Produce and genotype (with the new gene-based SNPs developed in [3.], above) a high-resolution mapping population of 4000 meioses to confine the target QTL to a 0.025cM (~5kb) interval. Retain a plant with a recombination breakpoint as close as possible to one side of the target, and from that plant produce a second high-resolution mapping population of 4000 meioses, finishing the genetic mapping with an average of 0.012cM resolution (~2.5kb) between breakpoints (*i.e.*, the longest average distance between a breakpoint and any position in the genome will be ~1.2kb). **This two-step high-resolution recombinational mapping will serve simultaneously to determine whether each QTL is composed of one or more genes, verify the identity of the gene(s) that is(are) the QTL, and yield a high-resolution NIL (hrNIL) containing an average of 0.025cM (~5kb) on either side of the target QTL – about the same size as the average single-gene interval (9kb) in *Mimulus*, as estimated from the preliminary annotation of the *M. guttatus* genome (D.2. Aim 2).** [*NB*: We also have the ability to validate QTL identity by transgenesis, but for practical reasons given in D.2. **Aim 2** (below) we will attempt single-gene resolution by recombinational mapping.]
5. **Alternative/supplementary positional cloning strategies.** If the markers flanking a target QTL are not located on a single BAC contig on the *M. lewisii* physical map (as described in [3.] immediately above), there are two options. First, create a new BAC

library (8-10x depth; 32-40K BACs @ 125kb) of the *M. lewisii* genome, using either random shearing, or a restriction enzyme other than *Hin*dIII (which was used to construct the existing BAC library). Screen (by filter hybridization) the new and existing BAC libraries with flanking markers, sequence the hybridizing BACs to develop new markers and candidate gene lists, and chromosome walk to the QTL. **We propose to create a new *M. lewisii* BAC library (see D.1. Aim 1, below) since we know that markers closely linked to at least one target QTL (*YUP*) are unrepresented in the current library.** Second, align the QTL map with the available *M. guttatus* genome sequence (which soon will be public at http://monkeyzome.phytozome.net/cgi-bin/gbrowse/mimulus2/) to develop new markers and candidate gene lists. We have found that the genomes of *M. lewisii* and *M. guttatus* are collinear, at least over the 1-5cM typical of our QTL intervals. **We have identified the orthologous region of the *M. guttatus* genome sequence at (or very near) 3 of the 4 flower color QTLs (*YUP*, *YLO*, *ROI*; details below).**

C.3.1. *YELLOW UPPER* (*YUP*). Vickery and Olson (1956) and Hiesey, Nobs, and Björkman (1971) described *YUP* as the only one of 24 traits obviously segregating in a Mendelian fashion in crosses between *M. lewisii* and *M. cardinalis*. We have found that *YUP* is on linkage group 7, and accounts for 90% of the segregating variance in yellow (carotenoid) pigmentation in the BC_1 to *M. cardinalis* (Table 1, above). The dominant *M. lewisii YUP* allele prevents carotenoid pigment deposition in the chromoplasts of the upper epidermis of the petal, as is apparent from the pink (rather than yellow, orange, or red) color of the F_1 (Fig 2). A *yup/yup* low-resolution NIL in the *M. lewisii* background is shown in Fig. 3.

Mimulus lewisii line LF10 | *yup* lrNIL

The effect on pollinator visitation of substituting (at low resolution) the *yup* allele from *M. cardinalis* into *M. lewisii* has been determined in field experiments (Bradshaw & Schemske 2003). The pale yellow-flowered "mutant" produced by the *M. cardinalis yup* allele in the *M. lewisii* background attracts hummingbird visits at ~70 times the rate of the pink-flowered wild-type *M. lewisii*, while simultaneously decreasing the rate of bumblebee visitation by ~6-fold. **Hummingbirds are responsible for 99.9% of visits to *M. cardinalis*, but only 0.1% of visits to the *YUP*/___ wild-type *M. lewisii* NIL. Hummingbirds represent 35% of the visits to the *yup*/*yup* lrNIL in the *M. lewisii* background – *i.e.*, *yup* confers 35% of the reproductive isolation between *M. lewisii* and *M. cardinalis* in sympatry!** Thus, alternative alleles at the *YUP* locus have the remarkable effect of recruiting an entirely novel pollinator guild, and it seems plausible that a loss-of-function mutation in *YUP* played a significant role in the evolutionary divergence that led to the origin of *M. cardinalis* as a reproductively isolated, hummingbird-pollinated species. *YUP* serves as both proof-of-concept for our proposed field experiments with NILs (D.3. **Aim 3**, below) and as a high-value target for positional cloning and characterization.

The current genetic map of *YUP* is shown in Fig. 4. Unfortunately, the marker most closely linked to *YUP* (MISTS5481) does not hybridize to the existing *M. lewisii* BAC library. This is not due to faulty overgo primer design or failed hybridization, since the same overgo, in the same

hybridization experiment, readily hybridizes with BACs in the heterologous *M. guttatus* libraries. Since our best evidence suggests that *YUP* is not represented in the current 8x *M. lewisii* library, we are proposing to create an additional 8-10x *M. lewisii* BAC library (D.1. **Aim 1**, below).

Until we can obtain a *M. lewisii* BAC library that covers the *YUP* locus, one alternative approach to candidate gene identification is to take advantage of the collinearity between the *M. lewisii* genome and the sequenced *M. guttatus* genome. The EPIC marker most closely linked to *YUP* (MISTS5481), and the nearest flanking marker (MISTS5212) have their orthologs on the ~3Mbp *M. guttatus* sequence scaffold_11 (Fig. 4). New markers developed (based on the *M. guttatus* sequence) for a MATE efflux protein (TRANSPARENT TESTA12) and a HEAT repeat protein have moved us within 0.18cM (*ca.* 50kb) of *YUP* (Fig. 4). Effort is now being focused on the R2R3 *MYB* transcription factor gene in the expected vicinity of *YUP* (Fig. 4). MYB transcription factors figure prominently in the regulation of anthocyanin pigmentation in plants (*e.g.*, Glover & Martin 1998; Schwinn *et al.* 2006), including acting as repressors of pigment deposition (Martin *et al.* 1991). Essentially nothing is known about the transcriptional control of the carotenoid pigmentation pathway in plants, but it seems worthwhile to test the hypothesis that *YUP* co-segregates with the *MYB* gene in the candidate region.

Figure 4. Genetic map of the *YELLOW UPPER* locus on a segment of linkage group 7 (gray bar). Numbers in red represent the number of observed recombinants between adjacent markers, of 1536 total meioses. The orthologous region of the *M. guttatus* sequence scaffold is shown (yellow bar) with some gene models listed below. Mapped markers/genes are indicated with vertical black lines. Physical:genetic distance ratio = 259kb/cM.

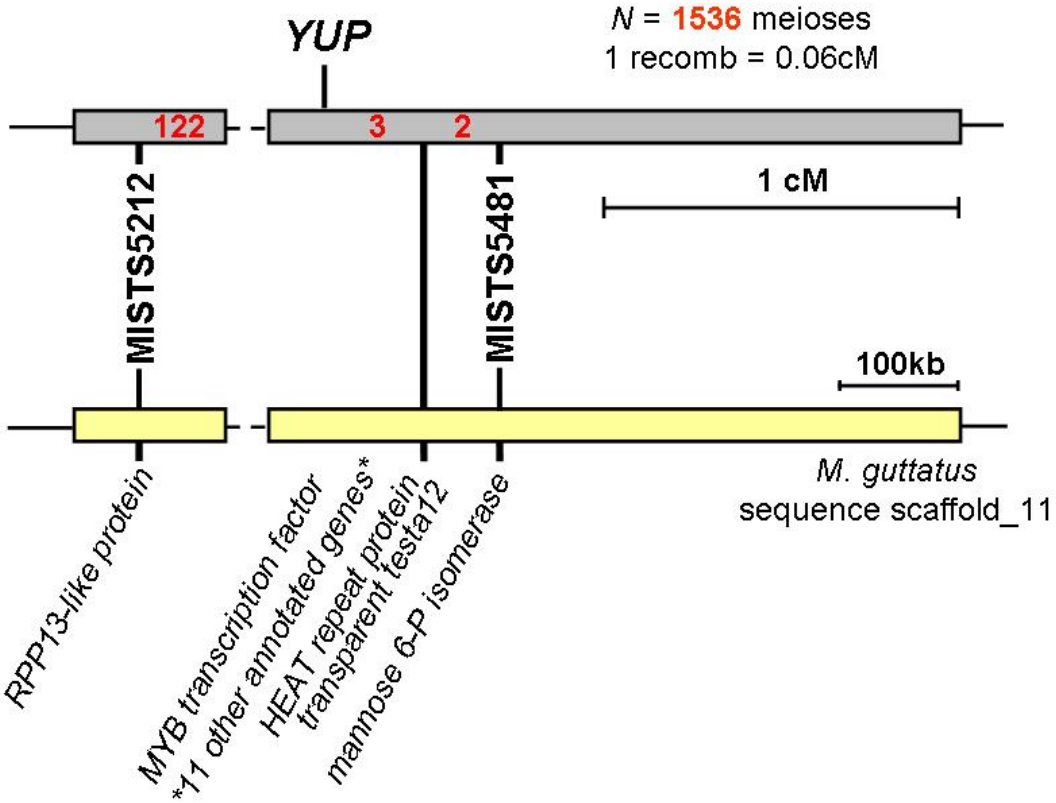


Figure 5. *YLO*/*ylo* and *ylo*/*ylo*

YLO/*ylo* *ylo*/*ylo*

C.3.2. *YELLOW LOWER* (*YLO*). Hiesey, Nobs, and Björkman (1971) described the YELLOW LOWER phenotype as genetically "complex," but we find that 86% of the phenotypic variance in YELLOW LOWER visual score among the BC_1 to *M. cardinalis* is accounted for by a single locus (*YELLOW LOWER*) on linkage group 1 (Table 1, above).

Two flowers from a BC_1 to *M. cardinalis*, differing in their genotypes at *YELLOW LOWER* (but having the dominant *M. lewisii YUP* allele, hence pink-flowered) are shown in Fig. 5. The dominant *YLO* allele from *M. lewisii* represses carotenoid pigment deposition in the lower epidermis of the petal.

The image in Fig. 6 (below), of a *YLO* lrNIL (in a *M. cardinalis* background) shows that the lack of carotenoids in the lower petal epidermis (left flower, left panel) produces a different, less intense salmon-pink (rather than red-orange) hue when viewed as a pollinator would see it, from the front (left flower, right panel). In a pink-flowered (*YUP*/___) plant, the addition of carotenoids to the lower epidermis (*ylo*/*ylo*) creates the "amber sheen" noted by Hiesey *et al.* (1971), who understood the histology but were unable to resolve the genetics of this phenotype.

Figure 6. *YLO*/*YLO* lrNIL in a *M. cardinalis* background

YLO lrNIL *M. cardinalis* line CE10 *YLO* lrNIL *M. cardinalis* line CE10

We do not expect *YLO* to have as dramatic an effect on pollinator discrimination as *YUP*, because our QTL mapping results show that *YLO* accounts for none of the BC_1 variance in yellow pigmentation as observed from the front of the flower. However, we do expect that the increased concentration of carotenoids produced by *ylo* will measurably reduce bumblebee visitation rate, since the yellow lower epidermis will obscure, to some degree, the contrast between the corolla throat (white in *M. lewisii*) and the yellow nectar guides. To the extent that reduced bumblebee visitation makes flowers more attractive to hummingbirds (perhaps by allowing more nectar to accumulate), the *ylo*/*ylo* NIL in the *M. lewisii* background should also increase hummingbird visitation rates.

The current genetic map of *YLO* is shown in Fig. 7. The *M. guttatus* ortholog of MlSTS5143 lies on a sequence scaffold that is probably too short (46kb) to contain *YLO*, since the physical:genetic distance ratio in this region is approximately 100kb/cM, and MlSTS5143 is about 1cM from *YLO*. Sequencing of *M. lewisii* BAC contig #326, and development of flanking SNP markers from genes on that contig, will allow us to determine whether *YLO* is found there.

Figure 7. Genetic map of the *YELLOW LOWER* locus on a segment of linkage group 1 (gray bar). Numbers in red represent the number of observed recombinants between adjacent markers, of 618 total meioses. A BAC contig from the *M. lewisii* physical map (pink bar) and the orthologous region of the *M. guttatus* sequence scaffold (yellow bar) are shown.

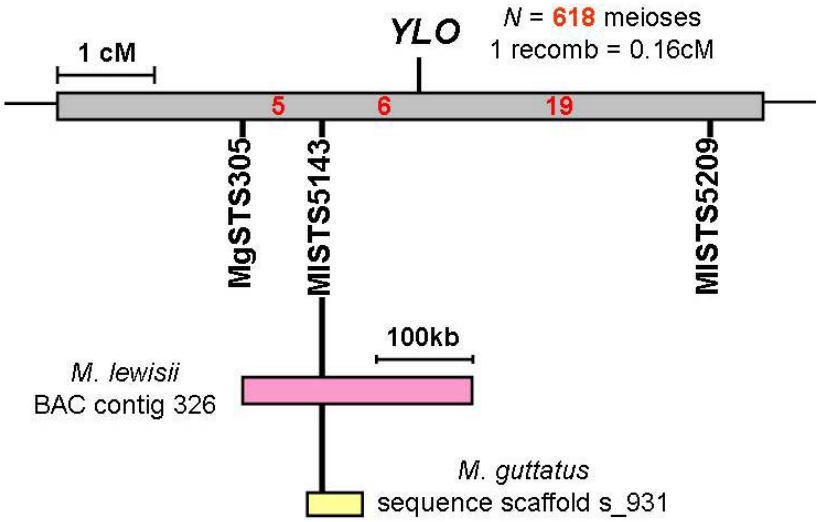


Figure 8. *roi*/*roi* lrNIL in a *M. lewisii* background

Mimulus lewisii line LF10 — *roi* lrNIL

C.3.3. *ROSE INTENSITY* (*ROI*). Hiesey, Nobs, and Björkman (1971) described the trait ROSE INTENSITY as the concentration of magenta "anthocyanins in the cell sap." Hiesey *et al.* (1971) stated that, "Obviously, no simple Mendelian interpretation suffices to account for the complex expression of this character." However, we find that 44% of the phenotypic variance in the BC_1 to *M. cardinalis* is accounted for by a single locus (*ROSE INTENSITY*) on linkage group 1 (Table 1). The *M. lewisii ROI* allele, which suppresses anthocyanin pigment deposition, is semidominant, with the *ROI*/*roi* heterozygote having an intermediate ROSE INTENSITY phenotype, as can be seen in the F_1 between *M. lewisii* and *M. cardinalis* (Fig. 2).

A *roi/roi* low-resolution NIL in the *M. lewisii* background is shown in Fig 8. The semidominant suppression of anthocyanin deposition produced by *ROI* is reminiscent of *Eluta* in *Antirrhinum* (Martin *et al.* 1991). Unfortunately, *Eluta* has not been cloned, and so cannot inform a candidate gene search for *ROI*. However, it has been suggested that *Eluta* may be an allele of *Rosea*, which is known to encode an R2R3 MYB transcription factor (Schwinn *et al.* 2006).

Increased anthocyanin concentration simultaneously decreases bumblebee visitation rate and increases hummingbird visitation rate in an F_2 of *M. lewisii* and *M. cardinalis* (Schemske &

Bradshaw 1999), so it is likely that allelic variation at *ROI* will have a marked effect on divergence in pollinator preference in the field experiments to be carried out in D.3. **Aim 3**. In combination with *yup*, the *roi* allele will produce an orange or red-orange flower, and this is expected to be very attractive to hummingbirds but unattractive to bumblebees.

We have flanked *ROI* with STS markers (MgSTS55 and 158) that lie on the same ~1Mbp *M. lewisii* BAC contig, #711 (Fig. 9, below). Using the orthologous region of the *M. guttatus* genome sequence scaffold_4 as a guide, we developed markers for two candidate genes potentially involved in anthocyanin transport: a *TRANSPARENT TESTA12* homolog, and an *ABC transporter*. These markers flank *ROI* very closely (even mapping to the same 125kb *M. lewisii* BAC clone 75E12), but are clearly separable from *ROI* by recombination on either side. In addition to possible regulatory regions of the *TT12* and *ABC transporter* homologs, there are two annotated genes in this 11kb interval: homologs of *HSP70* and an *Arabidopsis* gene of unknown function (*At1g11655*). By far the most direct way to locate *ROI* is to sequence *M. lewisii* BAC 75E12 and develop markers in this recombinationally active (20kb/cM) region.

Figure 10. Genetic map of the *ROSE INTENSITY* locus on a segment of linkage group 1 (gray bar). Numbers in red represent the number of observed recombinants between adjacent markers, of 2166 total meioses. The corresponding *M. lewisii* BAC contig, (#711) with markers flanking *ROI* (MgSTS55 and MgSTS158), is shown (pink bar). The single *M. lewisii* BAC clone 75E12 contains two genes flanking *ROI*. The orthologous region of the *M. guttatus* sequence scaffold_4, with all gene models known in the vicinity of *ROI*, is shown (yellow bar). Physical:genetic distance ratio = 20kb/cM.

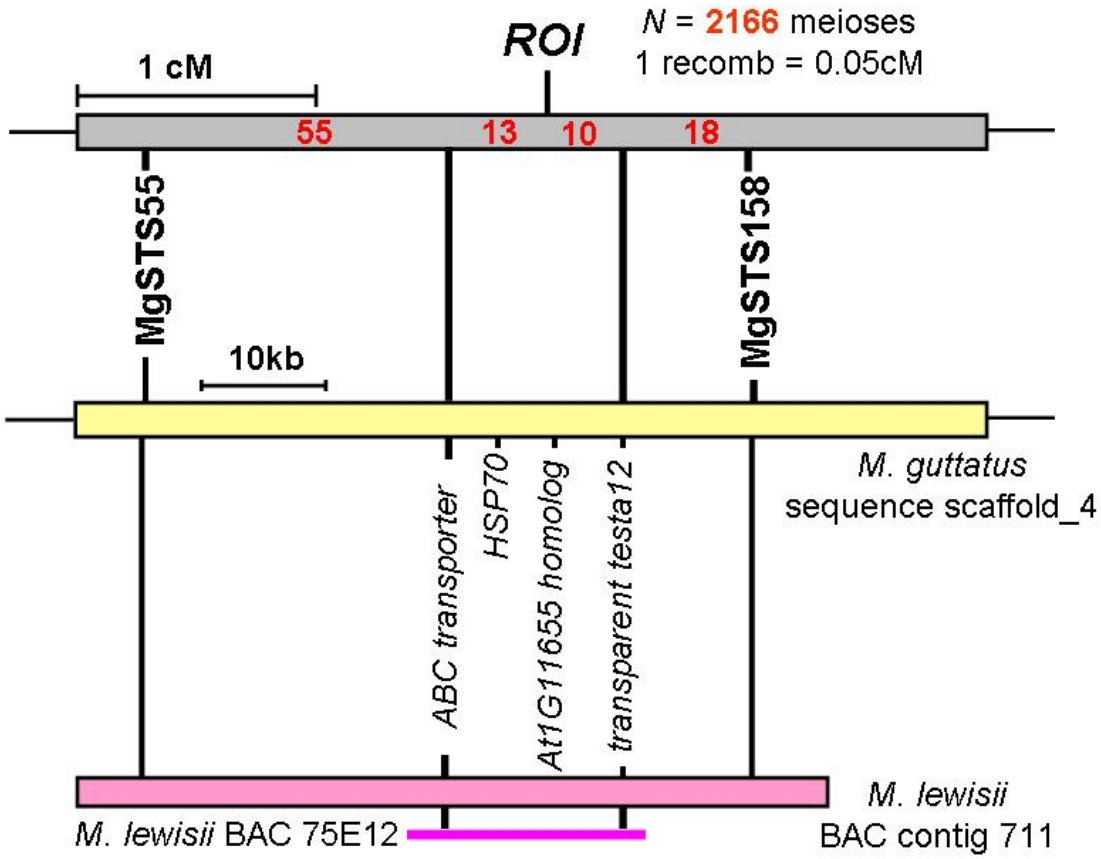


Figure 10. *lar*/*lar* lrNIL in a *M. lewisii* background

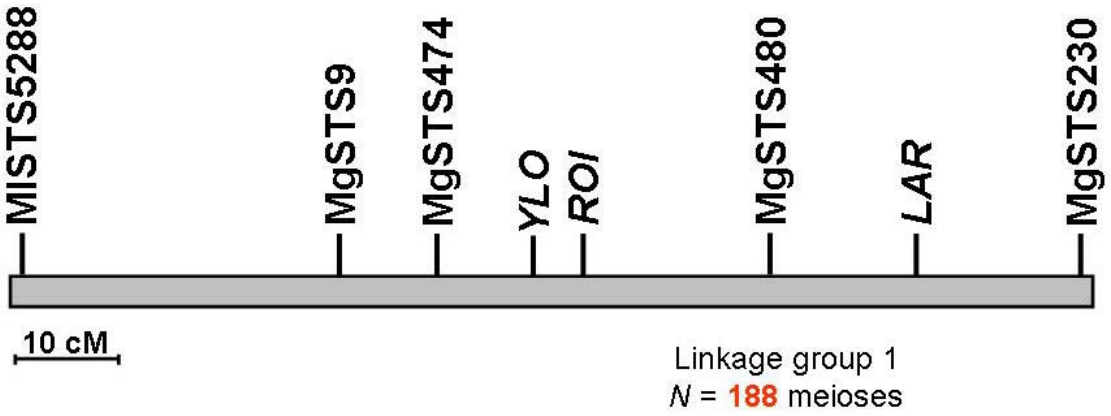
Mimulus lewisii line LF10 | *lar* lrNIL

C.3.4. *LIGHT AREAS* (*LAR*). Hiesey, Nobs, and Björkman (1971) defined LIGHT AREAS as "an unpigmented band about 2mm wide at the base of the petal lobes" (arrow in image of *M. lewisii* LF10 wild-type). Hiesey *et al.* (1971) suggested that this phenotype could be scored as present/absent in an F_2, with "ratios approximating 3:1 although some marked deviations have been observed in F_3 progenies …". LIGHT AREAS is easiest to score in *YUP*/*yup* (*i.e.*, pink-flowered) segregants in the BC_1 to *M. cardinalis*. When scored as a presence/absence trait, *LIGHT AREAS* maps to linkage group 1 (Table 1, above).

A *lar*/*lar* low-resolution NIL in the *M. lewisii* background is shown in Fig. 10. Unlike *YUP*, *YLO*, and *ROI*, *LIGHT AREAS* affects pigment pattern more than pigment intensity. The *M. cardinalis lar* allele causes the anthocyanin pigments to be more uniformly distributed within the petal, eliminating the darker pink stripes down the middle of each *M. lewisii* petal, as well as eliminating the white petal patches (LIGHT AREAS) adjacent to the yellow nectar guides. Both the dark anthocyanin stripes and the yellow nectar guides are likely to act as visual cues for bumblebees, so we expect that the reduced contrast produced by the *lar*/*lar* genotype in the *M. lewisii* background will reduce attractiveness to bumblebees, while the modest enhancement of overall anthocyanin pigmentation may increase hummingbird visitation.

LAR has been mapped to linkage group 1 (Fig. 11), but not yet with resolution adequate to locate it on the *M. lewisii* physical map nor the *M. guttatus* genome sequence scaffolds. The mapping seed stocks derived from a *LAR* lrNIL have been produced and are being grown now.

Figure 11. Framework genetic map of linkage group 1, showing the position of *LIGHT AREAS* (*LAR*).



D. Experimental Design and Methods

D.1. Aim 1. Produce high-resolution genetic and physical maps of the 4 major quantitative trait loci (QTL) controlling differences in flower color between bumblebee-pollinated *M. lewisii* and hummingbird-pollinated *M. cardinalis*. Beginning with our genotyped reciprocal BC_1 QTL mapping population (Preliminary Studies C.1.), we have identified "low-resolution NIL (lrNIL) progenitors" for each of the 4 target flower color loci (*YUP*, *YLO*, *ROI*, *LAR*). The lrNIL progenitors are heterozygous in the vicinity of the target locus but homozygous for the *M. lewisii* allele at marker loci across the remainder of the genome. The *M. cardinalis* flower color alleles were further introgressed into the *M. lewisii* background by up to four additional generations of backcrossing to *M. lewisii*. A final round of self-pollination yielded BC_nS_1 lrNIL progeny homozygous for each of the desired *M. cardinalis* flower color alleles (images in C.3., above). These lrNILs will serve as starting points for creating large (*N* = 8000 meioses) high-resolution mapping populations and the high-resolution NILs (hrNILs) that will be employed in field experiments to investigate pollinator preferences (D.3. **Aim 3**).

We will use the *M. lewisii* genetic background for carrying out the high-resolution mapping and hrNIL construction because phylogenetic analysis indicates that hummingbird pollination is the derived character state in the section of *Mimulus* (*Erythranthe*) containing *M. lewisii* and *M. cardinalis* (Beardsley *et al.* 2003). Bee → hummingbird pollination is also the predominant polarity of character state evolution in other plant taxa (Whittall & Hodges 2007, Smith *et al.* 2008) and in the western North American flora as a whole (Grant 1994), so our results can be generalized to large-scale evolutionary trends.

D.1.1. Genetic mapping of each QTL at 1-5cM (~200-1000kb) resolution. Low-resolution NILs will be backcrossed to *M. lewisii* inbred line LF10. The resulting heterozygotes will be self-pollinated to produce a 3:1 F_2 segregation and obtain two informative meioses for the cost of each DNA extraction and genotyping assay. Only recessive homozygous phenotypes ($N \geq 250$) are used for mapping.

We have used a variety of SNP genotyping platforms, primarily the Perkin-Elmer AcycloPrime single-base extension chemistry, which works well with liquid handling robots and plate readers. Other technologies, such as TaqMan and mass spec (*e.g.*, Sequenom) also are suitable for the scale of our project (*i.e.*, a few markers on dozens to tens of thousands of individuals), and will be evaluated for effectiveness (accuracy, cost, turnaround time) as needed.

Of the 4 flower color loci, only *LIGHT AREAS* remains to be mapped at 1-5cM resolution (C.3.4., above). The necessary *LAR* mapping crosses have been made, and the F_2 plants representing at least 500 meioses with the recessive phenotype are being grown in the greenhouse now. We have 42 STS/SNP markers on linkage group 1, with an average spacing of <2cM, so we foresee no difficulties in localizing *LAR* as we have already done for the other 3 flower color genes.

D.1.2. Genetic to physical map. Our STS markers have been placed (by overgo hybridization) on the *M. lewisii* BAC contigs that constitute the physical map (http://www.genome.clemson.edu/activities/projects/mimulus/pmap/mlwebfpc.shtml).

In the best case (*e.g.*, for *ROSE INTENSITY*, C.3.3., Fig. 9, above), we will find a single BAC contig that carries two or more flanking genetic markers, and therefore must contain the target flower color QTL, as well. We will proceed directly to sequencing the minimum tile path of the

BAC contig containing the target (details below), use the annotated sequence to create a catalog of candidate genes, develop new SNP markers in the candidate genes, and refine the map position of the target locus with recombination breakpoints derived from at least 8000 meioses (0.012cM resolution, ~2.5kb; described in D.2.1., below). This will reveal whether the QTL is composed of one gene or multiple linked genes, and identify the causal gene(s) as long as they are within the resolution provided by 8000 meioses.

In other cases, such as for *YELLOW LOWER* (C.3.2, Fig. 7, above), we have only one flanking marker (the most tightly linked) on a *M. lewisii* BAC contig. In such circumstances, we will sequence and annotate the contig, develop new SNP markers, and attempt to flank the target locus with the newly-discovered SNPs. This looks promising for *YLO*, given the length of the BAC contig and the proximity of MlSTS5143 (Fig. 7).

If we find flanking markers on two separate BAC contigs, this suggests that either there is a gap in the physical map, or that the genome-wide FPC assembly thresholds (Soderlund *et al.* 1997) were too stringent to recognize the overlap between the two (or perhaps more) separate flanking contigs. We will attempt a "local assembly" of all the BACs in the unjoined contigs using FPC with relaxed requirements for overlap of BAC fingerprints, appropriate for dealing with only a few dozen BACs. The FPC raw data are available from our NSF FIBR collaborators at the Clemson University Genomics Institute. If necessary, we will sequence all of the unjoined BACs to look for short regions of overlap that escaped detection by FPC.

If there appears to be a genuine gap in the current physical map (*e.g.*, for *YELLOW UPPER*, C.3.1., above), we will screen (by filter hybridization with flanking markers) a new 10x *M. lewisii* BAC library constructed by random shearing, or with a restriction enzyme other than the *Hin*dIII used for the existing library. The new *M. lewisii* BAC library, like the existing library, will be constructed at the CUGI BAC service facility for $0.30/clone (~$12K/library + ~$2K for a set of filters).

We have good evidence that constructing an additional BAC library will provide the needed genome coverage, based upon the *M. guttatus* physical map (also constructed at CUGI). The *M. guttatus* physical map was made with <u>two</u> 8x BAC libraries, and 34.5% of all loci mapped by overgo hybridization were mapped to just one of the two libraries (Jason Phillips and Todd Vision, UNC, pers. comm.), suggesting that the additional *M. lewisii* BAC library will be necessary to close the gaps in the *M. lewisii* physical map.

D.1.3. Physical map to DNA sequence. Once we have identified a BAC contig that contains one of the target flower color genes, we will sequence the minimum tile path for that contig. If we are chromosome walking in the new *M. lewisii* BAC library, at each step we will pool, sequence, assemble, and annotate all the BACs hybridizing to tightly linked markers.

We have considered the various next-gen sequencing technologies available to us on our campuses (U Washington and Michigan State). We prefer the longer read length of the 454 platform for sequencing *M. lewisii* genomic DNA, since longer reads will make assembly easier in the absence of a *M. lewisii* reference genome sequence. The *M. guttatus* genome sequence will be useful for assembling the coding regions in *M. lewisii*, but divergence in non-coding DNA would make it more risky to attempt assembly of entire *M. lewisii* BAC contigs using a short-read sequencing platform such as Illumina/Solexa. However, we will keep abreast of all available

technologies and use whichever platform, or combination of platforms, seems best suited our sequencing needs as they arise.

The 454 sequencing will be done in the laboratory of Prof. Roger Bumgarner (UW Microbiology; see attached letter of support). BAC DNA will be prepared and pooled in equimolar amounts, fragmented by nebulization, and sequenced.

We anticipate sequencing 1-2Mbp to 50-100x depth surrounding each of the 4 flower color loci. Even if we have identified the orthologous region of the existing *M. guttatus* sequence (*e.g.*, for *YELLOW UPPER*, C.3.1., above), given the low cost of 454 technology we will sequence the *M. lewisii* genome around each flower color locus to detect any subtle (but potentially important) evolutionary events (*e.g.*, gene duplication or transposon insertion/deletion) since the divergence of *M. lewisii* and *M. guttatus*.

The current 454 run yields 1.2M reads of 400nt (500Mnt) in Dr. Bumgarner's lab, enough to provide 50-100x depth for ***two*** minimum tile paths of 1-2Mbp (~5-10cM) each, for $10K in reagent cost + $1K of technician time. Barcoded sequencing libraries will make it possible to sequence two (or more, if feasible) target regions simultaneously, allowing us to combine BAC contig sequencing at some loci with chromosome walking experiments at other loci, keeping sequencing costs very reasonable.

D.1.4. Sequence to candidate gene(s). Sequence reads will be assembled and annotated in Prof. Bumgarner's lab (see attached letter). The *M. guttatus* sequence will serve as a useful reference for *M. lewisii* sequence assembly and annotation, at least in protein-coding regions where sequence divergence is just 6% (Toby Clarke and Todd Vision, UNC, pers. comm.).

Candidate gene lists (including putative miRNAs) will be compiled from the annotated sequence. Based upon the known distance from mapped genetic markers, we will choose genes for development of new SNP markers in the expected vicinity of the target QTL, using the same SNP discovery methods we have employed to make our current genetic maps (*i.e.*, sequencing the orthologous region from the *M. cardinalis* inbred line CE10). With a SNP frequency of 3% at neutral positions, we will be able to define recombination breakpoints with considerable precision. **At minimum, we will circumscribe each of the 4 flower color QTL positions with an average resolution of 0.012cM (~2.5kb) provided by 8000 meioses (D.2.1., below) and with SNPs in every gene close to the target in the candidate region.** We anticipate that the gene density in *M. lewisii* will be approximately 1 gene/9kb, based upon a gene density of 11 genes/100kb estimated from sequencing and annotating 14 *M. guttatus* BACs known to contain at least one gene (Eric Ganko and Todd Vision, UNC, pers. comm.) **Our genetic mapping resolution of 0.012cM (~2.5kb) is considerably better than the expected single-gene level.**

We wish to emphasize that it is not essential to the success of the work described in this proposal that we define (by recombination breakpoints) the 4 flower color QTLs with single-gene precision. Nevertheless, we anticipate that in the course of high-resolution mapping we will discover the identity of all or most of the genes that produce the major flower color differences between *M. lewisii* and *M. cardinalis*. These findings will add to the small number of "speciation genes" already identified (mostly from *Drosophila*, nearly all involved in postzygotic reproductive isolation), and contribute even more substantially to a genetic understanding of prezygotic isolation.

D.2. Aim 2. Construct and characterize high-resolution near-isogenic lines (hrNILs) for each of the 4 major loci controlling differences in flower color between *M. lewisii* and *M. cardinalis*. Low-resolution NILs are entirely adequate to Mendelize QTLs for mapping and positional cloning. But lrNILs are less than desirable for testing the effects of a single QTL allele on a phenotype because of linkage drag – the unwanted hitchhiking of alleles at loci linked to the target QTL.

Due to the relatively poor genetic markers (dominant RAPDs, useless for tracking recessive QTL alleles in repulsion phase) and low marker density available to us before the NSF FIBR project, our previous field experiment used low-resolution *YUP* NILs (Bradshaw & Schemske 2003). The 4 backcross generations (without any capability to do marker-assisted selection) we used to make the lrNILs for our prior work gave a nominal non-target genome fraction of ~3%, which likely represents at least 1000 unwanted genes. Linkage drag was detected for several of the phenotypic traits that differ between *M. lewisii* and *M. cardinalis* (Bradshaw & Schemske 2003), including petal reflexing and pistil length (a result that we have now explained by using our new markers and maps to locate major QTLs for these traits on linkage group 7, where *YUP* is also found).

Thanks to the genomics toolkit now available for *Mimulus*, we will develop proper high-resolution NILs at all 4 major flower color loci (**Aim 2**), and test them in the field (**Aim 3**).

D.2.1. Construction of hrNILs. We have chosen to construct high-resolution NILs for each of the 4 flower color loci. This approach has three critical advantages over transgenics for the proposed experiments: 1) hrNIL construction does not depend on verification of the identity of the individual flower color genes (although we do expect to define at least some of these genes by recombinational mapping); 2) there will be no USDA-APHIS or US Park Service restrictions on the deployment of hrNILs in field experiments in or near Yosemite National Park (**Aim 3**), where we have carried out experiments with lrNILs in the past (Bradshaw & Schemske 2003); and, 3) the hrNILs will be direct allele substitutions by recombination, so we will not have to deal with the copy number or position effects that can result from transgenesis.

Each of the hrNILs will be constructed in two steps of 4000 meioses. In the first step, a backcross population (*N* = 4000) segregating 1:1 for the *M. cardinalis* allele in a *M. lewisii* background (*i.e.*, [lrNIL x LF10] x lrNIL) will be screened at the seedling stage with two markers flanking the target QTL at 1-5cM. Only those 1-5% of plants that are homozygous for the *M. cardinalis* allele at one flanking marker and heterozygous at the other (*i.e.*, with an informative recombination in the region) will be retained for further genotyping at markers within the interval and for development of hrNILs. Pre-screening with the flanking markers at the seedling stage allows us to grow very large mapping populations at high density in the greenhouse, then transplant only the informative recombinants into larger pots until their precise recombination breakpoints and flower color phenotype can be determined. We have developed a high-throughput robotic DNA extraction protocol for this screening step.

Precise recombination breakpoints for each backcross plant (*N* = 40-200) retained from the pre-screening with flanking markers will be determined by genotyping with SNPs developed from the BAC contig sequence (D.1.4., above). If it becomes necessary to resolve the order among closely spaced breakpoints near the target (*e.g.*, breakpoints which map to a single gene in the candidate region), we will PCR amplify and sequence the region containing the breakpoint from each relevant recombinant plant and compare it to the orthologous region from *M. lewisii* LF10

and *M. cardinalis* CE10. At this point the target gene will be mapped with a resolution of 0.025cM, or approximately 5kb.

Half of the retained recombinants are expected to be homozygous for the *M. cardinalis* flower color allele desired for hrNIL construction. Among those recombinant genotypes with the desired flower color phenotype we will identify the single plant with a recombination breakpoint closest to one side of the target flower color allele. In the second step of hrNIL construction, this individual will be backcrossed to *M. lewisii* LF10, and the progeny screened to identify a backcross plant containing the breakpoint chromosome (rather than the non-recombinant chromosome which will also be present in a flower color allele homozygote). The heterozygous "close-breakpoint" recombinant will be backcrossed again to *M. lewisii* LF10, and 4000 progeny planted and screened at the seedling stage for recombinants in the target interval (previously resolved at 0.025cM breakpoint spacing). Since only half of these recombinants will be carrying the desired *M. cardinalis* allele at the flower color gene, each of the 5-10 closest recombinants will be self-pollinated and 36 of their F_2 progeny grown to determine their allelic state at the target locus. **The single heterozygous plant that has a recombination breakpoint closest to the other side of the desired *M. cardinalis* allele becomes the parent of the hrNIL population that will be field tested in (D.3. Aim 3).**

At the conclusion of these mapping/hrNIL construction experiments, the target flower color loci will be mapped with 0.012cM (~2.5kb) resolution. Since half of the meioses (*N* = 2000) at each of the two crossing steps yield plants with the desired *M. cardinalis* allele, the average distance between breakpoints at each step of hrNIL construction is expected to be 0.05cM. So, on average, the finished hrNILs will contain ~10kb of *M. cardinalis* DNA in a ~480Mbp *M. lewisii* genome – **approximately a single-allele replacement** by recombination, given the 9kb average gene spacing in *Mimulus* (supporting data in D.1.4. **Aim 1**, above). There is a >90% probability of producing hrNILs with less than 20kb of *M. cardinalis* DNA surrounding the target, and >98% probability that the introgressed segment will be shorter than 30kb (Durrett *et al.* 2002).

D.2.2. Characterization of hrNILs. Each hrNIL parent will be self-pollinated to yield an F_2 population segregating 1:2:1 for each of the three possible flower color genotypes. As the double, triple, and quadruple NILs are produced (D.3. **Aim 3**), the phenotypes of each genotype will be characterized, as well. Digital photographs of each genotype will be analyzed for reflectance of magenta (anthocyanin) and yellow (carotenoid) pigments, as we did for the QTL mapping described above (C.2.). A more refined biochemical analysis will be carried out by extracting anthocyanin and carotenoid pigments from disks punched out of the lateral petal, and pigment concentration measured spectrophotometrically (Bradshaw *et al.* 1995). To assess any differences in the distribution of pigments across cell layers (*e.g.*, for *YELLOW UPPER* and *YELLOW LOWER* hrNILs) or among individual cells within a cell layer (*e.g.*, for *ROSE INTENSITY* and *LIGHT AREAS*) we will make hand sections of the petal and use light microscopy (and digital image analysis, if needed) to determine the phenotypic effect of each allele substitution.

D.3. Aim 3. Determine the proportion of the total reproductive isolation between *M. lewisii* and *M. cardinalis* attributable to each of the 4 flower color loci, and to their combinatorial effects, by direct observation of pollinators in field experiments conducted in areas of natural sympatry. We know from field experiments with low-resolution NILs that the *M. cardinalis yup* allele can account for 35% of the total reproductive isolation between *M.*

lewisii and *M. cardinalis* in sympatry (Bradshaw & Schemske 2003). We now ask, in a much more genetically precise way: What are the effects of all 4 major flower color loci, individually and in combination, on reproductive isolation? Can an oligogenic change in flower color produce substantial (possibly *very* substantial) reproductive isolation mediated by pollinator preference – the same selective force that seems to underlie the massive adaptive radiation of flowering plants (Takhtajan 1986; Labandeira et al. 1994; Labandeira 1998)?

We have identified field sites along the South Fork of the Tuolumne River near Mather CA, where populations of *M. lewisii* and *M. cardinalis* have been in sympatry for 50 years or more (Hiesey *et al.* 1971; Angert & Schemske 2008), from which the parents of our mapping populations and hrNILs are descended, where all of the components of reproductive isolation between the two *Mimulus* species have been studied most intensively (Ramsey *et al.* 2003; Bradshaw & Schemske 2003), and which have the infrastructure (*e.g.*, a supply of water for irrigation) capable of supporting large-scale field experiments. We are very familiar with these sites, having used them for more than a decade. The sites are still managed by the Carnegie Institution of Washington where Clausen, Keck, Hiesey, Nobs, and Björkman began this series of investigations.

D.3.1. Experimental design. Each field experiment will be established in two sites separated by at least 500m, to minimize movement of pollinators between sites. We will test all 4 homozygous single-locus hrNILs at each site in the first field season (2012 – the first year that the single hrNILs will be available). The 6 two-locus "doubles" will be bred from the singles in 2012-13 and field tested in 2013. The 4 triples and the quadruple hrNIL will be bred from the doubles and singles in 2013-14 and field tested in 2014. In the final field season (2015), we will compare just 3 genotypes: the parental *M. lewisii*, the quadruple hrNIL (*yup ylo roi lar*), and the parental *M. cardinalis*. **By replicating the quadruple hrNIL across two sites and two years, we will get a robust (yet logistically tractable) estimate of the aggregate effect of the 4 flower color loci on the reproductive isolation between *M. lewisii* and *M. cardinalis*.**

We will use the same experimental protocol successfully employed for our original low-resolution *yup* NILs (Bradshaw & Schemske 2003). Potted plants of flowering age (*ca*. 2 months from seed) will be transported by truck from Seattle to Mather, and arrayed randomly on 1m centers within a rectangular grid. At each site, each genotype (including the "ancestral" *M. lewisii* parent) will be represented by 25 plants (75-175 plants per site, depending on how many hrNILs/combinations are being tested in a given field season).

A 2-person team will collect data at each site. At each site, the number of flowers on each plant will be counted daily at first light, and older flowers removed as necessary to make total flower number equal among all genotypes within a site (typically 4-6 flowers per plant, 100-150 flowers per genotype). This makes pollinator visitation opportunities comparable across days within a site. Pollinator observations will be carried out from dawn to dusk, with a break at midday when both bumblebees and hummingbirds are least active. Pollinator identity, plant identity (genotype and position), and number of visits at each plant will be recorded and transcribed for later analysis. Observations will be made for up to 4 weeks and pooled for each plant.

D.3.2. Data analysis. The effect of each introgressed genotype (hrNILs and combinatorials) on pollinator-mediated reproductive isolation (RI) from *M. lewisii* will be expressed as the difference in the ratio of hummingbird visits to total pollinator visits (hummingbirds + bumblebees):

$$RI = \frac{\text{hummingbird visits to hrNIL (or combinatorial)}}{\text{total visits to hrNIL (or combinatorial)}} - \frac{\text{hummingbird visits to } \textit{M. lewisii}}{\text{total visits to } \textit{M. lewisii}}$$

In prior experiments at Mather we have shown the proportion of hummingbird visits to be 0.999 for *M. cardinalis* and 0.001 for *M. lewisii* (Bradshaw & Schemske 2003), yielding an RI of 0.998 for *M. cardinalis* – concordant with the very low observed frequency of hybrids with *M. lewisii*.

RI values from individual plants (*N*=25 per genotype) will be analyzed by ANOVA. Genotype at each locus will be treated as a fixed effect, and site as a random effect. For logistical reasons, except for the "quadruple" NIL and the *M. lewisii* parent there will be no replication of genotypes across years, so any effect of field season will, of necessity, be ignored. Pollinator abundance across years makes this a reasonable strategy (Bradshaw & Schemske 2003). The ANOVA for the contribution of all 15 single- and multi-locus hrNIL genotypes to prezygotic reproductive isolation (RI) mediated by pollinators will take the form:

$$RI = \text{site} + yup + ylo + roi + lar + yup*ylo + yup*roi + yup*lar + ylo*roi + ylo*lar + roi*lar + yup*ylo*roi + yup*ylo*lar + yup*roi*lar + ylo*roi*lar + yup*ylo*roi*lar + \text{error}$$

Significant interaction terms (*i.e.*, multi-locus effects larger or smaller than the sum of the individual locus effects) indicate epistasis.

With 25 plants per genotype and 4-6 flowers on each plant, our experience indicates that we will observe many hundreds of pollinator visits per day. Power calculations show that differences in the proportion of hummingbird visits as small as 1% can be detected after 21 days of field observation, even making the conservative assumptions that: 1) the proportion of hummingbird visits to *M. lewisii* is 2% (20 times higher than the observed proportion in previous experiments); 2) at each site each genotype is represented by 100 flowers each day; and, 3) that each flower is visited only once each day by any pollinator. [*NB*: In our experience, wild-type flowers are visited >5 times per day by their preferred pollinators, and both bumblebees and hummingbirds have been abundant and ubiquitous in all of our years of fieldwork at Mather.]

By way of example, we know from prior work (Bradshaw & Schemske 2003) that 35% of visits to *yup/yup* NILs are by hummingbirds, a difference from *M. lewisii* that could be detected in less than an hour of observation in the experimental arrays we are proposing here. As the combinatorial hrNILs become increasingly attractive to hummingbirds with each additional *M. cardinalis* flower color allele, our power to detect differences will decline somewhat. For instance, with 4 weeks of observation (and including a sequential Bonferroni correction for multiple tests with an experiment-wise α=0.05; Rice 1989) we will be able to detect a 3% increase in the proportion of hummingbird visitation in a double hrNIL that is *yup*/*yup* (*i.e.*, has a baseline hummingbird visitation proportion of 35%). Even if the best triple hrNIL has a hummingbird visitation proportion of 90%, we will still be able to detect an effect of as little as 5% from the fourth added allele in the quadruple hrNIL.

The most critical experiment – the comparison of the quadruple hrNIL (*yup*, *ylo*, *roi*, *lar*) with *M. lewisii* – will be replicated in two years as well as in two sites. In the third field season (2014), the quadruple hrNIL will be in an experimental array with all 4 triples and *M. lewisii*. In the fourth and final field season (2015), the quadruple hrNIL will be arrayed in a three-way comparison with *M. lewisii* and *M. cardinalis*. This represents a final test of the aggregate effect of all 4

flower color loci on reproductive isolation in sympatry, calibrated with “ancestral” and derived genotypes (*i.e*., the parental species). The ANOVA model will specify genotype as a fixed effect, with site and year as random effects.

D.3.3. Expected outcomes. Our working hypotheses are that: 1) each *M. cardinalis* flower color allele introgressed into *M. lewisii* will increase hummingbird visitation and decrease bumblebee visitation; 2) each locus will have an additive effect on reproductive isolation; and, 3) the cumulative effect of all 4 loci on reproductive isolation will account for most of the reproductive isolation between *M. lewisii* and *M. cardinalis* in sympatry.

Hypothesis 1. We have some reason to suspect that increased anthocyanin and carotenoid pigmentation will discourage bumblebee visitation, based upon a field study with an interspecific F_2 population (Schemske & Bradshaw 1999). However, this same study predicted that increasing carotenoid pigmentation would have no effect on hummingbird visitation, yet when the *M. cardinalis yup* allele was introgressed into *M. lewisii* hummingbird visitation was increased 70-fold (Bradshaw & Schemske 2003)! It seems likely that the difference between the previous F_2 and lrNIL experiments is that there is vastly more segregating variation and linkage disequilibrium among the F_2s, producing unwanted trait correlations which are not relevant in nature, since hybrids between *M. lewisii* and *M. cardinalis* are essentially nonexistent. Clearly, a proper test of Hypothesis 1 requires the precision afforded by the hrNILs we propose to develop.

Hypothesis 2. Despite our working hypothesis that the effect of each flower color locus on pollinator preference is additive, exceptions to this are possible. In particular, the combination of carotenoid pigmentation (*yup*) and anthocyanin intensity (*roi*) should produce an orange flower that is much more attractive to hummingbirds, and much less attractive to bumblebees, than the simple sum of the individual allelic effects at each locus. If such a synergistic epistatic effect exists, it will be revealed as a significant interaction term (*yup*roi*) in the ANOVA.

Hypothesis 3. At the conclusion of our field experiments, we will be able to estimate the contribution of each of the 4 flower color loci, singly and in aggregate, to reproductive isolation mediated by pollinator choice, calibrated against the reproductive isolation between pure *M. lewisii* and *M. cardinalis*. We anticipate that most of the total reproductive isolation between these two species in sympatry will be accounted for by the alleles at the 4 flower color loci. We have already shown (Bradshaw & Schemske 2003) that introgression of the *M. cardinalis yup* allele increases the hummingbird contribution to visitation from 0.1% in *M. lewisii* to 35% in the *yup* NIL. It seems plausible that the other 3 flower color loci could push that number far higher, demonstrating that prezygotic reproductive isolation mediated by pollinator preference, a major driver of speciation among extant organisms, could evolve quickly from the fixation of just a few (mainly recessive, loss-of-function) mutations. Darwin’s “abominable mystery” would become much less mysterious, but remain every bit as interesting.

D.3.4. Potential pitfalls and alternative strategies.

Multi-gene QTLs. It is formally possible that one or more flower color QTLs contain more than a single gene affecting flower color. This seems unlikely based upon the fine-mapping we have done for *YUP* and *ROI*, however. In any event, fine-mapping will reveal which gene(s) within the locus contribute to the flower color difference between *M. lewisii* and *M. cardinalis*. If more than one gene within a locus is affecting flower color, we will make hrNILs containing only the single gene with the largest effect, to keep the field experiments logistically feasible.

Linkage drag. We expect that linkage drag will be eliminated during hrNIL construction, but it is possible that tight linkage or pleiotropy could affect other phenotypes relevant to pollinator preference or pollination efficiency (*e.g.*, petal reflexing, nectar volume, stamen length). We will measure a whole suite of pollination-related traits (Bradshaw *et al.* 1998) in each hrNIL.

Are introgressed alleles equivalent to mutational steps? The most desirable experiment to understand the genetic basis of reproductive isolation mediated by pollinator preference would be to identify the genes controlling flower color (and other) differences between *M. lewisii* and *M. cardinalis*, discover all of the mutations (*e.g.*, potential QTNs, gene duplications) that are fixed between the two species, and test each mutation for function (pollinator visitation). Since we don't yet know the identity of the flower color genes, nor the number (or nature) of mutations that distinguish the *M. lewisii* allele from the *M. cardinalis* allele, such experiments are not feasible at the moment. However, **it is worth noting that the derived *M. cardinalis* allele is completely recessive for 3 of the 4 flower color loci**, strongly suggesting that *any* knockout mutation in the *M. lewisii* allele is equivalent to the *M. cardinalis* allele, and that the null alleles in *M. cardinalis* are, for all practical purposes, equivalent to single mutations. We are testing this notion by carrying out a large induced-mutant screen in *M. lewisii* (not part of this proposal).

For the one locus – *ROI* – at which the *M. cardinalis* allele is partially dominant (and thus probably results from a gain of function mutation), we are only a BAC sequence away from identifying the gene(s) and their allelic differences. Within *ROI* the recombination rate is so high (20kb/cM) that our 8000 recombinants will map the causative mutation(s) with an expected precision of about ±125bp, and our hrNILs should have highly desirable intragenic recombinants that will help delimit the functional domain(s) of the *M. cardinalis* allele.

What do these experiments tell us about the role of flower color in the divergence of *M. lewisii* and *M. cardinalis* from their common ancestor? We do not (and cannot) know the circumstances under which *M. lewisii* and *M. cardinalis* diverged from their common ancestor (*e.g.*, in sympatry, parapatry, or allopatry; by reinforcement or genetic drift; the relative abundance of bumblebees and hummingbirds in the ancestral environment). Nor can we know the order of allelic substitutions during this divergence, though if "sign" epistasis is strong not all evolutionary trajectories are accessible by natural selection (Weinreich et al. 2006).

What we do know is that *M. lewisii* and *M. cardinalis* have diverged sufficiently to become distinct biological species by the most stringent criterion – they very rarely hybridize (<0.1%) in sympatry (Hiesey et al. 1973; Ramsey et al. 2003). Further, we know that this reproductive isolation in sympatry is mediated almost exclusively by pollinator preference in one of the very few systems where all components of reproductive isolation have been measured (Ramsey et al. 2003). Finally, we know that flower color is a major (perhaps *the* major) determinant of pollinator discrimination (Schemske & Bradshaw 1999; Bradshaw & Schemske 2003).

Timeline

Years 1-2. Finish mapping all 4 flower color loci at 1-5cM resolution. Create and screen new *M. lewisii* BAC library. Sequence and annotate BAC contigs containing target flower color genes. Finish mapping all 4 flower color loci at 0.012cM resolution. Develop "single" hrNILs.

Years 2-5. Characterize and field test "single" hrNILs. Create, characterize, and field test "double", "triple", and "quadruple" hrNILs.

Multiple PI Leadership Plan

Co-PIs Bradshaw and Schemske have worked together on the monkeyflower (*Mimulus*) system for 15 years, first as colleagues at the University of Washington and now as long-distance collaborators since Schemske moved to Michigan State University in 2001. This proposal is a continuation of that collaboration. We will meet annually, with all the personnel supported by this proposal, at our field sites in the central Sierra Nevada of California. Between field seasons communication among group members will be by email and telephone.

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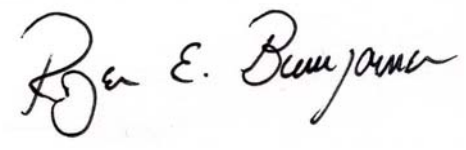
March 9th, 2009

Dear Toby,
I am writing in support of your proposal "Genetics of prezygotic reproductive isolation in natural populations of monkeyflowers (Mimulus)". In particular, as we have discussed, I have a 454 instrument in my lab and I now have considerable experience using this platform. In particular, we have sequenced more than dozen strains of the oral bacterium Actinobacillus actinomycetocomitans, Haemophilus aphrophilus, the golden delicious apple (presently at 3.0 X coverage), some cDNA sequences of baboon and 10's of other species in various collaborative projects.

I would welcome more use of the 454 platform by other groups in the Seattle area. I also have a specific interest in sequencing other plant genomes and would enjoy contributing my 454 expertise to the project you propose. As we discussed, we have recently upgraded our instrument and are currently running the "titanium" version of the platform. In this mode, a single run generates approximately 1.2M reads of 400bp average length for 500Mbp total/run. The reagent and supply costs/run are about $10k for the titanium runs. Each run takes approximately 1 FT week of technician effort, assuming there are no problems in library production, etc.

Assembly and gene annotation generally takes considerably more effort than the data production but we would also be willing to contribute to those efforts. We now have an excellent pipeline established for genomic assemblies and have built a good pipeline for gene finding/annotation in prokaryotes. We are currently building a pipeline for gene finding in eukaryotes as part of our apple genome work and would be happy to apply this to the monkeyflower project.

I look forward to working with you on this,



Roger E. Bumgarner
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Resource Sharing Plan

All inbred lines, NILs, seed stocks, markers, etc. we have developed are freely available to investigators who request them.

PHS 398 Checklist

OMB Number: 0925-0001
Expiration Date: 9/30/2007

1. Application Type:

From SF 424 (R&R) Cover Page. The responses provided on the R&R cover page are repeated here for your reference, as you answer the questions that are specific to the PHS398.

* Type of Application:

☐ New ☒ Resubmission ☐ Renewal ☐ Continuation ☐ Revision

Federal Identifier: R01GM088805

2. Change of Investigator / Change of Institution Questions

☐ Change of principal investigator / program director

Name of former principal investigator / program director:

Prefix:
* First Name:
Middle Name:
* Last Name:
Suffix:

☐ Change of Grantee Institution

* Name of former institution:

3. Inventions and Patents (For renewal applications only)

* Inventions and Patents: Yes ☐ No ☒

If the answer is "Yes" then please answer the following:

* Previously Reported: Yes ☐ No ☐

OMB Number. 0925-0001
Expiration Date: 9/30/2007

4. * Program Income

Is program income anticipated during the periods for which the grant support is requested?

☐ Yes ☒ No

If you checked "yes" above (indicating that program income is anticipated), then use the format below to reflect the amount and source(s). Otherwise, leave this section blank.

*Budget Period	*Anticipated Amount ($)	*Source(s)

5. Assurances/Certifications (see instructions)

In agreeing to the assurances/certification section 18 on the SF424 (R&R) form, the authorized organizational representative agrees to comply with the policies, assurances and/or certifications listed in the agency's application guide, when applicable. Descriptions of individual assurances/certifications are provided at: http://grants.nih.gov/grants/funding/424

If unable to certify compliance , where applicable, provide an explanation and attach below.

Explanation: Add Attachment | Delete Attachment | View Attachment