Arenaria paludicola (marsh sandwort: Caryophyllaceae) and *Rorippa gambelii* (Gambel's watercress: Brassicaceae) in Black Lake Canyon and the Oso Flaco Lake Region:

Field observations, greenhouse cultivation, molecular genetic variation, and trial reintroductions

Volume I (Field and lab observations)*

Joint Report for FG7590-R3 and FG8647-R3

Final Project Report to:

Deb Hillyard
California State Department of Fish and Game
P.O. Box 4003
Aromas, CA 95004

Principal Investigator: Dr. Susan J. Mazer
Department of Ecology, Evolution and Marine Biology
University of California
Santa Barbara, CA 93106
e-mail: mazer@lifesci.ucsb.edu
Telephone: 805-893-8011

With: Dr. Anuja Parikh
Dr. Nathan Gale
FLX
e-mail: ConsultFLx@aol.com
Telephone: 805-564-1352

*** See Volume II for accompanying photographs**

Table of Contents

I. Objectives 1

II. Field Studies 5

A. Summary of Activities 5

B. Field Observations of Natural Populations 9

1. *Arenaria paludicola* 9

Black Lake Canyon 1998
Local Extinction of *Arenaria paludicola*
Recolonization of *Arenaria paludicola* below Gurney property

Oso Flaco Lake 1998

2. *Rorippa gambelii* 11

Black Lake Canyon 1998
Oso Flaco Lake marsh 1998
Oso Flaco Lake causeway 1998
Vandenberg Air Force Base – Main Gate 1998 and 1999

C. Correspondence with Dr. Ihsan Al-Shehbaz 15

III. Preliminary Transplant Trials: *Arenaria paludicola* 19

A. Black Lake Canyon – Land Conservancy Tract 1998 19

B. Black Lake Canyon – Land Conservancy Tract 1999 24

C. Black Lake – Land Conservancy Easement site - 1998 28

D. Black Lake – Land Conservancy Easement site – 1999 32

IV. Greenhouse Cultivation..... 33

A. *Arenaria paludicola*..... 33

B. *Rorippa gambelii*..... 34

1. Seed Bank Study from Vandenberg Air Force Base (Main Gate) site..... 35
2. Results of Seed Bank Study from VAFB (Main Gate) site..... 37

V. Laboratory Observations..... 37

A. *Rorippa gambelii* and *R. nasturtium-aquaticum* fruit and seed morphology..... 37

B. Seed Mass Differences Between Vandenberg and Oso Flaco Lake Populations of *Rorippa gambelii*..... 38

C. Differences in Fruit Morphology Between Vandenberg Air Force Base, Oso Flaco Lake, and Little Oso Flaco Lake Populations of *Rorippa gambelii* and *R. nasturtium-aquaticum*..... 43

D. Differences in Seed Morphology Between Vandenberg Air Force Base, Oso Flaco Lake, and Little Oso Flaco Lake Populations of *Rorippa gambelii* and *R. nasturtium-aquaticum*..... 44

VI. Preliminary Survey of Genetic Variation in *Arenaria paludicola* and *Rorippa gambelii*..... 44

A. Introduction..... 44
B. Background..... 46

C. *Arenaria paludicola*..... 47
1. Materials and Methods..... 48
2. Results..... 50
3. Discussion..... 53

D. *Rorippa gambelii*..... 53
1. Background on *R. gambellii*, *R. nasturtium- aquaticum* and putative hybrids..... 53

2. Materials and Methods 54
3. Results 56
4. Discussion 60

VII. Considerations and Recommendations for Future Restoration Efforts 61

VIII. Literature Cited 62

IX. Maps 63

Map 1. Oso Flaco Lake: locations of *Arenaria paludicola* and *Rorippa gambelii*, indicating the "marsh" and the "causeway" populations 64

Map 2. Sketch by John Chesnut of *Arenaria paludicola* and *Rorippa gambelii* locations at Oso Flaco Lake: note the "marsh" and the "causeway" populations 65

Map 3. Location of Vandenberg Air Force Base (Main Gate) population of *Rorippa gambelii* 66

Map 4. Location of Black Lake Canyon Land Conservancy site of the introduction of *Arenaria paludicola* 67

X. Publications & Documents 68

Copy of Ihsad Al-Shehbaz' revision of *Nasturtium*, including *Rorippa gambelii* 69

Copy of letter from Dr. Anuja Parikh to Dr. Ihsan Al-Shebaz regarding *Rorippa* populations and the request for species confirmation for enclosed plant material and voucher specimens 72

Copy of Al-Shehbaz' e-mail message in response to our request for confirmation of our identification of *Rorippa gambelii* from Oso Flaco Lake and from Vandenberg Air Force Base (Main Gate) populations 73

Copy of Al-Shehbaz' e-mail message suggesting future directions of investigation of the Oso Flaco and VAFB populations of *Rorippa gambelii* 75

XI. Appendix: Protocols and Methods used for DNA fingerprinting 76

XII. Photographs (see Volume II)

1998 Photographs

Field Observations of Natural Populations
Arenaria paludicola
Oso Flaco Lake
Rorippa gambelii
Vandenberg Air Force Base (Main Gate)
Oso Flaco Lake

Preliminary Transplantation Trials
Black Lake Canyon Nature Conservancy site
Black Lake site

Greenhouse Cultivation
Arenaria paludicola
Rorippa gambelii

Microphotography of Fruits and Seeds of *Rorippa gambelii*
Lab Observations (Fruits, Seeds & Voucher Specimens)
Fruits (collected from field populations September 6, 1998)
Seeds (collected from field populations September 6, 1998)

1999 Photographs

Arenaria paludicola: Black Lake Canyon Restoration Site
Arenaria paludicola: Oso Flaco Lake marsh
A. paludicola flower with developing ovary, cultivated at UCSB
Arenaria paludicola ovary, with multiple aborting ovules, cultivated at UCSB
Rorippa gambelii (and putative hybrid) at field sites
Rorippa nasturtium-aquaticum: ditch near Little Oso Flaco Lake

I. Objectives

This study was initiated in April 1998, with section 6 funding (FG7590-R3; $11,200) covering the period from April 1998 – March 1999. A second contract (FG8647-R3; $20,000) was awarded for the period of May 1999 – December 1999. This report summarizes the objectives and accomplishments of both contracts.

The primary objectives were to implement several recommendations concerning the recovery of two endangered plant species now restricted to one or two wetland populations in California — Gambel's watercress (*Rorippa gambelii*: Brassicaceae) and Marsh sandwort (*Arenaria paludicola*: Caryophyllaceae) — and to learn more about the distribution of these taxa. These recommendations had been proposed in three reports, including a previous study that had been funded by the California State Department of Fish and Game and conducted by Dr. Susan Mazer, a draft recovery plan written by Drs. Nathan Gale and Anuja Parikh, and the final Draft Recovery Plan published in May 1997 by the U.S. Fish and Wildlife Service.

In accordance with these previous reports, the field, greenhouse and lab work conducted from April 1998 – December 1999 had the following goals:

1. To increase vegetative and seed reserves of both species through a combination of vegetative propagation, germination of seed from the soil seed bank, and a conservative degree of seed collection from field populations. This objective has been achieved. We have built up greenhouse populations of *Rorippa gambelii* and *Arenaria paludicola* from seed stock of *Rorippa gambelii* and from vegetative material of *Arenaria paludicola*.

2. To collect seed of *Rorippa gambelii* from extant populations. These populations now include: (a) a population at Vandenberg Air Force Base (VAFB) and (b) a population adjacent to the causeway at Oso Flaco Lake State Park. This second population is referred to here as the Oso Flaco Lake "causeway" population. Several hundred individuals are located in the understory adjacent to the causeway leading from the parking lot of Oso Flaco State Park to Oso Flaco Lake. This population was found by John Chesnut in 1998 as part of his work for the California Department of Parks and Recreation. We suggest in this report that this population represents a hybrid between *R. gambelii* and *R. nasturtium-aquaticum*). This objective has been achieved.

3. To explore the Dune Lakes region — if accessible — to locate additional populations *of Arenaria paludicola*. Also due to the work of John Chesnut, an additional population

of A. paludicola has been located along the margins of a marsh just north of Oso Flaco Lake. We refer to this location as the Oso Flaco Lake "marsh" site.

4. To visit the known site of an extant population of *Rorippa gambelii* at Vandenberg Air Force Base to determine whether seeds can be collected here and whether this is a potential site for future introductions of *Rorippa gambelii*. This objective has been achieved.

5. To transplant greenhouse-raised vegetative material of *Arenaria paludicola* to natural sites in Black Lake Canyon to determine whether such vegetatively reproduced transplants can become re-established in the field. This objective has been achieved. These transplants were monitored during the growing seasons of 1998 and 1999.

6. To transplant a small number of greenhouse-raised seedlings of *Rorippa gambelii* to Black Lake Canyon. The complete apparent extinction of the large population at Black Lake Canyon combined with the fragility of the greenhouse-raised seedlings served to caution us against this activity. Several dozen *R. gambelii* seedlings from both: (a) the Vandenberg Air Force Base population and (b) the Oso Flaco Lake causeway population are now growing in the UCSB greenhouse.

7. To monitor and — if available — to collect seeds from the population of *Rorippa gambelii* previously observed (in 1994, by Drs. Parikh and Nathan Gale of FLX) on the border of a drainage ditch adjacent to Little Oso Flaco Lake. We learned that this location is cited on private agricultural land owned by Alan Texiera. In addition, this population has been extirpated. The drainage ditch along which this population grew has been dredged and highly disturbed.

8. To germinate seedlings from field collections of *Rorippa gambelii* seeds in the UCSB greenhouse, and to transplant a few seedlings back to their home sites for subsequent monitoring. Seeds have been sampled from *Rorippa gambelii* growing on Vandenberg Air Force Base and from the Oso Flaco Lake causeway population. We present morphological and genetic evidence (below) that this population represents a hybrid *between R. gambelii* and *R. nasturtium-aquaticum*. The lack of a formal agreement between the Department of Fish and Game or the U.S. Fish and Wildlife Service and Vandenberg Air Force Base, and the absence of any other extant populations unambiguously identified as *R. gambelii*, prevented any transplantation of seedlings back into the field.

9. To conduct preliminary investigations concerning molecular genetic variation within and between populations of *A. paludicola* and *R. gambelii*. To date, there have been no published accounts of molecular genetic variation in either *Arenaria* or *Rorippa*. Adapting known molecular methods (e.g., the use of the polymerase chain reaction to create multiple copies of a DNA fragment) to a new genus or species can be a time-consuming process. The polymerase chain reaction is highly sensitive to the temperatures at which it is conducted and the exact concentrations of the reagents that they use (e.g., taq polymerase, dNTPs, primers, and Magnesium). Consequently, much preparatory work must be done to identify the conditions that will work for a given taxon. As described below, we have succeeded in demonstrating the use of molecular genetic fingerprinting using Inter-simple Sequence Repeat primers in *A. paludicola* to detect genetic variation within and between Black Lake Canyon and Oso Flaco Lake genotypes. In addition, we have used the polymerase chain reaction and known primers to amplify (i.e., to create multiple copies of) DNA sequences of the ITS (internal transcribed spacer) region of *R. gambelii* from both Vandenberg Air Force Base and the Oso Flaco Lake causeway population. Comparisons of DNA sequences from these taxa and from known sequencies of *R. nasturtium-aquaticum* support the hypothesis that the Oso Flaco Lake causeway population is of hybrid origin, representing a hybrid between *R. gambelii* and *R. nasturtium-aquaticum*.

Most of these objectives were fulfilled and are described in detail below. As briefly mentioned above, a few of these goals were not possible to fulfill due to biological or administrative constraints, as follows:

(1) We were not permitted by Duck Hunting, Ltd. to explore the Dune Lakes other than Black Lake. As mentioned above, however, with the help of Dr. John Chesnut, we are now aware of the locations of these species at Oso Flaco Lake (the "**marsh**" and the "**causeway**" populations). A small population of *Arenaria paludicola* and a few individuals of *Rorippa gambelii* are growing in a freshwater marsh on the northern boundary of Oso Flaco Lake (described below). This location is referred to as the Oso Flaco Lake "**marsh**" population. A population identifiable as *Rorippa gambelii* (but which probably represents a hybrid with *R. nasturtium-aquaticum*) appears adjacent to the causeway at Oso Flaco Lake. This location is referred to as the Oso Flaco Lake "**causeway**" population. Seedlings of *Rorippa gambelii* derived from seeds collected from the causeway population, and vegetatively propagated ramets of *Arenaria paludicola* from the marsh population are now being cultivated at UCSB.

(2) We did not sow seeds or transplant seedlings of *Rorippa gambelii* into Black Lake Canyon because we discovered that the population that had been extant in Black Lake

Canyon in 1994 has completely disappeared. We believe that some kind of hydrological or environmental change in the canyon has made it no longer suitable for *Rorippa gambelii*, and we did not want to waste any seedlings until we have accumulated a larger greenhouse population.

(3) We did not monitor or collect seeds from the 1994 population of *Rorippa gambelii* observed in the drainage ditch adjacent to Little Oso Flaco Lake because this population has also become extinct. Also, we learned through an encounter with Glenn Texiera on August 21, 1999 that this location, which we had previously thought to be part of Oso Flaco State Park, is in fact on Texiera private property. In addition, the Texiera brothers are very hostile towards any botanical observations being made on their property. Further collection from this site would depend upon negotiations between the California Department of Fish and Game, or the U.S. Fish and Wildlife Service, and the Texiera family.

(4) We have not yet transplanted any seedlings of *Rorippa gambelii* back into a field site. We have successfully germinated seeds in the greenhouse of *Rorippa gambellii* from seeds collected from Black Lake Canyon in 1994, from seeds collected from Vandenberg Air Force Base in 1998, and from seeds collected from the Oso Flaco Lake causeway and the Vandenberg Air Force Base populations in 1999. These seedlings are currently growing at UCSB and can be used for future transplantation or restoration efforts.

Seeds sown in August 1998 representing the Black Lake Canyon population did not germinate until September, 1998, which was after the point when we felt it was prudent to transplant them into the field. Also, we did not want to plant Black Lake Canyon genotypes into the site of any other population because of the possibility of producing a hybridization event. In addition, the Black Lake Canyon site appears to be unsuitable for *R. gambelii* as the large population (more than 400 ramets taller than 1.5 m) present in Black Lake Canyon in 1994 is now extinct.

A summary of our activities in 1998 and 1999 is provided in the tables below, followed by detailed descriptions and interpretations.

II. Field Studies

A. Summary of Activities

Table 1. Summary of field activities during 1998 growing season.

Date	Investigators[1]	Site Visited[2]	Activities[3]
4/5/98	AP, DG, NG, SM	BLCO	Observed David Gurney's *Arenaria paludicola* transplants and new *Arenaria paludicola* ramets that have appeared to spread from David's transplants under the pipeline crossing in Black Lake Canyon.
	AP, NG, SM		Looked for original population of *Rorippa gambelii* at Black Lake Canyon, which we could not locate.
		BL LOFL	Examined potential introduction sites at Black Lake and at Little Oso Flaco Lake
4/15/98	AP, DG, NG	BLCLC	Transplanted 3 *Arenaria paludicola* plants from David Gurney's property to the Black Lake Canyon Land Conservancy Tract as a preliminary test of transplant technique
		BLCO	Looked for original *Arenaria paludicola/Rorippa gambelii* populations, which still could not be re-located. Both species appear to be extinct at site in Black Lake Canyon where they occurred in 1994.
5/1/98	AP, NG, SM	VAFB	Meeting (USFWS, CDFG, VAFB)
5/15/98	AP, DK, NG	VAFB	Examined existing population of *Rorippa gambelii* Examined potential introduction sites at Vandenberg
5/16/98	AP, NG, SM	BLCLC BL	Transplanted 12 *Arenaria paludicola* plants from SBBG Transplanted 9 *Arenaria paludicola* plants from SBBG (methods described below)
		OFL	Looked for *Rorippa gambelii* population reported by DW, but this population was not found
6/13/98	AP, MM, NG, SM	BL BLCLC	Monitored 9 *Arenaria paludicola* plants Monitored 15 *Arenaria paludicola* plants (including the three plants initially placed at this site on 4/15/98).

Date	Investigators[1]	Site Visited[2]	Activities[3]
8/8/98	AP, NG, SM	BLCLC BL	Monitored 15 *Arenaria paludicola* plants Monitored.9 *Arenaria paludicola* plants
	AP, JC, NG, SM	OFL	Examined two reported populations of *Rorippa gambelii* and one of *Arenaria paludicola* Collected small samples of *Rorippa gambelii* from causeway population at OFL to confirm identification because AP thought the plants appeared similar to the common *R. nasturtium-aquaticum.* This was our first suggestion that the OFL understory populaiton of *R. gambelii* might be of hybrid origin.
8/14/98	AP, NG	SBBG	DW confirmed that the fruits of *Rorippa gambelii* from both OFL populations had seeds arranged in two rows per chamber rather than one, therefore these populations have seed arrangement similar to *R. nasturtium-aquaticum.* DW recommended sending samples to Dr. Ihsan Al-Shehbaz at the Missouri Botanical Garden (see below for a summary of our correspondence with Dr. Al-Shehbaz)
9/6/98	AP, NG, SM	VAFB BL BLCLC OFL LOFL	Collected seed, soil, and plant vouchers from population of *Rorippa gambelii* Monitored 9 *Arenaria paludicola* plants Monitored 15 *Arenaria paludicola* plants Collected seed and vouchers from causeway *Rorippa* population Looked for original population of *Rorippa gambelii*, which could not be found; Other arm of lake had *R. nasturtium-aquaticum*, which we collected for comparison and photographic documentation.

1	2	3
DG = David Gurney DK = David Keil JC = John Chesnut MM = Malcolm McEwen NG = Nathan Gale SM = Susan Mazer	BL = Black Lake BLCLC = Black Lake Canyon, Land Conservancy Parcel, Guadalupe Road Transplanting Site BLCO = Black Lake Canyon, Sites of Original (1994-1995) Populations LOFL = Little Oso Flaco Lake OFL = Oso Flaco Lake SBBG = Santa Barbara Botanic Garden VAFB = Vandenberg AFB	CDFG = California Department of Fish and Game DW = Dieter Wilken USFWS = U.S. Fish and Wildlife Service

Table 2. Summary of field activities during 1999 growing season.

Date	Investigators[1]	Site Visited[2]	Activities[3]
5/21/99	NG, AP	BLCO	Visited *Arenaria paludicola* site planted by Gurney near pipeline crossing. *Arenaria* present, although more *Typha* present in planting area; no *Roippa gambelii* present in original site.
		BL	Monitored 9 *Arenaria paludicola* plants; all plants disappeared or drowned; took photos. The water level in the lake appears to be higher than in 1998 by about 10-12 inches, the *Scirpus* and *Typha* have expanded shorewards, *Oenanthe* has dense cover at the shore, and live clumps of *Juncus effusus* are present at the lake edge.
		BLCLC	Monitored 15 *Ap* plants; took photos. Vegetation generally appears overgrown in the marsh and has expanded towards the edges, water levels have receded about 4 inches relative to end of 1998 season, and the planting areas appear to be more shaded and overgrown. All *Arenaria paludicola* and nurse *Juncus* plants were browsed.
6/5/99	SM, AP	UCSB	Propagated more ramets of *Arenaria paludicola* from Gurney/Chipping plants.
6/24/99	NG, AP	OFL	Visited *Arenaria paludicola* "marsh" population on the north shore and collected about 10-15 cuttings; many plants were flowering; also collected possible hybrid *Rorippa gambelii* voucher specimen from the same marsh; collected soil samples from this marsh from under *Arenaria paludicola* and from under *Rorippa gambelii*; took photos.
		UCSB	Propagated the collected OFL marsh *Arenaria paludicola* cuttings in trays; left soil samples at Mazer lab.
6/26/99	NG, AP	UCSB	Checked greenhouse *Arenaria paludicola* cuttings in trays; left soil samples at Mazer lab.and *Rorippa gambelii* plants and watered them. Identified and counted all plant seedlings growing in trays with soil from VAFB *Rorippa gambelii* marsh.
7/1/99	NG, AP		Sent message to Al-Shehbaz updating him with this year's site visit results and plans, and request to ID OFL *Rorippa gambelii* hybrid voucher from the north shore marsh. Awaiting reply.

Date	Investigators[1]	Site Visited[2]	Activities[3]
7/2/99	AP	UCSB	Checked greenhouse *Arenaria paludicola* and *Rorippa gambelii* plants and watered them.
7/16/99	AP	UCSB	Checked greenhouse *Arenaria paludicola* plants and watered them.
8/21/99	NG, AP	BLCLC	Monitored 15 *Ap* plants; took photos. Marsh and willow vegetation has expanded and is overgrown, and the planting areas appear to be more shaded and covered with vegetation.
		OFL	Collected seed and fruit, and photographed *Rorippa gambelii* x *nasturtium-aquaticum* hybrids from the "causeway" population.
		LOFL	Checked ditch near LOFL but property owner Glenn Texeira asked us to leave after informing us that we were on Texeira Farms property; asked us to have Mazer contact his brother Alan at 928-3801 for future access; showed us State Parks boundary; we collected seed and fruit from *Rorippa nasturtium-aquaticum* in a ditch on State Parks land.
		VAFB	Collected seed and fruit, soil samples, and about 8 rhizome cuttings from *Rorippa gambelii* population; took photos.
		UCSB	Left all materials in Mazer lab.
8/24/99	NG, AP	LOFL	Tried to contacted Alan at Texeira Farms to gain access to ditch area where *Rorippa gambelii* was observed in 1994; access refused.
9/27/99	AP		McEwen report on BLC reviewed and comments returned.
11/11/99	NG, AP	BLCLC	Monitored 15 *Arenaria paludicola* plants; took photos. Marsh vegetation continues to be overgrown, and the *Scirpus* appears much taller.
11/15/99	NG	VAFB	Collected fruit of *Rorippa gambelii* for comparison with *Rorippa* hybrid from LOFL; delivered to Mazer 11/20.

1 NG = Nathan Gale
SM = Susan Mazer
AP = Anuja Parikh

2 BL = Black Lake
BLCLC = Black Lake Canyon, Land Conservancy Parcel Guadalupe Road Transplanting Site
BLCO = Black Lake Canyon, Sites of Original (1994-1995) Populations
LOFL = Little Oso Flaco Lake
OFL = Oso Flaco Lake
VAFB = Vandenberg AFB

B. Field Observations of Natural Populations

1. *Arenaria paludicola*

Black Lake Canyon 1998

Local Extinction of *Arenaria paludicola*

Observations on April 5, 1998: Susan Mazer, Anuja Parikh, Nathan Gale and David Gurney surveyed Black Lake Canyon at the location where a single clonal individual of *Arenaria paludicola* had last been observed by Susan Mazer, Anuja Parikh and Nathan Gale in 1994. There is no evidence that this individual continues to survive in the wild (although many ramets representing this clone are being cultivated vegetatively at the University of California, Santa Barbara greenhouses).

In general, the portion of the canyon that used to support *Arenaria paludicola* appears to be more heavily vegetated with *Rubus ursinus* (Rosaceae), *Myrica californica* (Myricaceae) and *Salix lasiolepis* (Salicaceae) than it was in 1994. In addition, the slopes of Black Lake Canyon above and to the south of the previous location of *A. paludicola* are now characterized by a deeper layer of *Eucalyptus* leaf litter and a thicker fern understory than they were in 1994.

Recolonization of *Arenaria paludicola* below Gurney property

Observations on April 5, 1998: Susan Mazer, Anuja Parikh, Nathan Gale and David Gurney observed that in a small backed-up pond in Black Lake Canyon, just north of David Gurney's property, several ramets of *Arenaria paludicola* were growing vegetatively. This location can be identified as being adjacent to where the canyon has been cleared to make way for a pipeline crossing. Two clumps of shoots were emerging from the water, one comprised of stems about 0.25 meter in height; the other comprised of stems up to 1.5 meters in height, which were twining among the vegetation in the banks of the pond. These ramets illustrate a growth pattern that we have observed repeatedly in *Arenaria paludicola*: stems do not seem to be able to reach more than 0.25 meters in height unless they are able to climb and twine among stems of neighboring plants.

It was agreed among those present that these ramets were probably the result of the asexual production of a ramet that David Gurney had propagated in 1994 from a small stem of the clonal individual of *A. paludicola* that had previously occupied a site upstream from his property. In 1995, upon consulting with Susan Mazer (who was currently on

contract with the California State Department of Fish and Game to monitor the Black Lake Canyon population of *A. paludicola*), David Gurney transplanted parts of this ramet back onto his property to see if *A. paludicola* could be reestablished in this way. Through this single action, it is likely that David Gurney prevented the extinction of *A. paludicola* in this canyon.

Oso Flaco Lake marsh population 1998

Observations on August 8, 1998: Susan Mazer, Anuja Parikh, and Nathan Gale joined John Chesnut on a survey of the population of *A. paludicola* that he located at the northernmost marshy edge of Oso Flaco Lake (see **Maps 1 and 2**, provided by John Chesnut; see **Photographs 1 - 6**). This site and vegetation is as described in John Chesnut's June 4, 1998 report to Connie Rutherford (U.S. Fish and Wildlife Service). The approach to this site is quite difficult on foot, as there is a dense thicket of poison oak extending 25 - 50 meters in length between the dunes and the marsh margin.

The part of the northern fringe of Oso Flaco Lake that appears to be most suitable for *A. paludicola* is a marsh comprised of *Sparganium eurycarpum* (bur-reed; Typhaceae), *Typha* cf. *latifolia* (cattail; Typhaceae) and *Carex cusickii* (sedge; Cyperaceae). Individuals of *Scirpus microcarpus* (tule; Cyperaceae), *Mimulus guttatus* (monkey flower; Scrophulariaceae), *Berula erecta* (cutleaf water-parsnip; Apiaceae) and *Epilobium ciliatum* (fireweed; Onagraceae) share this part of the marsh with *A. paludicola*.

The small population of ~20 *A. paludicola* ramets found here is nestled among tussocks of *Carex cusickii*. It appears that each ramet of *A. paludicola* requires a *Carex cusickii* to support its root and shoot growth. We did not penetrate the marsh very deeply, as it is extremely difficult to walk on the wet mud and peat that supports *Carex cusickii* without causing tremendous physical disturbance to the vegetation. Also, the *Carex cusickii* tussocks are often separated at one-meter intervals by a depth of up to 0.5 m of water and mud, making progress on foot extremely slow. John Chesnut's success in finding this population is extremely impressive. Given that *A. paludicola's* success amid *Carex cusickii* is evident here, it is hoped that similar plant communities elsewhere will provide viable sites for the future reintroduction of *Arenaria* ramets.

It is possible that the population size of *A. paludicola* exceeds the 20 ramets that we observed here, but it will not possible to verify this without risking significant physical damage to the peat and tussocks that support it. A few shoot segments of *A. paludicola* were collected by Susan Mazer from this population for vegetative propagation in the

UCSB greenhouse. They are currently growing successfully (see section on **Greenhouse Cultivation**, below).

From a distance, the vegetation that supports *A. paludicola* is visible because the yellow-green appearance of *Sparganium eurycarpum* stands out from the background marsh and dune vegetation. Because the extent of this vegetation greatly exceeds what we were able to explore on foot, we are optimistic that the *A. paludicola* population is more extensive than that which we surveyed.

2. *Rorippa gambelii*

Black Lake Canyon 1998

Local Extinction of *Rorippa gambelii*

Observations on April 15, 1998: Anuja Parikh, Nathan Gale and David Gurney surveyed Black Lake Canyon at the location where a large population (N ~400 ramets) of *R. gambelii* had last been observed in 1995. No evidence of a current population of *R. gambelii* was found. The absence of *R. gambelii* was particularly surprising, as vegetative growth of this perennial species would normally have been evident at this time of year.

Oso Flaco Lake marsh 1998 and 1999

Observations on August 8, 1998: John Chesnut had reported (June 4, 1998) that a few individuals of *Rorippa gambelii* were also located among the *A. paludicola* ramets. On August 8, we observed a couple of these plants, which appeared to Susan Mazer, Anuja Parikh and Nathan Gale to be more robust (i.e., with thicker, darker green, and stiffer stems) than the *Rorippa gambelii* individuals that they had previously observed elsewhere (including Oso Flaco Lake causeway, Vandenberg Air Force Base, Little Oso Flaco Lake, and Black Lake Canyon). A positive determination of these *Rorippa* plants remains to be conducted (see **Photo 172**).

Observations on June 24, 1999: This population was revisited by Anuja Parikh and Nathan Gale, and several individuals of Rorippa gambelii were seen (**Photograph 172**).

Oso Flaco Lake causeway 1998 and 1999

Observations on August 8, 1998: Drs. Susan Mazer, Anuja Parikh and Nathan Gale accompanied John Chesnut to the population of a few hundred *Rorippa gambelii* ramets

that he had located in June 1998 growing in the understory of a willow grove adjacent to the causeway near the northeast shore of Oso Flaco Lake (see **Maps 1 and 2; Photographs 21 – 34**). Many of the plants in this population were flowering in June 1998 (evident in photographs taken by John Chesnut), and the population was still in flower in August 1998.

This population would be identified as *Rorippa gambelii* based on several characters that are diagnostic of the species. These include:

- There are 7 – 13 leaflets per leaf
- The flowers are larger than that of *R.nasturtium-aquaticum*, its closest living relative
- The seeds are generally aligned in a single row (uniseriate) in each locule
- The style is elongated and up to 2 mm long
- The seed coats are dark brown and shiny
- The surface of the seed coat ranges from being finely papillate to reticulate, with the reticulations smaller than those of *R. nasturtium-aquaticum*.

In spite of these diagnostic characters, Anuja Parikh noted several differences between the ramets in this population and those we had observed previously in Black Lake Canyon and at the Vandenberg Air Force Base (Main Gate) population (described below) (see **Table 3**). The leaflets and flowers in the Oso Flaco population are relatively large and the fruits were not always clearly uniseriate (the seeds are sometimes so tightly packed that they overlap, approaching a biseriate condition).

Observations on September 6, 1998 (See **Table 3**): Because we felt that the positive identification of this population might be in doubt, we collected samples of flowering material, fruits and seeds for comparison with those sampled from the Vandenberg Air Force Base (Main Gate) population. In addition, for purposes of comparison with the Oso Flaco Lake causeway and Vandenberg Air Force Base populations of *Rorippa gambelii*, we collected two specimens of the common watercress, *Rorippa nasturtium-aquaticum*, from an agricultural drainage ditch extending from Little Oso Flaco Lake. Photographs of these samples were taken in the Mazer lab (**Table 3**). Susan Mazer observed that seeds produced by the Oso Flaco Lake causeway population are typically larger than the seeds of the Vandenberg Air Force Base (Main Gate) population of *R. gambelii* and larger than the small sample *of R. nasturtium-aquaticum* seeds collected from the drainage ditch near Oso Flaco Lake.

As described in detail (below), samples of each of the three taxa were collected and loaned to Dr. Ihsan A. Al-Shehbaz, a curator at the Missouri Botanical Garden who has recently

published a revision of the genus *Nasturtium* (Al-Shehbaz and Price, 1998). According to Al-Shehbaz' view, *Nasturtium* is most accurately described as including the species *Rorippa gambelii*. (as *Nasturtium gambelii*) and *Rorippa nasturtium-aquaticum* (as *Nasturtium officinale*). We don not adopt this usage in this report, but maintain the practice of referring to these taxa as members of the genus *Rorippa*. Dr. Al-Shehbaz confirmed that our samples of *Rorippa gambelii* from Vandenberg Air Force Base are identical to the type specimens of *R. gambelii* (or *Nasturtium gambelii*). He also agreed that our samples from the Oso Flaco Lake causeway appear to show evidence of introgression or hybridization between *R. gambelii* and *R. nasturtium aquaticum*. (see **Correspondence with Dr. Ihsan Al-Shehbaz**, below).

Observations on August 21, 1999: This population was flowering and healthy (**Photograph 173**). At this time, photographs of *Roripppa nasturtium-aquaticum* in the drainage ditch near Little Oso Flaco Lake were also taken (**Photographs 174 and 175**).

Table 3. Key to photographs of three taxa of *Rorippa*.

Subject	*Rorippa gambelii* from Vandenberg Air Force Base Main Gate population	*Rorippa gambelii* from Oso Flaco Lake population	*Rorippa nasturtium-aquaticum* from agricultural drainage ditch on the border of Little Oso Flaco Lake
Voucher specimens	Photographs 89 -92	Photographs 93 -95	Photographs 96 – 101
Fruits	Photographs 102 - 107	Photographs 108 - 113	Photographs 114 - 119
Seeds	Photographs 120 - 127	Photographs 128 - 136	Photographs 137 - 143

Correction: Please note that the magnifications indicated in the photographs of the seeds (Photographs 120 – 143) are incorrect by a factor of 2. Where the photograph caption indicates "10X", the actual magnification is 20X. Where the photograph caption reads "20X", the actual magnification is 40X. Where the photograph caption reads 40X, the actual magnification is 80X.

Vandenberg Air Force Base – Main Gate 1998 and 1999

Observations on May 15, 1998 (by Drs. Anuja Parikh and Nathan Gale) and on September 6, 1998 (by Drs. Susan Mazer, Anuja Parikh and Nathan Gale):

On May 15, 1998, Anuja Parikh and Nathan Gale visited with Dr. David Keil the population of *Rorippa gambelii* that he had located near the Main Gate of Vandenberg Air

Force Base (VAFB) (see **Map 3; Photographs 7 and 8**). At this time, only a few individuals were flowering (**Photographs 9 – 11**).

As described in Dr. Keil's "Evaluation of Potential Introduction Sites for *Rorippa gambelii* (Gambel's Water Cress) on Vandenberg Air Force Base (a report prepared for the U. S. Fish and Wildlife Service), this site may be located by following Highway 1 (Vandenberg Road) 0.5 miles northeast of the VAFB Main Gate. At a sign that states, "Lompoc Left Lane", there is a marsh to the northwest of and below the elevation of the highway. The wetland is comprised of a marsh that is surrounded by a fringe of swamp-woodland dominated by willow. In this woodland are found *Salix lasiolepis* (Salicaceae), *Myrica california* (Myricaceae), *Lonicera involucrata* (Caprifoliaceae) and *Cornus sericea* (Cornaceae). Ringing the willow woodland is a thicket of poison oak that makes entry into the marsh very difficult on foot. Beyond the poison oak thicket is the coastal live oak woodland, Burton Mesa chaparral, and coastal dune scrub that characterize the region.

The marsh itself is comprised of a floating mat on a pond that reaches at least one meter in depth. In the marsh are small sites dominated by *Typha latifolia* (Typhaceae), *Scirpus microcarpus* and *S. acutus* (Cyperaceae), *Sparganium eurycarpum* (Sparganiaceae), and *Oenanthe sarmentosa* (Apiaceae). Scattered amidst the marsh are individuals of *Solidago confinus*, *Helenium puberulum* and *Cirsium brevistylum* (Asteraceae), *Epilobium ciliatum* (Onagraceae), *Hypericum anagalloides* (Hypericaceae), Urtica dioica (Urticaceae), and *Rumex occidentalis* (Polygonaceae). The water in the marsh is provided by a spring that occurs at the head of the drainage above the marsh. The drainage crosses Highway 1 and the spring is located about 0.4 miles northeast of the highway.

At the western edge of the marsh is a local, clonal population of *Rorippa gambelii*. Here, fragile, hollow-stemmed ramets grow up to 2m tall, but require adjacent vegetation in the willow-dominated edge of the marsh for support. Because of its clonal reproduction from subterranean rhizomes, it is not clear how many genetically distinct individuals are present in this population. In mid-May only one or two ramets in this population had begun to flower.

Susan Mazer, Anuja Parikh and Nathan Gale agree with David Keil's assessment that this marsh represents a potentially favorable site for the future introduction of additional *Rorippa gambelii* ramets. It is also possible that edges of the marsh just upstream and downstream of the current location of*R. gambelii's* may support a more extensive population of this species.

On September 6, 1998 Susan Mazer, Anuja Parikh and Nathan Gale returned to this site. At this time, approximately six plants were observed to be flowering and fruiting (**Photographs 12 – 20**). A total of three flowering branches were collected from three ramets as voucher specimens of flowering and fruiting *R. gambelii*. One of these voucher specimens was photographed (see **Photographs 89 – 92**). In addition, we collected two or three fruits from a total of 10 individuals. These fruits and the seeds they contained were photographed (**Photographs 102 – 107 and 120 - 127**) for comparison to the seeds of the Oso Flaco Lake causeway population of *R. gambelii*. The voucher specimens and several samples of the seeds and fruits were sent to Dr. Ihsan Al-Shehbaz (Missouri Botanical Garden) for positive determination.

On September 6, three 5-gallon buckets of peat were removed from the vicinity *of R. gambelii* population to cultivate for the purpose of observing the seed bank (see **Greenhouse Cultivation**, below).

Observations on August 21, 1999: Plants were observed to be healthy and flowering on August 21 (**Photographs 170 and 171**).

C. Correspondence with Dr. Ihsan Al-Shehbaz

In September 1998, Anuja Parikh initiated correspondence with Dr. Ihsan Al-Shebaz and sent him voucher specimens and plant material for positive determination (see October 2 letter from Anuja Parikh to Ihsan Al-Shehbaz, below).

Dr. Ihsan Al-Shehbaz is a curator at the Missouri Botanical Garden who has recently published a revision of those species of *Rorippa* that are closely related to *Nasturtium*.. A copy of this paper is included below (see section on **Documents and Publications**). In addition are included copies of his October 6 and 7, 1998 e-mail messages to Susan Mazer and to Anuja Parikh. The most important components of Al-Shehbaz' observations are as follows:

1. Al-Shehbaz and Price (1998) now classify as *Nasturtium officinale* the taxon that is referred to in this report as *Rorippa nasturtium-aquaticum*.
2. Al-Shehbaz and Price (1998) classify as *Nasturtium gambelii* the taxon that is referred to in this report as *Rorippa gambelii*.
3. Al-Shehbaz (e-mail message) confirms that our samples from Vandenberg Air Force Base conform to the type description of *Rorippa gambelii*. He has no doubt that these plants belong to *Rorippa gambelii*.

4. Al-Shehbaz (e-mail message) agrees with our view that the specimens from the Oso Flaco Lake causeway population appear to be hybrids between *Rorippa gambelii* and *Rorippa nasturtium-aquaticum*.

Table 4 summarizes our observations and classification of the three *Rorippa* taxa that we have sampled.

Notes on Table 4:

- Observations of Dr. Susan Mazer, Dr. Anuja Parikh, and Dr. Nathan Gale
- Observations of John W. Chesnut, based on collection JWC #2370, 2371, 2372
- Observations reported by Rollins, R., in Hickman, 1993
- Observations reported by Munz, 1974
- Observations reported by Al-Shehbaz and Price, 1998

Table 4. Comparative table of morphological differences between populations of *R. gambelii* and between *R. gambelii* and *R. nasturtium-aquaticum*. This table is a compilation of observations made by the current investigators and Dr. John Chesnut, with supporting data from published accounts (Hickman, 1993; Munz, 1974; Al-Shehbaz and Price 1998).

Trait	*Rorippa gambelii*: Vandenberg Air Force Base population:	*Rorippa gambelii*: Oso Flaco Lake population: shows some evidence of introgression or hybridization between *R. gambelii* and *R. nasturtium-aquaticum*	*Rorippa nasturtium-aquaticum*: Little Oso Flaco Lake drainage ditch population
Plant habit and stature	Erect from a rooting, decumbent base, 5 – 20 dm; Stem is hollow and brittle[1]	Trailing to erect (up to 20 dm); plants root at base; Stem is hollow and brittle[1,2]	Prostrate to ascending stem 1 – 6 dm, rooting freely on stem; Stem is pliable[1]
Flower size	Large; petals 6 – 8 mm long[1,3]	Relative large; petals 8 mm long[1,2]	Small; petals 3-4 mm long[3]
Individual seed mass			
Range among plant means	0.051 – 0.176 mg	0.181 – 0.316 mg	No data available
Mean seed mass among all sampled plants	0.107 mg	0.265 mg	
Seed arrangement within fruits	Uniseriate: One row of seeds in each locule; Seeds 20 – 36 per fruit[3,4]	Uniseriate: One row of seeds in each locule, but the seeds are so tightly packed that they occasionally overlap, appearing to exhibit two rows.	Biseriate: Two rows of seeds in each locule; seeds are tightly packed and overlap
Seed Coat Color	Dark brown with shiny surface	Dark brown with shiny surface	Lighter brown with dull surface

Trait	*Rorippa gambelii*: Vandenberg Air Force Base population:	*Rorippa gambelii*: Oso Flaco Lake population	*Rorippa nasturtium-aquaticum*: Little Oso Flaco Lake drainage ditch
Seed coat morphology	Seed coat surface is very finely reticulate[1]; Reticulations on seed coat are barely visible at 80X magnification (see Photographs, below)* *these are the true magnifications, not the ones listed in the figure captions, which are off by a factor of 2.	Seed coat surface ranges from being finely papillate[2] to being more coarsely reticulate than the Vandenberg population[1]; Reticulations on seed coat of Oso Flaco Lake plants are highly visible at 20 x and 80X magnification (see Photographs, below)*	Seed coat surface is coarsely reticulate[1,4,5]; Reticulations on seed coat are highly visible at 20X and 80X* magnification (see Photographs, below) *these are the true magnifications, not the ones listed in the figure captions, which are off by a factor of 2.
Fruit morphology	Fruits are elongated and narrow: 0.8 – 1.0 mm x 15 - 25 mm[5]; Fruits exhibit constrictions between the seeds	Fruits are elongated and narrow: ~1 (-1.5) mm x 15 – 28 mm[1,2]; Fruits exhibit constrictions between the seeds	Fruits are relatively oblong: 2 – 2.5 mm x 10-15 mm; Fruit is fleshy and subterete.
Style appearance	Present and distinct; 1.5 – 2(2.5) mm[1,3,5]	Present, > 2 mm in length[1,2]	Absent or < 0.5mm[1,3]; Fruit apex is blunt
Trait	*Rorippa gambelii*: Vandenberg Air Force Base population:	*Rorippa gambelii*: Oso Flaco Lake population	*Rorippa nasturtium-aquaticum*: Little Oso Flaco Lake drainage ditch
Leaf Margin	Dentate to entire, with acute tip[1,3]	Dentate to wavy[2]	Entire to wavy[1,3]
Number of leaflets	7 – 13[1,2]	7 – 13[1,2]	3-7[3], 3 – 11[4]
Leaflet shape	±Linear to ±Round[3]	Narrow to obovate[1,2]	Rounded, oblong to ovate[3]

III. Preliminary Transplantation Trials – *Arenaria paludicola*

A. Black Lake Canyon – Land Conservancy Tract 1998

In the spring of 1998, a trial introduction of greenhouse-raised plants of *Arenaria paludicola* was initiated in Black Lake Canyon. The location chosen for this introduction is the site of a Land Conservancy easement in Black Lake Canyon where Guadalupe Road intersects with the canyon (**Photographs 36 and 37**). To reach this site, one drives north on Guadalupe Road from Highway 1 towards Black Lake Canyon. Within one mile, Guadalupe Road becomes a dirt road and then curves towards the northeast to run parallel to Black Lake Canyon. The location of reintroduction corresponds to the eastern edge of "Basin G", described in the McClelland 1988 Environmental Impact Statement (See **Map 4**).

At this time, the vegetation at the transplanting site for *Arenaria paludicola* is composed mostly of freshwater marsh species. *Salix lasiolepis*) (arroyo willow: Salicaceae) is the main canopy species distributed along the edges of the marsh; scattered small trees of *Myrica californica* (wax myrtle: Myricaceae) also occur in and near the marsh. The freshwater marsh is dominated by *Sparganium eurycarpum* ssp. *eurycarpum* (giant bur-reed: Sparganiaceae) and by *Scirpus microcarpus* (small-fruited bulrush; Cyperaceae); in areas with shallow water, *Juncus effusus* var. *brunneus* (common bog rush: Juncaceae) dominates.

Three plants raised in 8-inch pots by David Gurney and twelve plants that had been raised in 4-inch pots at the Santa Barbara Botanic Garden by Ms. Caroline Cooper were used for the spring 1998 introduction. All of these plants were originally derived from the single clone *of A. paludicola* that was previously located in Basin H of Black Lake Canyon (see **Map 4**).

A photograph of a typical individual raised by Caroline Cooper at the Santa Barbara Botanic Garden is shown in **Photograph 38**. These plants were not highly vigorous because they had recently suffered from a *Phytophthora* fungal infection (Caroline Cooper, personal communication with Susan Mazer), and their successful treatment by Ms. Cooper had only occurred within the past few months.

<u>First Introduction: April 15, 1998</u>

Anuja Parikh, Nathan Gale and David Gurney introduced three ramets raised in 8-inch pots by David Gurney to this location. Each ramet was placed in the center of a *Juncus effusus* tussock, which served to support the root mass of *Arenaria paludicola* and to

provide a matrix of vertical stems into which the shoots of *A. paludicola* could grow. The root mass was fully submersed in the water in which the *Juncus effusus* was growing (**Photograph 39**).

Second Introduction: May 16, 1998

Susan Mazer, Anuja Parikh and Nathan Gale introduced to this location the twelve ramets raised in 4-inch pots at the Santa Barbara Botanic Garden. Because there were not sufficient numbers of *Juncus effusus* tussocks to serve as nurse plants, these ramets were individually placed either on a *Juncus effusus* tussock or amid *Salix lasiolepis* or *Scirpus microcarpus* nurse plants. **Photographs 40, and 43 – 47** illustrate typical transplanted individuals of *A. paludicola*.

The 15 ramets were monitored monthly as recorded in **Table 5**.

Observations on June 13, 1998:

Photographs 41 and 48 - 53 show typical, vigorously growing plants before they were subject to herbivory.

Observations on August 8, 1998:

Photographs 42 shows a *Juncus effusus* tussock heavily predated (note where ~30 vertical shoots have been severed). **Photographs 54 – 58** show other transplanted ramets that have been heavily predated. **Photograph 59** shows a heavily predated *Juncus effusus*. Note that the tips of the severed stems have been severed by diagonal cuts. Rodent feces were observed among the root masses of the transplanted Arenaria paludicola plants, along with small mounds composed of four-inch long stems of *Juncus effusus*.

Observations on September 6, 1998:

Of the 15 ramets that were introduced to this site, 14 survived from April to September. Although most of the plants experienced a healthy and rapid growth spurt between May 15 and June 16, they experienced severe herbivory between June 16 and August 8. Although most plants seemed to recover somewhat from this herbivory, most of the the survivors were much smaller in August and September than when they were initially transplanted. We recommend that future reintroductions at this site include physicial protection for these plants against herbivory.
These results are summarized in the Table 5.

Table 5. Black Lake Canyon Land Conservancy site. Condition of transplanted *Arenaria paludicola* monitored over four months during the spring, summer and fall of 1998

MONITORING OF *Arenaria paludicola* TRANSPLANTS

Date	5/16/98	6/13/98	8/8/98	9/6/98
Plant identification number			**Note: Plants suffering greatly from browsing, apparently by rodents; August water level is ~6 inches lower than in June**	**Partial recovery from browsing evident**
1	Doing very well In *Juncus effusus* Water 10" deep	2 buds, 3 flowers and 3 fruits are developing; shoots up to 18" tall. Water 10" deep	Plant heavily browsed; root ball has 1" shoots emerging. The *J. effusus* nurse plant is also heavily grazed; *Juncus* stems are severed on the bias (severed tip is cut diagonally across).	Partial recovery from herbivory; ~6 4-inch shoots plus 2 6-8-inch shoots + 10-15 small 1-2-inch shoots
2	Doing OK, in *Juncus effusus* and *Scirpus microcarpus*, shoots present; Water 18" deep	Vigorous growth, with two climbing 2 shoots 24" tall; One fruit developing; Additional growth at base of root mass is evident. Water 18" deep	Few stems have survived grazing; a few *Arenaria paludicola* stems emerging from root ball are 4 – 6 " tall; *Juncus* plants without *Arenaria paludicola* are also being browsed.	4 4-inch shoots amid a mat of ~10 1 –2-inch shoots
3	Not too healthy, few branches, 3 feet west of *Salix lasiolepis* in *Scirpus microcarpus* (within 3' deep water). Water 24" deep	Still surviving, but only a few sparse shoots are growing; The two tallest shoots are 14" in height; The root ball has become completely submersed. Water 24" deep	The root ball has almost entirely disappeared; 3 – 4 short shoots of *A. paludicola* are sprouting; *Juncus effusus* nurse plant also browsed.	No plant evident; note oily smell and oily surface to water

Date	5/16/98	6/13/98	8/8/98	9/6/98
4	W side of log in *Salix lasiolepis, Juncus effusus, Sparganium eurycarpum* Water 10" deep	Submerged root ball; 3 small shoots emerging, 3" tall; Water 10" deep	*Arenaria paludicola* transplant has nearly disappeared; One 1" shoot remains, but no root ball is evident; No evidence of browsing.	Root ball not evident; Observe one 3-inch shoot.
5	E side of log in *Salix lasiolepis, Juncus effusus,* and *Sparganium eurycarpum.* Water 10" deep	Roots above ground, with 6" shoots present; Appearance is slightly more vigorous than Plant #4; We spread out the *Juncus* stems a bit to provide more light. Water 10" deep	*A. paludicola* and *Juncus effusus* are browsed; *Arenaria paludicola* includes only one 8" shoot plus several small emerging stems 1/2 inch in height.	Root ball intact with several 1" shoots; 2 shoots (4" and 8" in height) emerging about 8" away from root ball
6	Under *Salix lasiolepis* in shade with *Scirpus microcarpus* and *Juncus effusus* Water 9" deep	Not very vigorous; Plant still entirely shaded; No new growth evident; Vegetative material somewhat brown, except for 5-6 shoots 6" tall. Water 9" deep	*A. paludicola* and *Juncus effusus* are browsed; *Arenaria paludicola* includes only ~6 small emerging shoots, 1/2 inch in height.	No new browsing evident; 6 small shoots, 1-2" in height
7	Among *Scirpus microcarpus*, no *Salix lasiolepis* canopy Water 6" deep	Alive but with weak-looking stems; A few 6" shoots emerging from root mass. Water 6" deep	*Arenaria paludicola* located in *Sparganium* nurse plant is heavily browsed; *Sparganium* is free from browsing; *A. paludicola* appears as root ball with several tiny shoots	No new browsing evident; ~6 1-2-inch shoots; one shoot 12" in height

Date	5/16/98	6/13/98	8/8/98	9/6/98
8	In *Juncus effusus* beyond small log to E of Plants 6 and 7; Soil saturated to surface	Vegetative material brown but with a few very thin green shoots 6" in height; Soil saturated to surface	*Arenaria paludicola* is heavily browsed; Root ball present but covered with heavily browsed short shoots of *A. paludicola*	No new browsing evident; 6 shoots, up to 15" in height; many new short shoots emerging from root ball
9	In *Juncus effusus* and *Scirpus microcarpus* beyond small log to E of 6 and7 Water 10" deep	Brown root ball and shoots, with a few green shoots up to 10" in height Water 10" deep	*Arenaria paludicola* is heavily browsed; Root ball present but covered with heavily browsed short shoots of *A. paludicola.*	No new browsing evident; 5 shoots, 6-12-24" in height
10	In *Juncus effusus* clump near *Myrica californica* Water 10" deep	20" main stem dying, but there are new 3" long green shoots extending from its nodes; Basal shoots emerging from root mass remain 5 – 6" in height. Water 10" deep	*Arenaria paludicola* is heavily browsed; Root ball present with recovering short shoots of *A. paludicola;* *Arenaria paludicola* shoots are buried under pile of severed *Juncus effusis* stems ~3 inches in length.	No new browsing evident; ~3 4-inch shoots plus a mat of ~24 1-2-inch shoots
11	In *Juncus effusus* with less *Scirpus microcarpus* Water 12" deep	Half of plant material brown and dead, with new shoots emerging from root mass up to 8" in height. Water 12" deep	*Arenaria paludicola* is heavily browsed; Root ball present with emerging short shoots of *A. paludicola;* Rodent scat detected on and around *A. paludicola* root ball.	No new browsing evident; One 6" shoot and ~10 1" shoots
12	W of *Salix lasiolepis* near 3 in shallower water, in *Juncus effusus* Water 12" deep	Half of vegetative material is brown,; New shoots up to 6" in height. Water 12" deep	*Arenaria paludicola* and *Juncus effusus* are heavily browsed; Root ball present with a few emerging short shoots of *A. paludicola.*	Two 9-10" shoots and ~20 1-2-inch shoots

Date **Plant:**	5/16/98	6/13/98	8/8/98	9/6/98
13	W of *Salix lasiolepis* near 3 inshallower water, in *Juncus effusus* Water 9" deep	Stems in deep shade, but not very brown; New shoots up to 6" in height. Water 9" deep	*Arenaria paludicola* and *Juncus effusus* are heavily browsed; Root ball present with numerous emerging short shoots	Root ball a mat of half-inch shoots and one 4-inch shoot
14	W of *Salix lasiolepis* near 3 in shallower water, in *Juncus effusus* and *Scirpus microcarpus* Water 9" deep		*Arenaria paludicola* and *Juncus effusus* are heavily browsed; Root ball present with a few emerging short shoots .	10 1-2-inch shoots emerging from root mass
15	W of *Salix lasiolepis* near 3 in shallower water, in *Juncus effusus* and *Scirpus microcarpus* Water 6" deep	Lot of brown material,new green 10" shootsfrom dead stem like 10 Water 6" deep	*Arenaria paludicola* heavily browsed; Root ball present with numerous emerging short shoots (~1.5" in height) of *A. paludicola*	10 1-2-inch shoots emerging from root mass

B. Black Lake Canyon – Land Conservancy Tract 1999

These plants were monitored again during the 1999 growing season (see **Photos 144 and 145**).

Observations on May 21, 1999

Eleven of the 15 plants that were transplanted in May 1998 were successfully re-located. Plants #4, 7, 13, and 15 had disappeared between September 1998 and May 1999, although the basal root mass of plant #15 appeared to be somewhat intact. Plant stems emerged from the water surface to heights of between 2" and 20", although most stems were no more than 6" – 10" long. Each original ramet consisted of 1 – 25 individual shoots. The water at this time was approximately 0 to 12 inches deep, with most ramets at least partially submerged. No evidence of browsing was observed. See **Photos 146 – 152** to observe examples of surviving plants.

Observations on August 21, 1999

Ten of the 11 plants were still alive; plant #8 showed no sign of vegetative growth although the basal root mass was located. The water level had receded by about four inches, leaving several plants emerging directly from saturated soil (i.e., the root mass was not submerged). All surviving plants showed evidence of serious browsing, alhtough plant #5 exhibited 15–10 2-inch to 20-inch shoots and five developing fruits (See **Photos 153 – 157** for examples of surviving plants). Plant #9 also continued to grow vigorusly, with eight 6-inch to 24-inch shoots (**See Photo 155**).

Observations on November 11, 1999

Eight of the 11 plants present on May 21 were still alive; plant #3 disappeared between August and November 1999. Most plants did not seem to suffer from recent browsing. Plant #1 had partially recovered from the effects of browsing observed in in August. Plant #2 had also increased in size since August. Plant #5 was reduced in size since August. Plant #6 exhibited no vegetative growth (the one shoot present in August had disappeared). Plant #9 did not appear to be vigorous in spite of its relatively large size in August; five of its eight shoots had disappeared. Plant #10 increased substantially in size from August to November (from one to 10 shoots). Plant #11 exhibited increased shoot length. Plant #12 showed no growth between August and November (one of its shoots disappeared). Plant #14 showed minor growth between August and November. See Photos **158 – 166** for examples of surviving plants)

Comparison of plants in September 1998 and November 1999.

We can ask whether plants appear to be growing or diminishing by comparing the number of shoots produced by each plant in September 1998 to the number of shoots produced in November 1999. Plant #1 decreased from approximately 20 shots to 10-15 shoots. Plant #2 decreased from four shoots to two shoots. Plants #3 and 4 disappeared before September 1998. Plant #5 increased in size (from eight short shoots in 1998 to 20 short shoots plus three 10-15" shoots in 1999). Plant #6 disappeared between August and November 1999. Plant #7 disappeared between September 1998 and May 1999. Plant #8 disappeared between May and August 1999. Plant #9 declined from five shoots to three shoots. Plant #10 decreased from ~27 shoots to 10 shoots, although the maximum shoot length increased from four inches to 10 inches. Similarly, Plant #11 decreased from ~11 shoots to three shoots, although the maximum shoot length increased from six inches to 10 inches. Plant #12 declined from 22 shoots to 3 shoots. Plant #13 disappeared between September 1998 and May 1999. Plant #14 declined from 10 to

two shoots, although the maximum shoot length increased from two inches to 12 inches. Plant #15 disappeared between September 1998 and May 1999. In sum, the condition of virtually all plants declined between September 1998 and November 1999.

These data are summarized in Table 6.

Table 6. Black Lake Canyon Land Conservancy site. Condition of transplanted *Arenaria paludicola* monitored over four months during the spring, summer and fall of 1999.

Date	5/21/99	8/21/99	11/11/99
1	No browsing 15 2-18" shoots Water 10" deep 2 photos	Browsing present 10 2-12" shoots Water 6" deep 1 photo	Old browsing seen 10-15 2-20" shoots Water 6" deep 1 photo
2	No browsing 1 6" shoot; 1 1" shoot Water 12" deep	Browsing present 2 3" shoots Water 8" deep	Some browsing nearby 2 4" shoots Water 12" deep 1 photo
3	No browsing 1 2" shoot Water 12" deep	Browsing present 1 8" shoot Water 8" deep	Gone
4	Gone	Gone	Gone
5	No browsing 20-25 1-10" spreading shoots; 1 20" shoot Water 6" deep 1 photo	Browsing present 15-20 2-30" shoots; 1 flower; 5 fruit Water at surface 1 photo	No browsing 3 10-15" shoots; 20 1-2" shoots at the bottom Soil saturated at surface 1 photo
6	No browsing 2 2" shoots Water 4" deep	Browsing present 1 4" shoot Water at surface	Browsing at the edges Shoots gone Soil saturated at surface 1 photo
7	Gone	Gone; overgrown with *Scirpus*	Gone
8	No browsing 8 6-10" shoots Water at surface 1 photo	Gone; old root ball seen No water present	Gone Soil saturated at surface
9	No browsing 4 10-20" shoots	Browsing present 8 6-24" shoots	No browsing; plant not very healthy

Date	5/21/99	8/21/99	11/11/99
	Water 4" deep 1 photo	Water at surface 1 photo	1 20" shoot; 2 4" shoots Soil saturated at surface 1 photo
10	No browsing 8 6-10" shoots; 10 1-6" shoots Water 10-12" deep 1 photo	Browsing present 1 5" shoot Water 8" deep	Old browsing seen 10 4-10" shoots Water 10" deep 1 photo
11	No browsing 12 10-15" shoots Water 12" deep 1 photo	Browsing present 2 6-8" shoots Water 8" deep 1 photo	Old browsing seen 3 10" shoots Water 12" deep 1 photo
12	No browsing 10 10-15" shoots Water 10" deep 1 photo	Browsing present 4 6-12" shoots Water 6" deep 1 photo	Old browsing seen 3 6-8" shoots Water 6" deep 1 photo
13.	Not found	Not found	Not found
14	No browsing 2 10" shoots Water 6" deep	Browsing present 2 6-10" shoots Water 2" deep	Old browsing seen 2 12" shoots Water 6" deep 1 photo
15	Root ball remains seen No shoots Water 3" deep	Browsing on *Juncus* Root ball remains seen No shoots Soil saturated at surface	Root ball remains seen No shoots Water at surface

C. Black Lake – Land Conservancy Easement site - 1998

In the spring of 1998, a trial introduction of greenhouse-raised plants of *Arenaria paludicola* was initiated along the southwestern margin of Black Lake. The location chosen for this introduction is the site of a Land Conservancy easement on Black Lake (**Photographs 60 - 65**). At this time, the areas of the transplanting site where the water was deepest were occupied mostly by *Scirpus californicus* (California bulrush: Cyperaceae). In shallower locations, the *S. californicus* was bordered by *Typha latifolia* (broad-leaved cattail: Typhaceae) and *Juncus effusus* var. *brunneus* (common bog rush: Juncaceae). At the edges of the lake where water was very shallow, the dominant species were *Polypogon monspeliensis* (annual beard grass: Poaceae), *Oenanthe sarmentosa* (water-parsley: Umbelliferae), and *Baccharis douglasii* (marsh baccharis: Asteraceae). *Eucalyptus globulus* (blue gum: Myrtaceae) trees form a canopy on the slopes surrounding the lakeshore.

Nine plants that had been raised in 4-inch pots at the Santa Barbara Botanic Garden by Ms. Caroline Cooper were used for this introduction. All of these plants were derived originally from the single clone *of A. paludicola* that was previously located in Basin H of Black Lake Canyon (see **Map 4**).

Introduction: May 16, 1998

Susan Mazer, Anuja Parikh and Nathan Gale introduced the nine ramets to this location. Most plants were transplanted into *Juncus effusus* tussocks. **Table 7** describes the microenvironment in which each ramet was placed.

These ramets were monitored monthly as recorded in **Table 7**.

Observations on June 13, 1998:

Photographs 66 – 72 show examples of the plants that had become established since their May 16 introduction. Some plants show vigorous growth, but most stems had not emerged more than six inches above the surface of the water. Note that the surface of the water was relatively clear of duckweed.

Observations on August 8, 1998:

Photographs 73 – 77 show examples of plants monitored on August 8, 1998. Two plants had floated away from their original transplant site (e.g., Plants #1 and #2; Photographs 73

and 74). Other plants show vigorous growth (e.g., Plants #4 and #9; Photographs 75 and 76). Note that the density of duckweed on surface of water greatly increased since June.

Observations on September 6, 1998:

Photographs 78 – 81 show Plant #4 and habitat quality on September 6. Plant #4 (Photograph 78) was still growing vigorously. At this time, the surface of the water was densely occupied by duckweed.

Summary: Of the nine ramets that were introduced to this site, six survived from April to September. The largest plants that survived here are notably larger than the largest plants in Black Lake Canyon, presumably due to the difference in intensity of herbivory. We observed no evidence of herbivory on either *Arenaria paludicola* or on *Juncus effusus* at Black Lake.

Table 7. Black Lake introduction site. Condition of transplanted *Arenaria paludicola* monitored over four months during the summer of 1998

Date	5/16/98	6/13/98	8/8/98	9/6/98
Plant	**BL**		**No evidence of browsing on these plants**	
1	Near log/debris Water 6" deep	Doing OK, floated East of previous location; Consists of a few shoots up to 2" high; Three fruits, 3 flowers and 3 buds observed. Water 6" deep.	Root ball intact, but no emergent growth	Plant cannot be located
2	In *Juncus effusus* Water 6" deep	Surviving; floated East of previous location; Not not too many shoots but emerging 2" from water Water 6" deep	Plant floated away, but the root ball is still anchored by a flag. The root ball has nearly entirely disintegrated.	Plant cannot be located
3	In *Juncus effusus* in *Scirpus californicus* area. Floating in water 3'deep	Was submerged but is still alive, was re-anchored Floating in water 3'deep	10 – 15 shoots emerging, 6 – 8" tall; Tallest shoot = 15" tall; Water ~20" deep This plant had been elevated and re-anchored last month following complete submergence.	10 – 15 emerging shoots, up to 15" in height; Root ball submerged; *Juncus effusus* nurse plant is dead, evidently due to flooding; *Arenaria paludicola* now climing up *Scirpus*; New shoots growing over dead *Juncus* stems.
4	In *Juncus effusus* and *Polypogon monspeliensis* among dying/drowned *Baccharis pilularis* Water 10" deep	Many shoots 6" tall Water 10" deep	Most vigorously growing plant in sample; 12" shoots, climbing *Juncus* stems; Both *Juncus* and *Arenaria* show recent growth.	

Date Plant:	5/16/98	6/13/98	8/8/98	9/6/98
5	In *Juncus effusus* near *Typha latifolia* area. Water 2' deep	Submerged but 6" shoots present Water 2' deep	*Juncus* nurse plant has died; Root ball of *Arenaria* is evident with 10 – 15 shoots 2 – 6" tall; We propped the remaining root ball on the remaining *Juncus* and tied dead *Juncus* stems around it for support.	*Arenaria paludicola* still present but appears to be drowning; ~4 shoots emerging 2" above water surface; no vigorous growth
6	In *Juncus effusus* near *Typha latifolia* Water 2' deep	OK with 6" shoots Water 2' deep	Flag completely submerged; Root ball appears enmeshed in dead *Juncus*; There are some living green vegetative shoots emerging from root ball, up to 9" in height; Root ball pulled out of water and propped onto a *Typha* plant.	*Arenaria paludicola* still attached to *Typha*; Root ball still intact; ~8 shoots, each about 2" in height.
7	In *Juncus effusus* and *Polypogon monspeliensis* Water 12" deep	OK with 8" shoots Water 12" deep	Root ball still in Juncus nurse plant; ~10 – 15 shoots, ~8" in height	*Arenaria paludicola* not visible except for 2 small shoots, ~3" in height; *Juncus* is in ~12" of water; Root ball disintegrating.

Date Plant:	5/16/98	6/13/98	8/8/98	9/6/98
8	In *Juncus effusus* with *Polypogon monspeliensis* Water 14" deep	OK with 5 shoots 4-5"tall Water 14" deep	All shoots of *Arenaria paludicola* have disappeared, but flag remains; Rootball is disintegrating; Perhaps the Juncus nurse plant was too small; Disintegrating root ball was propped onto a dead *Baccharis* plant.	Plant cannot be located
9	In *Juncus effusus* with *Typha latifolia* and*Baccharis douglasii* Water 2-1/2' deep	Submerged but with 20 new shoots, 6" shoots above water Water 2-1/2' deep	The flag and nurse plant have disappeared, but the *Arenaria paludicola* plant is spreading vegetatively; There are two clumps of shoots ~12" apart; In main clump, ~25 shoots are ~10" in height; Larger clump is presumably the site of the original root ball; In secondary clump are ~10 shoots; Plant co-occurring with *Polygonum* punctatum; Water depth ~2 feet.	*Arenarila paludicola* plant seems to be spreading vegetatively from completely submerged root mass; ~20 shoots 4 – 6" tall in main clump, plus ~10 scattered shoots emerging 18" away, growing in *Polyonum punctatum*; additional isolated shoots ~4" tall appear here and there.

D. Black Lake – Land Conservancy Easement site - 1999

None of the plants introduced to this site in 1998 survived into 1999, in spite of their relative vigor at the end of 1998.

IV. Greenhouse Cultivation

A. *Arenaria paludicola*

At regular intervals from June – August 1998, *Arenaria paludicola* ramets were cultivated from shoot cuttings in the greenhouse at the University of California (Santa Barbara) (Photographs 82 – 86). Cuttings were used from two sources:

(1) A large, vegetatively growing individual given to Susan Mazer by David Chipping in the spring of 1997 (**Photograph 84**). This was originally propagated from a ramet growing at Black Lake Canyon.

(2) Cuttings sampled by Susan Mazer on August 8, 1998 from several ramets of *Arenaria paludicola* at the Oso Flaco Lake population

One successful method of cultivating this species is as follows:

- Drill drainage holes in a plastic tray (21" x 12" x 2.5" seedling trays were used here)
- Fill the tray up to 2.5 inches in depth with a saturated mixture of vermiculite and perlite.
- Cut growing stems of *A. paludicola* 6 – 8 inches from the shoot tip. Dip ends in water.
- Coat the base of the stem with a root-promoting agent such as "Rootone" (available in most gardening centers).
- Place stems vertically in the vermiculite/perlite mixture at 3-inch intervals.
- Mist tray at regular intervals to maintain moisture of rooting medium.

The stem will root successfully and begin to grow. Additional stems will form at the nodes of these stems. **Photographs 82 and 83** show a few of the 14 trays of *A. paludicola* from Black Lake Canyon that are currently being cultivated at UCSB. One tray of *A. paludicola* from Oso Flaco Lake is also being cultivated. This contains multiple ramets representing at least three genotypes with distinct molecular markers (see **results of molecular genetic study, below**).

In addition to these trays, 16 large plants of *A. paludicola* are currently being raised in 2-gallon pots at UCSB (**Photographs 84 – 86**). These plants are all derived from 4-inch diameter potted plants given to Susan Mazer by Caroline Cooper in April 1998. Caroline Cooper raised these plants from vegetative cuttings originally given to the Santa Barbara

Botanic Garden in 1994 by Susan Mazer (Caroline Cooper was working under a contract with the U. S. Fish and Wildlife Service to cultivate these cuttings vegetatively).

In June and in September 1998, the 4-inch pots of *A. paludicola* were transplanted into 8-inch pots filled with a mixture of peat, loam, perlite, and Osmocote fertilizer pellets. In June through mid-August, these plants exhibited exceptionally vigorous growth and flowering in 1-gallon plots (**Photograph 85**). In spite of this flowering, no flowers appeared to develop successfully into fruits — when plants were visited weekly, no mature capsules could be found.

In mid-August 1998, all plants (except for the original plant from David Chipping, which remained healthy in the five-gallon pot in which it has been growing for the last 1.5 years) experienced a synchronous and unexplained die-back (**Photograph 86**). By late August, ~90% of the green shoots had turned brown in the 15 plants in the 8-inch diameter pots, although the nodes still appeared to be active and the roots appeared to be healthy.

On September 14 and 15, 1998, these struggling plants were transplanted into 12-inch diameter pots, where they recovered completely. **As of February 2000, they are once again growing extremely vigorously (with a few flowers) as they appeared in Photograph 85.**

The cause of the August 1998 die-back is still unexplained. Upon transplanting these plants in September, Susan Mazer observed that, although the top two-inches of the soil in the 8-inch diameter pots was densely packed with *A. paludicola* roots, the roots had not penetrated more deeply into the pot. This kind of unexplained die-back has been observed occasionally before in the UCSB greenhouse trays of *Arenaria paludicola*, where its cause could not be accounted for.

In the absence of hand-pollinations (which we have not performed), we have not observed the production of viable seeds in the greenhouse or lathehouse. *A. paludicola* flowers typically contain aborted ovules (see **Photographs 168 and 169**).

B. *Rorippa gambelii*

Rorippa gambelii has been successfully cultivated from seed to the rosette stage in the UCSB greenhouse. The specimens shown in **Photographs 87 – 88** are derived from seeds collected at Black Lake Canyon in 1994, but we currently have seedlings growing representing the populations at Vandenberg Air Force Base, the Oso Flaco Lake marsh,

and the Oso Flaco Lake causeway. As of February 2000, we have continued efforts to propagate these specimens with the aim of producing flowers, fruits, and seeds in the greenhouse.

The most successful propagation has been from the eight four-inch rhizomes collected from Vandenberg Air Force base population on August 21, 1999. These were placed in 10-inch pots in the UCSB outdoor lathehouse. As of March 19, 2000, two of these plants have begun to bolt. If they flower, we will cross-pollinate them in an effort to produce seeds at UCSB.

1. Seed Bank Study from Vandenberg Air Force Base (Main Gate) site

On September 19, 1998, a seed bank emergence study was initiated for two purposes:

(1) To determine whether seeds of *Rorippa gambelii* would germinate from peat collected from their native site.

(2) To evaluate the soil seed bank community at the VAFB site of *R. gambelii*. Because the soil samples were collected at the end of the summer and before the autumnal germination period, this means that these soil samples would potentially contain both dormant seeds produced during and prior to the 1998 reproductive season **and** non-dormant seeds that had just been produced during the spring and summer of 1998.

This seed bank study was set up as follows:

- In each of 18 21" x 12" x 2.5" plastic trays, a 1.5" layer of perlite was placed.
- On top of the perlite, 1000 ml of saturated peat collected at the site of the VAFB population of *R. gambelii* was spread out to produce a 0.25" thick layer of peat.
- These trays were mist-watered daily.
- As of December 19, 1998, hundreds of seedlings were emerging from these trays.
- On June 26, 1999, the cumulative counts of all identifiable seedlings emerging from these trays were completed (**Table 8**).

Table 8. Cumulative seedling counts for 18 trays containing soil samples from VAFB location of Rorippa gambelii.

JUNE 26, 1999 Data collected by ANUJA PARIKH and NATHAN GALE

SPECIES OBSERVED	TRAY NUMBER 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	TOTAL NUMBER
Athyrium filix-femina												1							1
Cirsium occidentale					2														2
Conyza bonariensis								1											1
Epilobium canum	18	19	7	67	65	143	25	57	12	5	2	17	2	14	38	4	16	12	523
Juncus effusus	5		3	4	3	2	14	2	7	4	2	1	2	1	1	1	2	1	55
Myrica californica							2												2
Oenanthe sarmentosa	28	3	4	11	8	1	17	6	1	2	5	9	3		6	6	4	2	116
Oxalis corniculata				4										7		2	5		18
Pellaea breweri X P. bridgesii												1							1
Rorippa gambelii	7	2	3				1								1		5		19
Rumex crispus					1		2												3
Scirpus microcarpus	34	22	54	69	41	50	36	46	32	39	38	9	49	24	63	27	37	13	683
Urtica dioica ssp. holos	1	1			1	1					1								5
Unknown (Moss?)					2												1		3
TOTAL NUMBER OF SEEDLING	93	47	71	155	123	197	97	112	52	50	48	38	56	46	109	40	70	28	1432

2. Results of Seed Bank Study from Vandenberg Air Force Base (Main Gate) site

A total of 12-13 species were identified among the 1432 emerging seedlings (It is possible that the one specimen observed of the fern *Pellaea* was a greenhouse contaminant). Of the 1432 seedlings collected from the 18 soil samples, the vast majority belonged to three species: *Epilobium canum* (523 seedlings), *Scirpus microcarpus* (583 seedlings) and *Oenanthe sarmentosa* (116 seedlings). *Epilobium canum* and *Scirpus microcarpus* appeared in all 18 of samples and *Oenanthe sarmentosa* emerged from 17 of the samples, indicating that the seeds of these species are very widespread thorughout the soil.

A total of 19 seedlings (1.3%) of *Rorippa gambelii* emerged; these were distributed among 6 of the 18 soil samples.

V. Laboratory Observations

A. *Rorippa gambelii* and *R. nasturtium-aquaticum* fruit and seed morphology

Seeds sampled from each of 16 individuals of *R. gambelii* from Vandenberg Air Force Base (Main Gate) (VAFB) and 14 individuals of *R. gambelii* from Oso Flaco Lake were brought back to the Mazer lab on September 6, 1998. These seeds were counted and weighed so that we could compare these taxa with respect to their mean individual seed mass (i.e., the mean mass of individual seeds). Seeds that appeared to be inviable (i.e., seeds that were dried, shriveled up, or empty) were excluded from this analysis.

A subset of these fruits and seeds from the VAFB and Oso Flaco Lake populations of *R. gambelii* as well as the fruits and seeds of two specimens of *R. nasturtium-aquaticum* from the Little Oso Flaco Lake drainage ditch were photographed in the Mazer lab at 20X – 100X magnification using a dissecting microscope with a photographic attachment (**Photographs 102 – 143**). These photographs allow these taxa to be compared with respect to:

- The surface texture of the seed coat.
- The color of the seed coat.
- The shape and length of the style.
- The arrangement of the seeds (i.e., arranged in a single or a double row in each half of the fruit).

B. Seed Mass Differences Between Vandenberg and Oso Flaco Lake causeway Populations of *Rorippa gambelii*

Table 9 provides the raw data analyzed for the comparison of seed mass differences between plants sampled from the Vandenberg Air Force Base (Main Gate) and Oso Flaco Lake causeway populations.

Table 10 shows descriptive statistics for the seed samples from each population. The mean individual seed mass produced by the individuals sampled from Vandenberg Air Force Base is 0.107 mg. The mean individual seed mass produced by the individuals sampled from Oso Flaco Lake causeway population is 0.285 mg. See **Figure 1** for graphical representation.

Table 11 shows the results of an analysis of variance conducted to detect a significant difference between the mean individual seed mass produced by VAFB vs. Oso Flaco Lake individuals. The mean mass of individual seeds produced by plants sampled from Oso Flaco Lake causeway population is significantly greater than that of seeds produced by plants sampled from VAFB. Fisher's PSLD, Scheffé's Test, and the Bonferonni-Dunn test all confirm that the difference between these two populations in mean individual seed mass is statistically significant at the $p < 0.0001$ level.

Figure 2 shows clearly that even the seeds from the smallest-seeded plants in the Oso Flaco Lake causeway population are of higher mass than the seeds from the largest-seeded plants at VAFB. There is almost no overlap in mean individual seed mass among the plants sampled from the two populations.

Table 9. Raw data used in the statistical analyses below. Individuals whose seeds appeared dried, shrivelled, and likely to be inviable were excluded from these analyses.

	Species	Population	Maternal Family	Number of Seeds Weighed	Total Seed Weight (mg)	Mean Individual Seed Mass (mg)	Notes
1	gambelii	Vandenberg	1	54	9.496	.176	
2	gambelii	Vandenberg	2	91	12.413	.136	
3	gambelii	Vandenberg	3	68	6.554	.096	
4	gambelii	Vandenberg	4	85	3.887	.046	seeds looked dried & shriveled
5	gambelii	Vandenberg	5	70	6.402	.091	
6	gambelii	Vandenberg	6	50	5.601	.112	
7	gambelii	Vandenberg	7	40	2.661	.067	seeds looked dried & shriveled
8	gambelii	Vandenberg	8	42	1.285	.031	seeds looked dried & shriveled
9	gambelii	Vandenberg	9	22	1.125	.051	
10	gambelii	Vandenberg	10	95	15.592	.164	
11	gambelii	Vandenberg	11	32	1.041	.033	seeds looked dried & shriveled
12	gambelii	Vandenberg	12	38	.987	.026	seeds looked dried & shriveled
13	gambelii	Vandenberg	13	54	5.439	.101	
14	gambelii	Vandenberg	14	37	3.491	.094	
15	gambelii	Vandenberg	15	18	2.093	.116	
16	gambelii	Vandenberg	16	41	4.416	.108	
17	gambelii	Vandenberg	17	51	7.047	.138	
18	gambelii	Vandenberg	18	34	1.198	.035	seeds looked dried & shriveled
19	gambelii	Vandenberg	19	87	11.718	.135	
20	gambelii	Vandenberg	20	13	.768	.059	
21	gambelii	Vandenberg	21	30	1.068	.036	seeds looked dried & shriveled
22	gambelii	Vandenberg	22	23	1.268	.055	
23	gambelii	Vandenberg	23	23	1.804	.078	
24	gambelii	Oso Flaco Lake	1	50	13.743	.275	
25	gambelii	Oso Flaco Lake	2	50	13.520	.270	
26	gambelii	Oso Flaco Lake	3	50	15.817	.316	
27	gambelii	Oso Flaco Lake	4	50	12.231	.245	
28	gambelii	Oso Flaco Lake	5	50	14.413	.288	
29	gambelii	Oso Flaco Lake	6	50	15.575	.311	
30	gambelii	Oso Flaco Lake	7	50	12.648	.253	
31	gambelii	Oso Flaco Lake	8	52	10.130	.195	
32	gambelii	Oso Flaco Lake	9	52	12.015	.231	
33	gambelii	Oso Flaco Lake	10	50	15.657	.313	
34	gambelii	Oso Flaco Lake	11	52	16.067	.309	
35	gambelii	Oso Flaco Lake	12	52	13.163	.253	
36	gambelii	Oso Flaco Lake	13	54	9.791	.181	
37	gambelii	Oso Flaco Lake	14	61	16.564	.272	

Table 10. Seed mass variation among 16 sampled individuals of *Rorippa gambelii* from Vandenberg Air Force Base (Main Gate) and among 14 sampled individuals from the Oso Flaco Lake causeway population.

Descriptive Statistics
Split By: Population
Row exclusion: Rorippa Seed Weights

	Mean	Std. Dev.	Std. Error	Count	Minimum	Maximum	# Missing
Mean Individual Seed Mass (mg), Total	.181	.089	.016	30	.051	.316	0
Mean Individual Seed Mass (mg), Vandenberg	.107	.037	.009	16	.051	.176	0
Mean Individual Seed Mass (mg), Oso Flaco Lake	.265	.043	.011	14	.181	.316	0

Figure 1. Bar graph illustrating differences between the mean individual seed mass produced by individuals from Vandenberg vs. Oso Flaco Lake causeway populations of *Rorippa gambelii*.

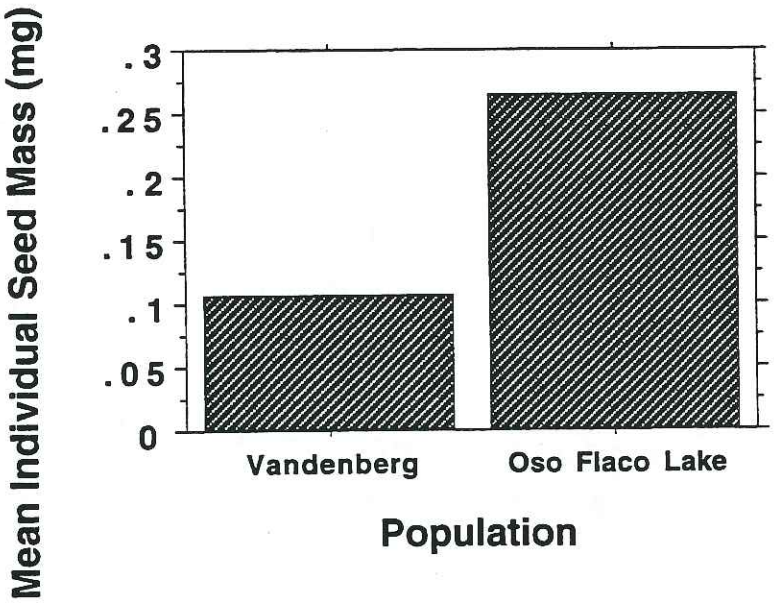


Table 11. Summary of one-way Analysis of Variance to detect significant differences between populations in mean individual seed mass. The results indicate that seeds sampled from the *R. gambelii* plants in the Oso Flaco Lake causeway population are significantly heavier than those sampled from *R. gambelii* at Vandenberg Air Force Base.

ANOVA Table for Mean Individual Seed Mass (mg)
Row exclusion: Rorippa Seed Weights

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Population	1	.187	.187	119.643	<.0001	119.643	1.000
Residual	28	.044	.002				

Fisher's PLSD for Mean Individual Seed Mass (mg)
Effect: Population
Significance Level: 5 %
Row exclusion: Rorippa Seed Weights

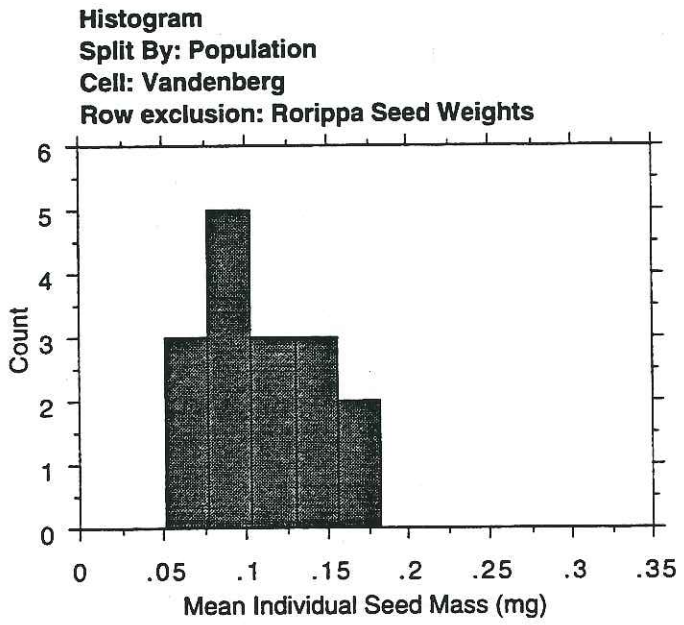
	Mean Diff.	Crit. Diff	P-Value	
Vandenberg, Oso Flaco Lake	-.158	.030	<.0001	S

Scheffe for Mean Individual Seed Mass (mg)
Effect: Population
Significance Level: 5 %
Row exclusion: Rorippa Seed Weights

	Mean Diff.	Crit. Diff	P-Value	
Vandenberg, Oso Flaco Lake	-.158	.030	<.0001	S

Bonferroni/Dunn for Mean Individual Seed Mass (mg)
Effect: Population
Significance Level: 5 %
Row exclusion: Rorippa Seed Weights

	Mean Diff.	Crit. Diff	P-Value	
Vandenberg, Oso Flaco Lake	-.158	.030	<.0001	S



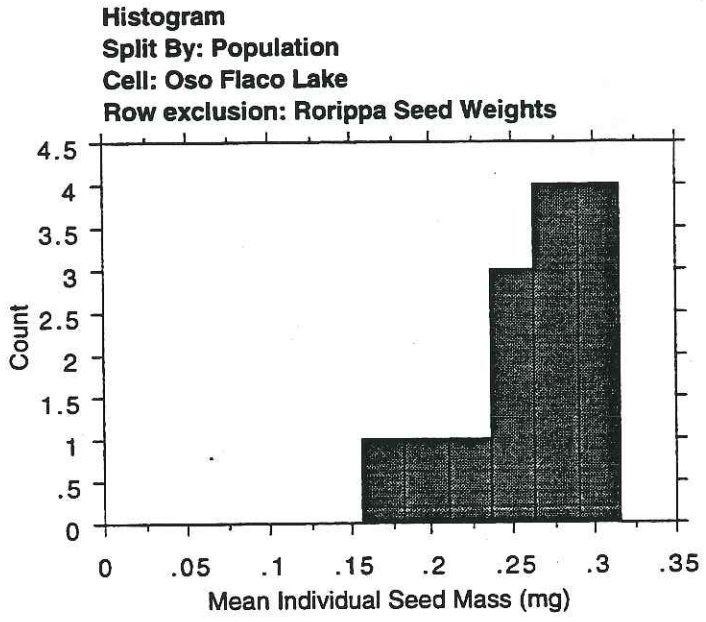


Figure 2. Frequency distributions of seed mass variation among individuals sampled on September 6, 1998 from Vandenberg and Oso Flaco Lake causeway populations of *Rorippa gambelii*. The largest seeds produced by the Vandenberg population are barely heavier than the smallest seeds produced by the Oso Flaco Lake causeway population.

C. Differences in Fruit Morphology Between Vandenberg Air Force Base, Oso Flaco Lake causeway, and Little Oso Flaco Lake Populations of *Rorippa gambelii* and *R. nasturtium-aquaticum*

Photographs 102 – 107 illustrate three basic features of the fruits from the Vandenberg Air Force Base (Main Gate) population of *Rorippa gambelii*. First, the styles are elongated at the tip of the fruit, extending to a length of 2 – 3 mm. Second, the points of attachment of the seeds to the edge of the fruit wall clearly appear in an alternating fashion. This means that, when mature seeds are aligned within the fruit wall, they appear as a **single row**. Third, a large number of seeds that begin development in the fruits of VAFB plants abort before reaching their final size. This can be seen in **Photograph 106**, which includes several flattened and dried aborted seeds still attached to the fruit wall.

Photographs 108 – 113 illustrate the similarities between the fruits from Vandenberg Air Force Base and those from the Oso Flaco Lake causeway population. As shown in **Photograph 108**, the fruits from the Oso Flaco Lake causeway also bear an elongated style at the tip of the fruit. Similar to the VAFB fruits, the Oso Flaco Lake causeway fruits exhibit points of attachment between the fruit wall and the seeds that appear in an alternating basis along the length of the fruit wall. In contrast to the VAFB plants, however, some of the fruits from the Oso Flaco Lake causeway population contain seeds that are so closely packed together that they sometime appear side by side in the fruit (See **Photograph 113**). Note the rich, dark brown color of the seeds.

Photographs 114 – 119 illustrate the fruits and the alignment of seeds in *Rorippa nasturtium-aquaticum* from the agricultural drainage ditch near Little Oso Flaco Lake. There are three clear differences between these fruits and those from the two populations conforming to *Rorippa gambelii*. First, the fruits of R. *nasturtium-aquaticum* contain no elongated style; the tip of the fruit is blunt in comparison to *R. gambelii*. Second, the seeds of *R. nasturtium-aquaticum* are tightly packed within the fruit in two rows instead of in a single row. Third, the seeds of R. *nasturtium-aquaticum* are a much lighter, golden shade of brown than those of *R. gambelii*. We observed evidence that seed abortion can also be fairly common in R. *nasturtium-aquaticum*; note the very dark brown shriveled seeds in **Photograph 115**.

D. Differences in Seed Morphology Between Vandenberg Air Force Base, Oso Flaco Lake causeway, and Little Oso Flaco Lake Populations of *Rorippa gambelii* and *R. nasturtium-aquaticum*

Photographs 120 – 127 represent seeds from the VAFB population of *R. gambelii*. Note the dark brown color of the seed coat. Also, note that at 20X magnification (**Photographs 120 – 123**) it is not possible to distingush the reticulations of the seed coat. At 80X magnification (**Photographs 124 – 127**), it is possible to see the reticulate network of ridges that appears on the surface of the seed coat.

Photographs 128- 136 represent seeds from the Oso Flaco Lake population *of R. gambelii.* Note that, in contrast to the VAFB population of *R. gambelii*, at 20X magnification it is possible to observe the surface reticulations on the seed coats of these seeds (**Photographs 128 – 133**). At 80X magnification (**Photographs 134 – 136**), the spaces bounded by the network of ridges are two to four times as large as they are on the seeds sampled from the VAFB population.

Photographs 137 – 143 represent seeds from the Little Oso Flaco Lake drainage ditch population of *R. nasturtium-aquaticum*. Note the light honey-colored seed coat in comparison to the dark brown seed coat of *R. gambelii*. At 20X magnification (Photographs 137 – 138), the reticulations are even easier to observe than in either of the sampled populations of *R. gambelii*. As is evident at 40X and 80X magnification (Photographs 139 – 143), the spaces bounded by the network of ridges are about twice as large as they appear in the seeds of the Oso Flaco Lake population of *R. gambelii*.

VI. Preliminary Survey of Genetic Variation in *Arenaria paludicola* and *Rorippa gambelii* (lab work and interpretation conducted by Dr. Lee Miller; Current Address: Department of Integrative Biology, University of California, Berkeley, California)

A. Introduction

A component of our efforts to preserve *Arenaria paludicola* has been to bring cuttings into cultivation in the greenhouses at UCSB. This effort has been successful and it appears that greenhouse propagation can continue to proceed. However, before undertaking any significant restoration of natural sites, it is important to determine the degree of genetic variability within and between populations. The detection of significant molecular genetic variation between populations (e.g., the Oso Flaco Lake

population and cultivated ramets representing the formerly extant Black Lake Canyon population) might suggest that the provenance of source material (e.g., vegetatively cultivated ramets) should be of concern when augmenting resident populations with additional vegetative material. For example, if molecular genetic differentiation between populations reflects adaptive ecological differences, it would be prudent to augment a given population only with ramets representing the residents of that population. Although *A. paludicola* appears to spread predominantly by vegetative growth within populations, information concerning the degree of genetic variation within populations may indicate that sexual reproduction (or somatic mutation) is likely to have occurred in the past.

In either case, a survey of molecular genetic variation can provide a sense of the geographic pattern of existing genetic variation. For example, is there more variation between *Arenaria paludicola* populations than within them? If so, attempts to preserve the genetic diversity of *Arenaria paludicola* would require maintaining genetic stocks from multiple populations.

Because funding for this component of the project was extremely limited, our goals for the current study have been very modest. The aims of the molecular genetic study conducted here have been:

(1) To develop a protocol that can be used to detect molecular genetic variation within and between populations. To date, there have been no published accounts of molecular genetic variation in either *Arenaria paludicola* or *Rorippa gambelii*. Adapting known molecular methods to a new species can be a very time-consuming process, as these methods can be highly sensitive to the temperatures at which they are conducted and the exact concentrations of the reagents that they use (e.g., taq polymerase, dNTPs, primers, and Magnesium).
(2) To use this protocol to detect molecular markers unique to the formerly extant Black Lake Canyon and currently extant Oso Flaco Lake populations of *Arenaria paludicola*, indicating genetic differentiation between these geographically separated populations,
(3) To detect, if present, evidence for genetic diversity within each of these populations, and
(4) To detect molecular evidence for the hybrid origin of *R. gambelii x nasturtium-aquaticum*.

The detection of genetic differences between populations would not necessarily indicate that there are significant differences between them in their ecological tolerances or

preferences, but it would indicate that genetic drift and/or natural selection had generated these differences.

B. Background: Review of fingerprinting techniques and our selection of an appropriate method: Genetic fingerprinting methods based on the polymerase chain reaction (PCR) have developed rapidly in the last decade, and they now offer attractive sensitivity and price-performance. The methods that have the highest rates of polymorphism detection (i.e. sensitivity) are all based upon the simultaneous amplification of numerous anonymous segments or regions of DNA. A region of DNA is considered anonymous if nothing is known concerning its sequence, function or chromosomal location. Perhaps the first anonymous PCR method was the random amplified polymorphic DNA, or RAPD, approach in which short (usually 10 base pair), arbitrary primers are employed in a modified PCR reaction using very low annealing temperatures (roughly 40C) and long extension times. When this approach is applied to genomic DNA, from 1 to 40+ bands, corresponding to individual anonymous gene sequences (fragments of DNA), are produced.

This technique is quite sensitive, as different individuals in outcrossed populations usually display different banding patterns. Each band corresponds to a segment of DNA of a particular molecular weight (correlated with its length), and is scored simply as present versus absent. A genetic matrix that illustrates the similarities and differences between all sampled individuals is then constructed by combining data from numerous bands that were produced using a number of different RAPD PCR primers. In this matrix, the first column lists the identify of each individual, and each subsequent column represents a distinct band. Within each column, each individual is represented as either a "+" (to indicate that the band is present) or a "-" (indicating that the band is absent). RAPD bands are dominant genetic markers because heterozygotes cannot be discriminated from homozygotes that possess a certain band. While RAPDs are still used extensively, many studies have shown that the banding patterns are very sensitive to all components of the PCR reaction, including the quantity and quality of genomic DNA, the thermocycler employed, and the brand of Taq polymerase. Thus, reproducibility of RAPD patterns is somewhat problematic and different laboratories are unable to compare data sets.

A completely different approach to genotyping has been to clone microsatellite regions and then to design PCR primers in regions flanking the microsatellite. Microsatellites, such as dinucleotide repeat motifs, have a high mutation rate and are therefore usually highly polymorphic within populations. This technique is less efficient than RAPDs in that only a single locus is amplified in a single PCR reaction. It is also much more costly

due to the requirements for cloning, sequencing and synthesizing novel primers. On the other hand, it offers the advantages of high reproducibility and co-dominance of the bands, i.e. each homozygote and the heterozygote has a different banding pattern.

A third technique, called inter-simple sequence repeat (ISSR), is similar to RAPDs, but specifically targets highly variable microsatellite regions. This approach takes advantage of the fact that microsatellites are strewn with imperfections in their repeat motifs. For example, a series of AG's will now and then have AA instead of AG, or some other aberration. ISSR uses PCR primers designed to bind to microsatellites at specific aberrancies. Generally, these primers consist of a series of repeats with one or two non-repeat bases at the 5' or 3' end of the primer. The non-repeat, or so-called "anchor" bases are more often placed at the 3' end of the primer due to the greater sensitivity of Taq polymerase to mismatches at the 3' end, thereby leading to more specific and reproducible amplification. As with RAPDs, single primers are used in the reactions, so that the primer must bind to opposing DNA strands within several hundred bases in order to produce a fragment. Due to the fact that these primers amplify regions near or within "junk" DNA, they reveal very high levels of polymorphism, usually equal to or surpassing RAPDs. In addition, the primers are longer (18-22 bp) than those used in RAPDs and standard PCR conditions including higher annealing temperatures are used. These two factors are thought to contribute to the fact that ISSRs have proven significantly more reproducible than RAPDs. **They offer similarly attractive levels of sensitivity and price-performance as do RAPDs, and this is the method that we have applied to *Arenaria paludicola* and to *Rorippa gambelii*.**

C. *Arenaria paludicola*

Arenaria paludicola is presently known to occur as a wild population only in the *Sparganium* marsh at Oso Flaco Lake (OFL: See **Photo 167**). Approximately ten ramets from this site were brought into cultivation at UCSB. In addition, one ramet from the now extinct population at Black Lake (BLC) Canyon has been extensively vegetatively cloned at UCSB and at the Santa Barbara Botanic Garden since 1994. Due to the spreading vine-like growth of *A. paludicola*, the number of distinct genetic individuals from OFL was unknown at the time of collection. The goals of the present genetic investigation were to determine: (1) the efficacy of ISSR fingerprinting for detection of genetic variation in the marsh sandwort, (2) how many genotypes ("clones") are present among the cultivated ramets from OFC, and (3) whether the cloned ramet from BLC is distinct from all genotypes at OFC.

1. Materials and Methods

DNA Extraction

DNAs were extracted from individual *Arenaria* ramets using the DNeasy Plant Mini Kit® (Qiagen) according to the manufacturers instructions, as outlined below. From one to four growing shoot tips, as necessary to provide 0.1g of material, were harvested from each ramet. These tips included the shoot meristem and 1 to 3 whorls of developing to full sized leaves. The material from each ramet was deposited in a 2ml screw cap eppendorf tube (Quality Scientific Plastics), along with two 0.25-inch diameter sterile steel balls (they were sterilized by soaking them in a 10% bleach solution). The tubes were frozen in liquid nitrogen for 5-10 minutes. The frozen material was crushed by shaking in a bead-beater (Biospec Products Mini-Bead Beater) set at 7 rpm for 2 minutes. 400uLs of heated (65C) extraction buffer was immediately added to each tube, and the material dispersed into the buffer by vortexing for ~2 seconds. 4uLs of RNase A was then added to each tube, which was then incubated at 65C for 10-20 minutes. Next, 130uLs of AP2 (a Qiagen kit reagent) was added to each tube, and the steel balls were removed with bleach-sterilize weighing spatulas. The tubes were vortexed for ~ 2 seconds and put on ice for 5-10 minutes. The chilled cell suspensions were then transferred to and spun through QiaShredder columns in a micro centrifuge at full speed (~12,000G) for one minute. The shredder was discarded and the liquid above the cell debris pellet was moved to a new 1.5ml eppendorf tube. One half volume of AP3 (a Qiagen kit reagent) and one volume of 95% ethanol were then added to each tube. The contents of each tube were mixed by gentle vortexing. DNA was immobilized and cleaned by pipetting 650uLs of the contents of each tube onto QiaCleaner columns sitting in new 2ml tubes. The contents were spun through the membrane, leaving the DNA attached, by centrifuging at 8000G for one minute. The liquid in the tube below the column was discarded, and the remaining contents from the 1.5ml tube were added onto the same column. The centrifugation and liquid discard were repeated, then the DNA holding column was moved to a new 2ml tube. The DNA was cleaned by spinning 500uLs of AP Wash buffer (a Qiagen kit reagent) through each column twice. The first centrifugation was for one minute at 8000G, after which the liquid was discarded, while the second spin was for 2 minutes at full speed. The dry columns were then moved to new 1.5ml tubes, and 75uLs of 65C AE buffer were added to each column. The solution was allowed to sit in the column for 5 minutes, then was centrifuged through at 8000G for one minute. Lastly, the elution step was repeated with an additional 75uls of AE buffer (a Qiagen kit reagent).

The stock DNAs obtained by this method were then quantified using a Hoescht TKO fluorimeter. The fluorimeter was calibrated by adding 10uLs of a 10ng/uL stock solution of lambda DNA to 2mls of 1X Hoescht TKO dye, and setting the fluorimeter reading to 50 (the reading of 50 is half the actual quantity of DNA in the cuvet, which contains 100 ng of lambda DNA). From 1 to 5uls of each stock DNA were added to 2mls of Hoescht dye, and the fluorescence was recorded. The values were doubled to account for the calibration of the machine, then divided by the number of uLs used to give the actual DNA concentration in ng/uL. These stock DNAs were then diluted down to 50ng/uL by adding an appropriate volume of TE (10mM Tris-base, 1mM EDTA). Working 5ng/uL stocks of these DNAs were made by diluting an aliquot in double distilled H_2O. The remaining stock DNAs were kept frozen at -20C.

ISSR Amplification

The first task was to evaluate the effects of varying certain PCR parameters on the ISSR banding patterns. While most previous studies report high reproducibility of ISSR banding patterns, they also state that the patterns depend upon the PCR conditions employed. Because previous studies make differing recommendations concerning optimal PCR conditions, we set out to evaluate the efficiency and reproducibility of band generation under several conditions. For example, some workers have reported that smears of DNA, in which few discrete bands can be distinguished, were obtained unless they added 2% formamide. Formamide alters the annealing characteristics of DNA, thereby reducing secondary structure of the target DNAs and influencing the specificity of primer annealing.

We employed the following standard set of ISSR-PCR conditions in all experiments: 10mM Tris, 50mM KCl, 1.5mM MgCl, 200uM of each dNTP, 1.25 units of Taq polymerase, 1uM ISSR primer, in 50uL reaction volumes and a Hybaid Express thermocycler set to 2min denaturing step at 94C followed by cycles of 1min at 94C, 1min at 32C, 2.5min at 72C, finished with a 15min extension step. We compared results from the following alternatives: (1) 2% formamide versus no formamide, (2) 35 versus 45 cycles, (3) Taq polymerase from Fisher Biosciences versus Amplitaq Gold (automatic hotstart) polymerase from Perkin Elmer Applied Biosystems, and (4) 5, 10, 25 and 50ng of genomic DNA.

Electrophoresis

Detection and separation of ISSR bands presents a further set of alternative approaches. The most common combination has been detection *via* labeling of the PCR primers or

product with P32 and separation via vertical non-denaturing polyacrylamide gel electrophoresis (PAGE). This approach offers the advantages of the detection of extremely small amounts of DNA (i.e. bands with few copies) and precise discrimination of bands differing by only a few base-pairs in length. However, the disadvantages include the high cost of the materials and the dedicated equipment required for radioactive methods. The most commonly used alternative approach has been detection with ethidium bromide and separation on horizontal agarose gels. This approach is much less expensive, but offers significantly less sensitivity of band detection and precision of band separation.

We have compared three non-radioactive approaches: (1) ethidium bromide/agarose gels, (2) ethidium bromide/acrylamide gels, and (3) GelStar fluorescent stain/acrylamide gels. We used Sigma High Resolution agarose in a 15 x 15cm format horizontal gel containing 0.01mg/ml ethidium bromide run in 0.5X TBE at 100-120 volts for 3-4 hours. We compared the patterns obtained from these agarose gels to patterns seen on 20cm x 20cm 8% FMC Long Ranger (modified acrylamide) gels, which were 0.75 or 0.4mm thick, run vertically at 40-60 volts for 12-16 hours. **Recipes for gels and stains are provided in Appendix I.**

2. Results

PCR Conditions

We tested the impacts of varying PCR parameters using the primer GA2. When bands were detected using the ethidium bromide/PAGE approach, we did not detect any differences in the patterns produced when a single sample of DNA (Black Lake ramet No.1) was added at 10, 25 or 50 ng. However, on agarose gels, the quantity of PCR products appeared significantly less for reactions containing only 5 or 10ng of DNA. We therefore used 25 or 50ng for all subsequent PCRs. We also did not observe differences depending on the presence of formamide. We continued to use formamide in subsequent reactions.

In contrast to DNA concentration and formamide, we did observe differing GA2 banding patterns, using ethidium stained PAGE, depending upon the number of cycles and the polymerase used. By increasing from 35 to 45 cycles, 2 new small bands appeared. Similarly, Amplitaq Gold resulted in 3 new bands, one of which was seen with Fisher Taq only after 45 cycles. However, all the bands produced by Fisher Taq were present (usually with much greater intensity) in the Amplitaq products. Thus, despite some

differences, the patterns were quite similar across these differing conditions, suggesting a potential for high reproducibility. We used Fisher Taq in all subsequent PCRs.

Ethidium/PAGE gels offered attractive resolution of bands that ran together or created only a smear on agarose gels. However, many bands stained weakly with ethidium. For this reason, we next tested the fluorescent GelStar stain. The sensitivity of detection afforded by GelStar was far superior to that of ethidium in agarose or acrylamide applications, and tens of additional bands were seen on GelStar gels. In some cases, we observed that samples lacking a particular band on an agarose, in fact, contained a weak band on GelStar/PAGE. However, most bright bands that were both present and absent in multiple samples on agarose had concordant patterns on GelStar/PAGE.

In further tests of reproducibility, we obtained three separate DNA extracts from the Black Lake ramet, and compared separate amplifications of these extracts. While the banding patterns were always quite similar, they were not identical among the three extracts for the two primers tested, CA1 and GT2. Several small, and usually relatively weak, bands differed between extracts. This result suggests either stochastic processes during extraction and PCR or somaclonal genetic variation (somatic mutations that cause genetic differences between different samples of tissue from the same ramet). Fortunately, the bright, polymorphic bands that appeared to provide the most unambiguous genetic data, were consistent across the three extracts. In addition, other weak bands appeared or disappeared when separate PCRs of a single DNA extract were performed. In tallying polymorphic bands, we have therefore restricted ourselves to consideration of bright bands that were: (1) consistent in the Black Lake extracts, (2) consistent across two separate PCRs, and (3) consistent when 25 and 50ng of DNA were used.

Genetic Variation

Variation in banding patterns was observed for every primer, whether assayed by agarose or acrylamide electrophoresis. There were usually 2-3 polymorphic bands on agarose gels and many more when including light bands on GelStar/acrylamide gels. However, due to questions concerning reproducibility of some of these bands, we only scored bright, consistent bands according to the criteria listed above. One qualifying polymorphic band was detected for each of the 3 primers characterized at this level (**Table 12**).

Table 12. ISSR Primer Sequences and Band Summaries

Primer Name	Sequence	Annealing Temp. Used (Celsius)	Approx. Mean # of Bands	# of Reliable Polymorphic Bands
GA1	GAGAGAGAGAGAAY	32	15	N/A
GA2	GAGAGAGAGAGAYG	32	9	N/A
GT1	GTGTGTGTGTGTGTGTYC	50	N/A	N/A
GT2	GTGTGTGTGTGTGTGTYG	52	9	1
CA1	CACACACACACACACARG	52	11	1
CT1	CTCTCTCTCTCTKA	32	8	1

Degenerate bases are as follows: Y = C + T, R = A + G, K = G + T. "Mean # of bands" refers to those identified and counted on agarose gels; many more were seen via GelStar/PAGE.

Table 13 provides a matrix of presence/absence of these validated bands across ramets. This matrix indicates the following: (1) the sample from Black Lake was distinct from all Oso Flaco samples, and (2) the ramets from Oso Flaco display variation and, therefore, represent several genotypes. Data from these three reliable bands divided the OFL ramets into 4 genotypes; one genotype was represented by 4 ramets, another by 2 ramets, and the last two genotypes by a single ramet each.

Table 13. Genetic Composition at Validated Polymorphic Loci Among *A. paludicola* Ramets

Ramet	**GT2**	**CA1**	**CT1**	**Genotype**
Black Lake 1	-	-	-	1
Oso Flaco 1	+	+	-	2
Oso Flaco 2	+	+	+	3
Oso Flaco 3	+	-	-	4
Oso Flaco 4	+	-	-	4
Oso Flaco 5	+	-	-	4
Oso Flaco 6	+	-	-	4
Oso Flaco 7	+	+	-	2
Oso Flaco 8	-	+	-	5

3. Discussion

While the complex ISSR banding patterns that we were able to detect using the sensitive approach of acrylamide electrophoresis and GelStar staining were not 100% consistent across multiple DNA extracts from the same individual, they were largely consistent. By using a conservative band scoring algorithm, we feel that the ISSR approach can provide useful and reliable information on genetic variation in *Arenaria paludicola.*

Based upon variation in highly reproducible bands, we conclude that the populations from Black Lake and Oso Flaco differ genetically, and that variation is also represented among the Oso Flaco ramets under cultivation. Banding patterns from other primers and bands, that we did not include in the final data matrix because they were not validated by repeated analyses, suggest that each of the ramets collected from Oso Flaco may, in fact, be unique.

This information may prove valuable in further propagation and conservation efforts. For example, it will clearly be of importance to maintain clones of each genotype in cultivation. In addition, the variation within Oso Flaco suggests that these plants have undergone outbreeding in the recent past, and that controlled crosses among genotypes may aid in producing seeds with higher fitnesses than would selfing.

D. *Rorippa gambelii*

1. Background on *R. gambellii, R. nasturtiu- aquaticum* and putative hybrids.

The internal transcribed spacer (ITS) region of the nuclear ribosomal repeat has become the first choice for molecular systematic studies of closely related plant species and genera for several reasons. The ITS evolves extremely rapidly relative to most known protein coding genes, thus providing informative, variable characters for systematics of close relatives. This gene can also be easily amplified by the PCR using conserved primers because it is flanked by highly conserved ribosomal coding regions (Hillis and Dixon 1991). In addition, the thousands of tandem repeats that occur in each cell make the gene an efficient target for the PCR (Zimmer et al. 1988). If there was significant variation in sequences among the ITS repeats present within a single individual or cells, phylogenetic interpretation of the data would become difficult. However, in general, there appears to be little or no variation within individuals, and also little variation within species (Baldwin 1992). This is due to the process of concerted evolution in which

members of the same gene family are homogenized by unequal crossing over and gene-conversion (Arnheim 1983).

Concerted evolution appears to be quite effective in homogenizing gene sequences over evolutionary time (Arnheim 1983). However, when genomes carrying divergent ITS sequences are united, especially by hybridization, this variation does not immediately vanish. Of course, F1 hybrids are expected to carry alleles from both parents, and F2 individuals are expected to begin to loose this heterozygosity *via* segregation. This simple scenario is complicated by the multicopy nature of the ITS gene and by the process of concerted evolution. One early consequence of the multicopy gene structure is that sequences derived from both parents should be maintained in additional hybrid generations. That is, given that there are hundreds of thousands of loci, and sequences specific to each parent, segregation would require many hundreds of generations to randomly fix all loci in favor of one parent or the other.

For these reasons, ITS sequences can be used as molecular markers of hybridization, and offer the advantage of clearly demarcating even advanced hybrids provided that the putative parents differ in ITS sequences. Methods based on multilocus genotypes derived from numerous single-copy loci can also detect advanced hybrids, but require large sample sizes and considerable statistical inference. While most molecular systematic studies have employed direct sequencing of ITS PCR products, and have assumed homogeneity of ITS repeats within the individuals sampled, a handful of studies have considered intra-individual ITS sequence variation (Jobst *et al.* 1998, Suh *et al.* 1993), and have even used such data to detect hybridization (Quijada *et al.*, 1997).

Here, we test the putative hybrid status of a *Rorippa* population by examining ITS sequence variation.

2. Materials and Methods

DNA Extraction

Seeds were stratified at 55C for 7 days, then planted in potting soil 1-inch diameter (2-inch deep) wells in seedling trays. DNA was extracted from 6 week to 3 month old seedlings as follows. Whole plants were washed, then separate extractions were conducted using the top (mostly meristems and young leaves) and bottom (mostly roots and basipetal lateral meristems) portions of a single plant. Approximately 1mg of fresh tissue was deposited in a 2ml screw top eppendorf tube with 2 steel balls, and frozen in liquid nitrogen. The rest of the DNA extraction was performed as for *Arenaria paludicola* (above).

ITS PCR Amplification

The ITS region was amplified using a new plant-specific primer, "ITS 1P", located at the 3' end of the 18S ribosomal gene, together with the universal primer ITS4, located at the 5' end of the 28S gene. The primer ITS 1P is located in the identical position as the fungal specific primer ITS 1F, described in Gardes and Bruns (1993), but substitutes the fungal-specific bases for plant-specific alternatives.

PCR reactions contained final concentrations of 200 uM of each dNTP, 0.5uM of each primer, 1X Fisher buffer B, 25 mM MgCl, 0.5 units of Taq DNA polymerase (Fisher Biotech). Amplifications consisted of 40 cycles in a Hybaid Express thermocycler and employed a 2 minute initial denaturation at 95 C prior to thermocycling, followed by 40 cycles of: 45 second denaturation at 95 C, a 1 minute annealing step at 50 C, and 2 minute elongation at 72 C. The last cycle was followed by extension at 72 C for 10 min.

Amplified products were observed in 1% agarose minigels stained with ethidium bromide and recorded with a UVP CCD camera over a Fisher UV transilluminator.

ITS Sequences

An ITS amplicon from a Vandenberg Airforce Base individual of *Rorippa gambellii* was cleaned with the Qiagen Quick PCR kit. The cleaned product was quantified using a Hoescht TKO fluorimeter. The fluorimeter was calibrated by adding 10uLs of a 10ng/uL stock solution of lambda DNA to 2mls of 1X Hoescht TKO dye, and setting the fluorimeter reading to 50. The cleaned product was diluted to 15ng/uL, and 4uLs were used in a 20uL cycle-sequencing reaction employing the Applied Biosystems BigDye terminator kit, according to manufacturers instructions. The cycle-sequence product was purified by centrifugation at 800g through a Sephadex G50 minicolumn (Princeton Separations). 1uL of the purified DNA was loaded onto an Applied Biosystems 310 Genetic Analyzer capillary automated sequencer. The resulting ABI sequence files were corrected by hand in Sequence Navigator (a software program produced by Applied Biosystems). ITS sequences of related species were downloaded from GenBank, the international on-line database of genomic sequences, as listed below.

Taxon	Accession Numbers	Source, as listed in GenBank
Nasturtium microphyllum	AF078029, AF078030	not listed
Nasturtium officinale	AF078027, AF078028	not listed
Rorippa nasturtium-aquaticum	RN58RRN17	Japan
Rorippa nasturtium-aquaticum	X98645	Tyler
Rorippa nasturtium-aquaticum	X98643	Hurd's Summer
Rorippa nasturtium-aquaticum	X98642	Ballyogan
Rorippa nasturtium-aquaticum	X98641	American WS
Rorippa nasturtium-aquaticum	X98640	American_1
Rorippa palustris	AF100795, AF100796	not listed
Rorippa palustris	X98639.1	not listed
Rorippa amphibia	AF078025, AF078026	not listed
Rorippa sylvestris	AF078023, AF078024	not listed
Rorippa heterophylla	X98638.1	not listed
Cardamine microphylla	X98636.1	not listed
Cardamine flexuosa	X98634.1	not listed
Hilliella paradoxa	AF100827, AF100828	China?

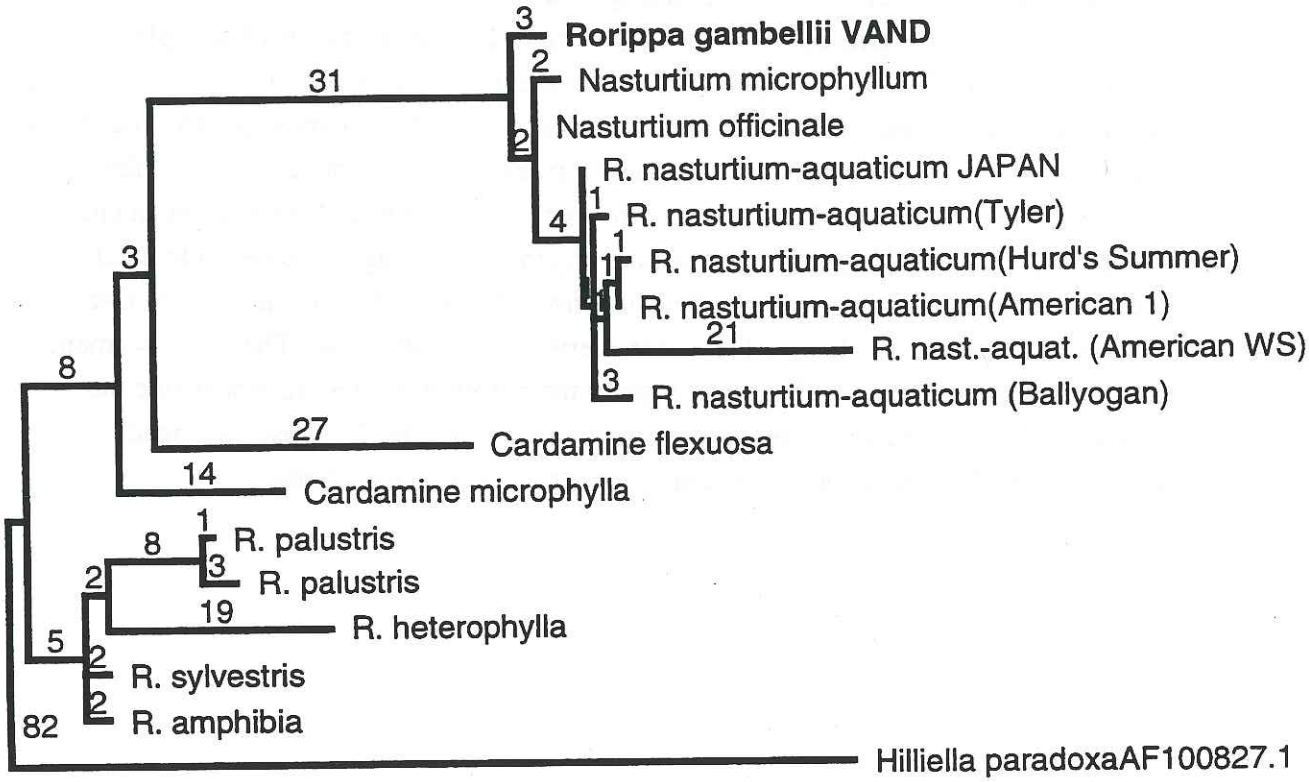
ITS RFLP Analysis

ITS amplicons were digested with the restriction enzyme Dde I as follows. A digestion cocktail containing (per reaction) 1uL of 10X Dde I buffer, 0.5uLs of the restriction enzyme and 8.5uLs of H2O were added to 20uL of each PCR product, vortexed, and maintained at 37C overnight. The digested PCR products were separated on a 2% NuSieve (FMC) agarose gel.

3. Results

The ITS sequence obtained from the Vandenberg *Rorippa gambellii* individual was quite similar to ITS sequences in GenBank obtained by other workers. The sequences of *R. gambellii* and related species displayed only moderate sequence divergence and were easily and unambiguously aligned by eye. Relationships among these taxa were estimated by performing an unweighted parsimony analysis in PAUP 3.1 (Swofford 1990), which employed 100 random addition heuristic searches under TBR branch swapping. The resulting gene-tree is shown in Fig. 1. Branch lengths (numbers of inferred nucleotide changes) are shown.

Figure 1. One of 135 most-parsimonious ITS gene-trees for *Rorippa* and relatives.

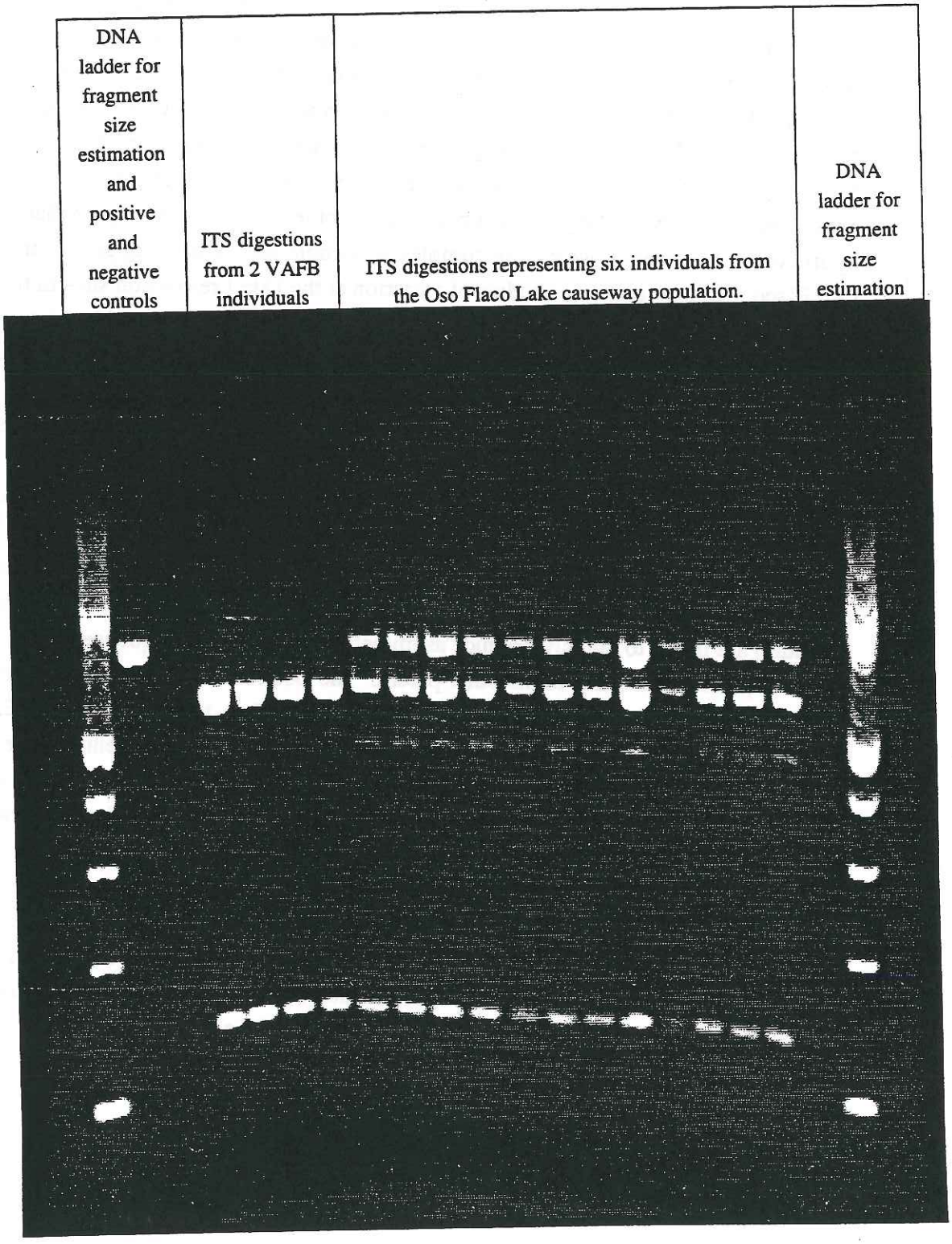


As expected, *R. gambellii* appears to be closely related to *R. nasturtium-aquaticum*, the other putative parent of the Oso Flaco individuals. However, considering the consensus sequence from the multiple *R. nasturtium-aquaticum* sequences (the low level of apparent variation within this taxon could be due to sequencing error), these two taxa are separated by 4 base transitions/transversions and 1 single base insertion/deletion. We mapped restriction sites in the ITS sequences of *R. nasturtium-aquaticum* (consensus) and *R. gambellii* using the software program DNA Strider 1.1, and found that two of the four base differences occurred in Dde I restriction sites. *R. gambellii* had only a single Dde I site, occurring at approximately base 120, while R. *nasturtium-aquaticum* lacked this site, but contained a Dde I site at approximately base 515. Since the entire ITS amplicon was about 800 bases, *R. gambellii* was predicted to produce Dde I ITS restriction fragments of 680 and 120 bases, while R. *nasturtium-aquaticum* was predicted to produce fragments of 515 and 285 bases.

We amplified and digested the ITS from separate root and shoot DNA extracts from two Vandenberg and 6 Oso Flaco individuals. The Dde I restriction fragment patterns obtained from these amplicons are shown in **Figure 2**. The restriction fragments seen in

both root and shoot amplicons from Vandenberg agreed with predictions based upon the ITS sequence we obtained. All 6 individuals from Oso Flaco, including root and shoot amplifications, had identical restriction fragment patterns that were much more complex. Instead of the two fragments seen in Vandenberg *R. gambellii*, the Oso Flaco plants displayed 4 clear ITS Dde I fragments. This result indicates the presence of multiple ITS sequences within these individuals, since the total sizes of the 4 bands sum to about twice the total size of the ITS amplicon. The largest fragment was identical in size to the undigested ITS amplicon, demonstrating the existence of some ITS sequences lacking any Dde I sites, in these plants. The second and the fourth fragments were identical to the two fragments seen in *R. gambellii,* showing that some ITS sequences in the Oso Flaco plants had the single Dde I site characteristic of *R. gambellii.* The third fragment was about 500 base pairs, which may suggest the presence of ITS sequences like those predicted for *R. nasturtium-aquaticum.* However, the smaller 285 base pair band predicted for *R. nasturtium-aquaticum* was not evident in these plants.

Figure 2. The Dde I restriction fragment patterns obtained from the amplified ITS gene representing root and shoot extracts from two VAFB individuals of Rorippa gambelii and from six individuals of putative hybrids from the Oso Flaco Lake causeway population.



Discussion

The surviving *Rorippa* population at Oso Flaco has a number of morphological characters that are intermediate between those of *R. gambellii* and *R. nasturtium-aquaticum* (summarized in **Table 3, above**). We have found here that the Oso Flaco plants contain multiple ITS sequences within individuals, based on analysis of mapped, polymorphic restriction sites. In contrast, the morphologically "pure" *R. gambellii* individuals from Vandenberg sampled here did not display sequence polymorphism at the same restriction sites. Thus, the ITS data strongly supports the hypothesis that the Oso Flaco plants are of hybrid origin. However, we cannot completely account for the banding pattern in the Oso Flaco plants, at present. Studies of variation at the Dde I restriction sites in the ITS regions of local *R. nasturtium-aquaticum*, or other putative parents, would assist in interpreting the Oso Flaco RFLP patterns. However, one relatively simple hypothesis can be suggested. The brightness of the ITS restriction fragments on the gel should be a function of two parameters. First, brightness decreases in smaller fragments because smaller fragments bind less ethidium bromide than an equivalent number of larger fragments. Second, the number of any particular restriction fragment depends directly on the number of ITS repeats in the individual that have the sequence producing that fragment. An F1 hybrid should have equal numbers of the ITS sequences corresponding to each parent. However, in succeeding generations, backcrossing and or concerted evolution are likely to quickly produce asymmetries such that the sequence of one parent is over-represented relative to the other parent. The Oso Flaco restriction fragments that are identical to *R. gambellii* fragments are quite bright. Thus, the *R. gambellii* ITS sequence may be predominant in these plants. The 500 base pair fragment is quite dim in the Oso Flaco plants. Thus, the 285 base pair fragment that is also expected from an *R. nasturtium-aquaticum* background, may be invisible due to its smaller size and lower representation of this sequence in these plants. Finally, the uncut fragment (and corresponding sequence) is abundant, but less abundant than the *R. gambellii*-like bands. Recombination between ITS sequences containing the two Dde I sites would be expected to produce a fraction of sequences lacking both restriction sites. Thus, the Oso Flaco patterns could have been produced by hybridization between *R. nasturtium-aquaticum* and *R. gambellii*, followed by backcrossing to *R. gambellii* and subsequent recombination.

VII. Considerations and Recommendations for Future Restoration Efforts: Possible sites for the reintroduction of *Arenaria paludicola* and *Rorippa gambelii*

To date, our introduction of populations of *A. paludicola* at Black Lake Canyon are sufficiently promising to warrant continued reintroduction of *A. paludicola* at this site. Black Lake does not appear to be a viable site for the long-term survival of transplanted *A. paludicola*; it is not clear whether the poor survivorship of the ramets we planted at Black Lake was due to poor water quality, to fluctuations in water level, or to other biotic or abiotic factors not studied here.

The Vandenberg Air Force Base (Main Gate) population of *R. gambelii* is vigorous. It should be noted, however, that the population of *R. gambelii* that was viable in Black Lake Canyon as recently as 1995 was much larger (N ~ 400 ramets) than the population at Vandenberg Air Force Base. Given that the Black Lake Canyon population of *R. gambelii* appears to have become extinct in the last few years, the fate of the small population at Vandenberg Air Force Base is of great concern. We would suggest that a small sample of seeds be collected from plants at this site and then raised in the greenhouse for reintroduction to this site. This would avoid the introduction of alien genotypes and allow us to monitor the fate of introduced seedlings. If this method is successful, future efforts could concentrate on increasing the size (and total seed production) of this population. This prospective reintroduction would depend upon the availability of this site from the Vandenberg Air Force Base administration.

A second promising site of potential reintroduction of *R. gambelii* is the Oso Flaco Lake site of the extant population of *A. paludicola* (described above). Here, the question would be one of which seed source to use for this reintroduction. The population of *R. gambelii* that is geographically the most proximate is the putative hybrid between *R. gambelii* and *R. nasturtium-aquaticum* described here. Whether it would be preferable to use seeds or ramets from this apparent hybrid population instead of seeds or ramets from the "purer" population of *R. gambelii* from Vandenberg Air Force Base (Main Gate) is an issue that should be discussed and resolved among botanists at the California State Department of Fish and Game and the U.S. Department of Fish and Wildlife. This prospective reintroduction would depend upon the availability of this site from administrators responsible for Oso Flaco Lake State Park

VIII. Literature Cited

Al-Shehbaz I. A., and R. A. Price. 1998. Delimitation of the genus *Nasturtium* (Brassicaceae). Novon 8: 124 – 126.

Arnheim, N. 1983. Concerted evolution of multigene families. Pages 38-61 in R. K. Koehn and M. Nei, eds. *Evoltuion of Genes and Proteins*. Sinauer Associates, Sunderland.

Baldwin, B. G. 1992. Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: an example from the Compositae. *Molecular Phylogenetics and Evolution* 1: 3-16.

Gardes, M., and T. D. Bruns. 1993. ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. *Mol. Ecol.* 2: 113-118.

Hickman, J. C. 1993. The Jepson manual: higher plants of California. University of California Press, Berkeley.

Hillis, D. M., and M. T. Dixon. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. *Quart. Rev. Biol.* 66: 411-446.

Jobst, J., K. King, and V. Hemleben. 1998. Molecular evolution of the internal transcribed spacers (ITS1 and ITS2) and phylogenetic relationships among species of the family Cucurbitaceae. *Molecular Phylogenetics and Evolution* 9: 204-219.

Munz, P. A. 1974. A California Flora. University of California Press, Berkeley.

Quijada, A., A. Liston, W. Robinson, and E. Alvarez-Buylla. 1997. The ribosomal ITS region as a marker to detect hybridization in pines. Molecular Ecology, 6: 995-996.

Suh, Y., L. B. Thien, H. E. Reeve, and E. A. Zimmer. 1993. Molecular Evolution and Phylogenetic Implications of Internal Transcribed Spacer Sequences of Ribosomal Dna in Winteraceae. *American Journal of Botany* 80: 1042-1055.

Swofford, D. L. 1990. PAUP: Phylogenetic Analysis Using Parsimony. Illinois Natural History Survey, Champaign.

Zimmer, E. A., E. R. Jupe, and V. Walbot. 1988. Ribosomal gene structure, variation and inheritance in maize and its ancestors. *Genetics* 120: 1125-1136.

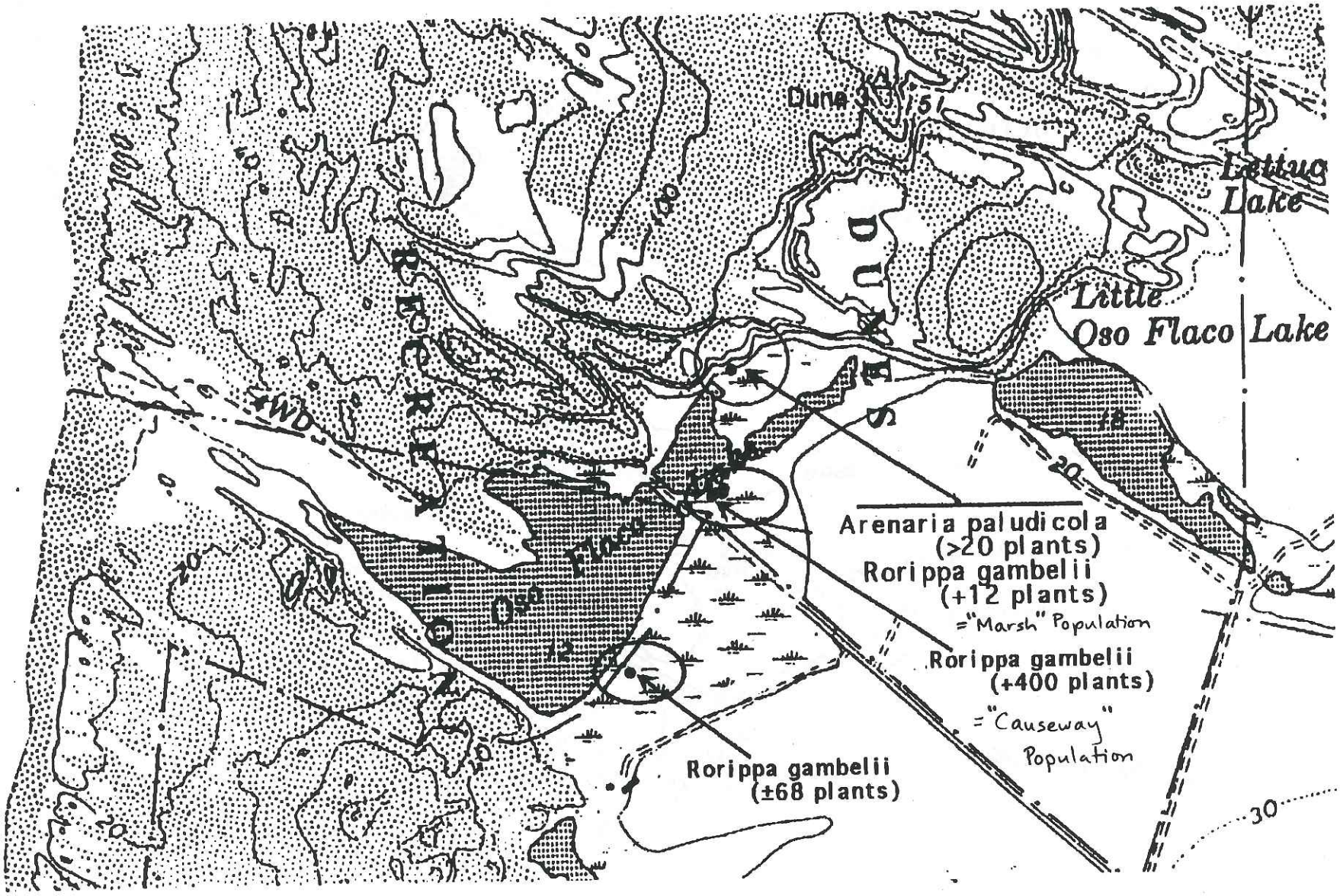
IX. Maps

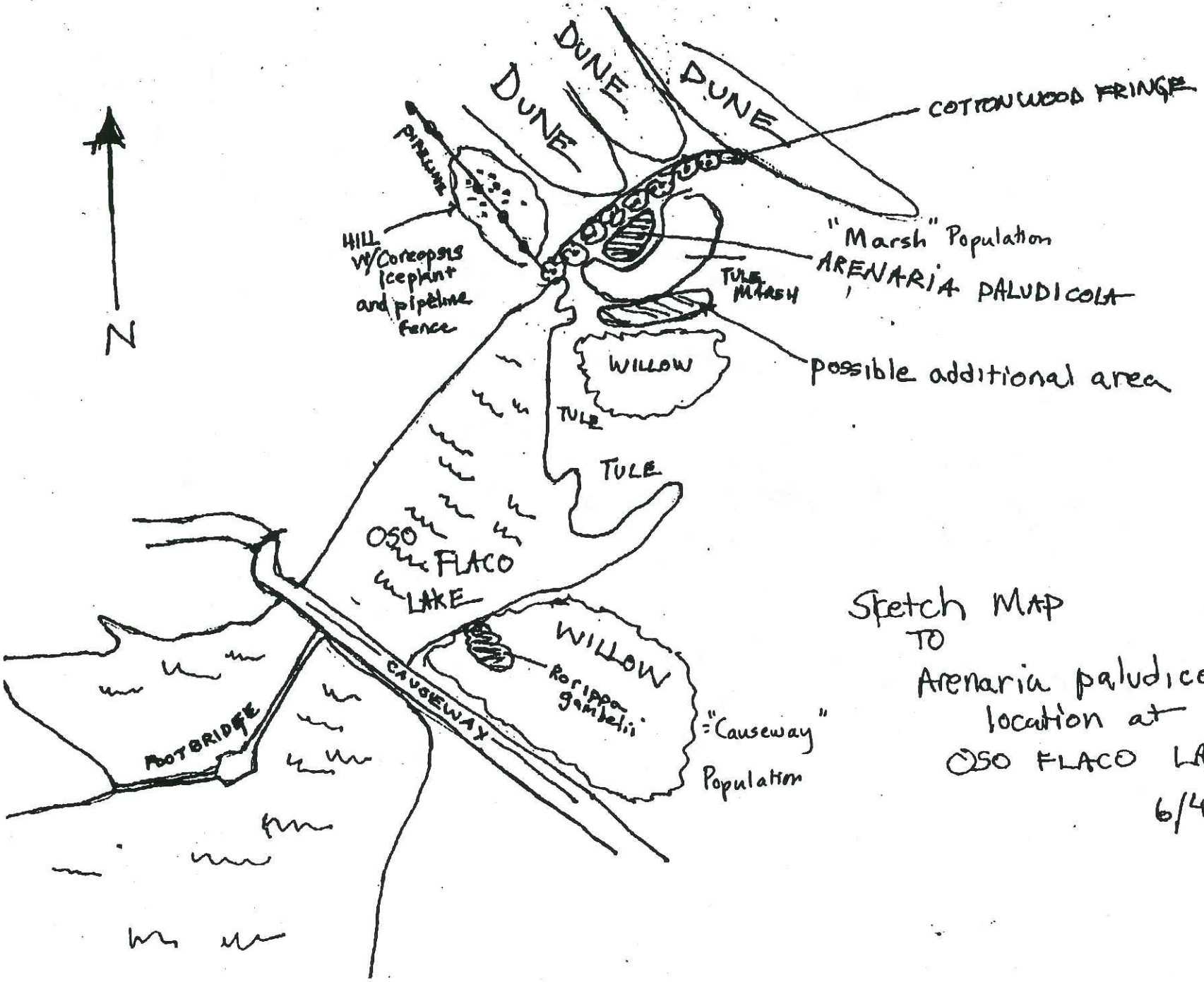
Map 1. Oso Flaco Lake: locations of *Arenaria paludicola* and *Rorippa gambelii*, indicating the "marsh" and the "causeway" populations..... 64

Map 2. Sketch by John Chesnut of *Arenaria paludicola* and *Rorippa gambelii* locations at Oso Flaco Lake: note the "marsh" and the "causeway" populations.,,,,,,,,,, 65

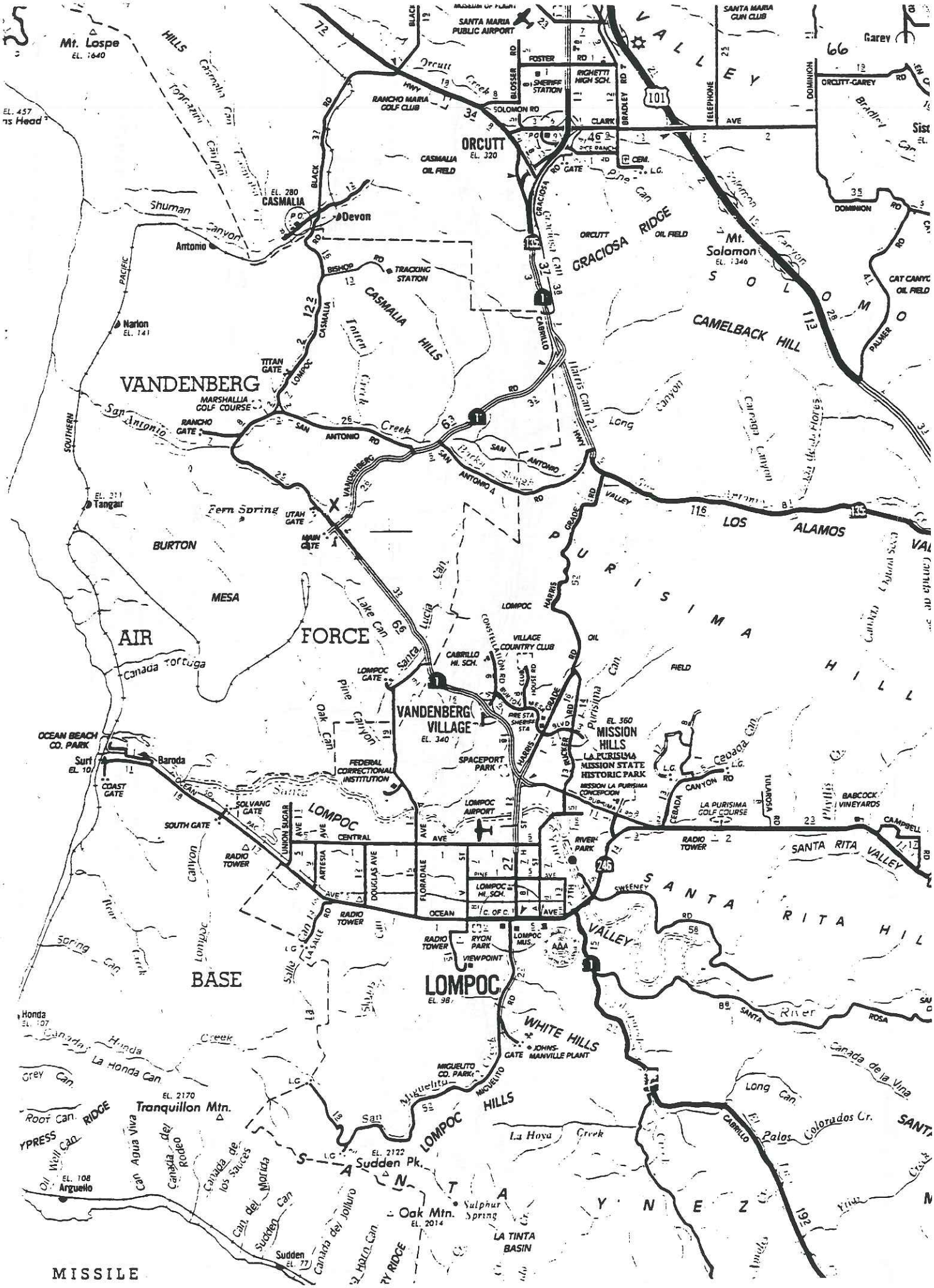
Map 3. Location of Vandenberg Air Force Base (Main Gate) population of *Rorippa gambelii*..... 66

Map 4. Location of Black Lake Canyon Land Conservancy site of the introduction of *Arenaria paludicola*..... 67

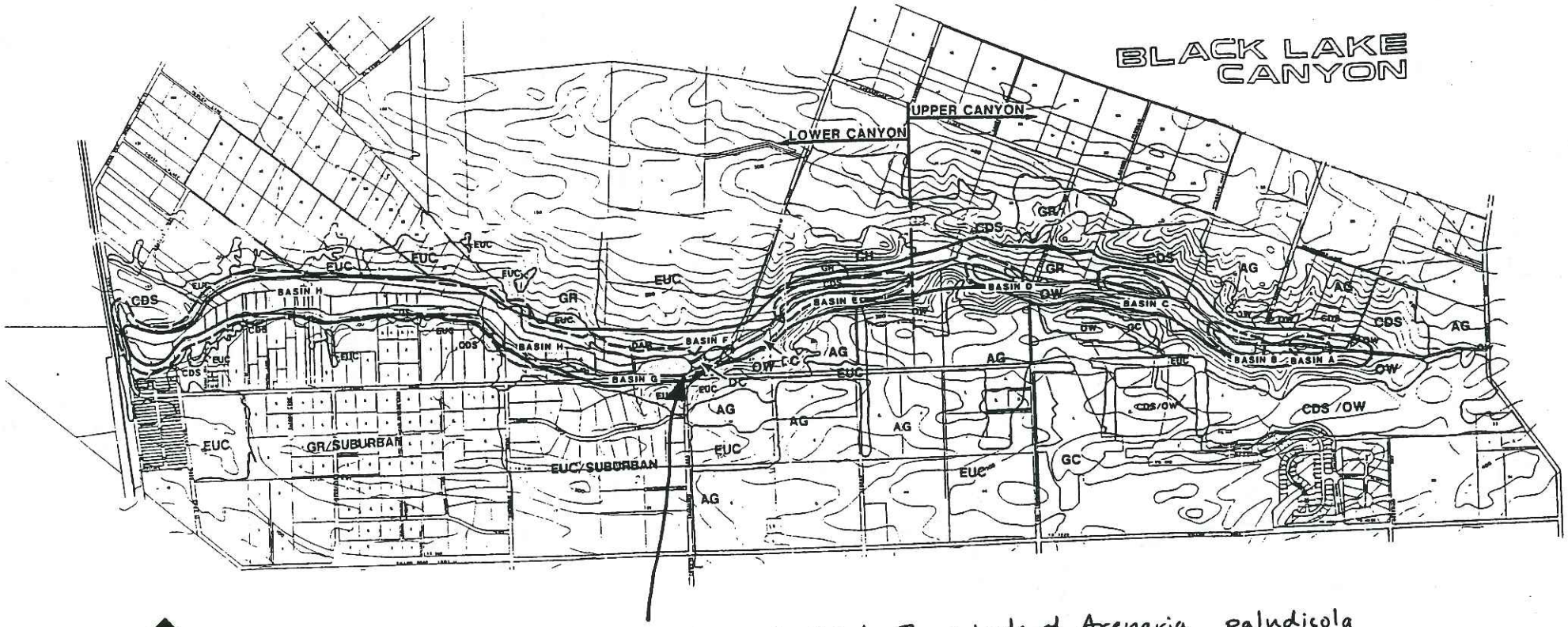

Dune
Lettuce Lake
Little Oso Flaco Lake
Arenaria paludicola
(>20 plants)
Rorippa gambelii
(+12 plants)
="Marsh" Population
Rorippa gambelii
(+400 plants)
="Causeway" Population
Rorippa gambelii
(±68 plants)
20
30



Sketch MAP
TO
Arenaria paludicola
location at
OSO FLACO LAKE
6/4/98



Mt. Lospe
EL. 1640
HILLS
Casmalia Can
Tognazini Canyon
EL. 457
ns Head
SANTA MARIA PUBLIC AIRPORT
SANTA MARIA GUN CLUB
VALLEY
Garey
66
FOSTER RD
BLOSSER RD
SHERIFF STATION
RIGHETTI HIGH SCH.
BRADLEY RD
TELEPHONE
DOMINION
ORCUTT-GAREY RD
Orcutt Creek
RANCHO MARIA GOLF CLUB
SOLOMON RD
CLARK
101
ORCUTT
EL. 320
CASMALIA OIL FIELD
CEM.
L.G.
GATE
Pine Can
EL. 280
CASMALIA
Shuman Canyon
Devon
Antonio
BLACK RD
GRACIOSA RD
ORCUTT OIL FIELD
GRACIOSA RIDGE
Mt. Solomon
EL. 1346
SOLOMON
CAT CANYON OIL FIELD
PALMER RD
BISHOP RD
TRACKING STATION
CASMALIA HILLS
Totten Creek
CABRILLO
CAMELBACK HILL
Narlon
EL. 141
PACIFIC
TITAN GATE
LOMPOC
VANDENBERG
MARSHALLIA GOLF COURSE
RANCHO GATE
San Antonio Creek
SAN ANTONIO RD
Harris Can
Long Canyon
Careaga Canyon
SOUTHERN
EL. 211
Tangair
Fern Spring
UTAH GATE
MAIN GATE
VANDENBERG
VALLEY
LOS ALAMOS
BURTON
MESA
PURISIMA HILL
Lake Can
Santa Lucia Can
LOMPOC
HARRIS GRADE RD
AIR FORCE
Canada Tortuga
VILLAGE COUNTRY CLUB
CABRILLO HI. SCH.
CONSTELLATION RD
OIL FIELD
LOMPOC GATE
Pine Canyon
Oak Can
VANDENBERG VILLAGE
EL. 340
FIRE STA SHERIFF STA
Purisima Can
EL. 360
MISSION HILLS
LA PURISIMA MISSION STATE HISTORIC PARK
MISSION LA PURISIMA CONCEPCION
OCEAN BEACH CO. PARK
Surf
EL. 10
Baroda
COAST GATE
FEDERAL CORRECTIONAL INSTITUTION
SPACEPORT PARK
Cebada Can
CANYON RD
TULAROSA RD
BABCOCK VINEYARDS
SOLVANG GATE
SOUTH GATE
LOMPOC
LOMPOC AIRPORT
LA PURISIMA GOLF COURSE
CAMPBELL RD
RADIO TOWER
UNION SUGAR
CENTRAL AVE
RIVER PARK
SANTA RITA VALLEY
ARTESIA AVE
DOUGLAS AVE
FLORADALE AVE
246
SWEENEY RD
SANTA RITA HILL
LOMPOC HI. SCH.
OCEAN
C. OF C.
RADIO TOWER
RYON PARK
LOMPOC MUS.
VALLEY
Spring Can
BASE
VIEWPOINT
LOMPOC
EL. 98
WHITE HILLS
SANTA ROSA
River
Honda
EL. 107
Canada Honda Creek
La Honda Can
JOHNS-MANVILLE PLANT
GATE
Canada de la Vina
MIGUELITO CO. PARK
MIGUELITO
Long Can
EL. 2170
Tranquillon Mtn.
LOMPOC HILLS
La Hoya Creek
CABRILLO
Colorados Cr.
Roof Can
RIDGE
YPRESS
Oil Well Can
Can Agua Viva
Canada del Rodeo
Canada de los Sauces
Can del Morida
EL. 2122
Sudden Pk.
SANTA YNEZ
EL. 108
Arguello
Sudden Can
Sulphur Spring
Oak Mtn.
EL. 2014
LA TINTA BASIN
Canada del Jolluro
Sudden
EL. 77
MISSILE



Location of Trial Transplants of *Arenaria paludicola*

N·O·R·T·H

LEGEND

- ▭ = WETLAND BASINS A–H
- CDS = COASTAL DUNE SCRUB
- OW = OAK WOODLAND
- CH = CHAPARRAL
- GR = GRASSLAND
- GC = GOLF COURSE
- EUC = EUCALYPTUS WOODLAND
- AG = AGRICULTURE
- SUBURBAN = SCATTERED HOMES
- (·····) DC = DRAINAGE CHANNEL CONNECTING BASINS
- ——— EXISTING SRA BOUNDARY
- — — PROPOSED PROJECT BOUNDARY

BIOLOGICAL CONSTRAINTS

(from McClelland 1988)

IX. Publications & Documents

Copy of Ihsad Al-Shehbaz' revision of *Nasturtium*, including *Rorippa gambelii*..... 69

Copy of letter from Dr. Anuja Parikh to Dr. Ihsan Al-Shebaz regarding *Rorippa* populations and the request for species confirmation for enclosed plant material and voucher specimens..... 72

Copy of Al-Shehbaz' e-mail message in response to our request for confirmation of our identification of *Rorippa gambelii* from Oso Flaco Lake and from Vandenberg Air Force Base (Main Gate) populations..... 73

Copy of Al-Shehbaz' e-mail message suggesting future directions of investigation of the Oso Flaco and VAFB populations of *Rorippa gambelii*..... 75

Delimitation of the Genus *Nasturtium* (Brassicaceae)

Ihsan A. Al-Shehbaz
Missouri Botanical Garden, P.O. Box 299, St. Louis, Missouri 63166-0299, U.S.A.

Robert A. Price
Department of Botany, University of Georgia, Athens, Georgia 30602, U.S.A.

ABSTRACT. *Nasturtium*, which is often reduced to synonymy of *Rorippa*, is recognized as a distinct genus of five species. It is more closely related to *Cardamine* than to *Rorippa*, and its distinguishing characters from these genera are given. The new combination *N. floridanum* is proposed, and a key to the species is provided.

The generic status and limits of *Nasturtium* R. Brown have been the subject of considerable controversy. In some of the recent accounts (e.g., Czerepanov, 1995; Hedge, 1968; Jonsell, 1993; Mabberley, 1997; Stuckey, 1972; Wannenmacher, 1986) the genus has been maintained, whereas in others (e.g., Al-Shehbaz, 1988; Al-Shehbaz & Rollins, 1988; Green, 1962; Jonsell, 1988; Rich, 1991; Rollins, 1993) it is reduced to synonymy of *Rorippa* Scopoli. Schulz (1936) also united the two genera, but he adopted *Nasturtium* for the combined genus, instead of the earlier-published *Rorippa*.

The basic disagreement among these treatments is whether or not the morphological differences between *Nasturtium* and *Rorippa* are sufficient to clearly distinguish the genera. There are numerous other examples of generic pairs in the Brassicaceae with similar controversial boundaries. Because of convergence in almost every conceivable character, emphasis on a small number of morphological characters can often result in artificial generic groupings within the family. The use of molecular data, along with critical evaluation of morphology, can often help resolve conflicts between competing hypotheses about the limits and relationships of genera of the Brassicaceae (Al-Shehbaz & Warwick, 1997; O'Kane & Al-Shehbaz, 1997). A case in point is the delimitation of *Nasturtium* and *Rorippa*.

Sequence comparisons of chloroplast DNA consistently support the separation of *Nasturtium* as a genus very distinct from *Rorippa*, and indicate that *Nasturtium* is most closely related to the cosmopolitan genus *Cardamine* L. This result was obtained by Les (1994), who compared sequences of the gene *rbc*L for six species in the cardaminoid group of Brassicaceae and found relatively strong support for a grouping of *N. officinale* R. Brown with *C. pensylvanica* Muhlenberg ex Willdenow, while two species of *Rorippa*, *R. sylvestris* (L.) Besser (the generic type) and *R. amphibia* (L.) Besser, formed a separate clade more closely related to lakecress (*Neobeckia aquatica* (Eaton) E. L. Greene) and horseradish (*Armoracia rusticana* P. Gaertner). Recent comparisons of the more rapidly changing chloroplast gene *ndh*F and the *trn*L-F intron and spacer regions (Price & Sweeney, in prep.) indicate that the endangered species *N. gambellii* (S. Watson) O. E. Schulz forms a well supported clade with *N. officinale* and that the genus *Nasturtium* is much more closely related to *Cardamine* than to other genera in the cardaminoid group, including *Armoracia* Gaertner et al., *Rorippa*, and *Barbarea* R. Brown.

As presently delimited, *Nasturtium* is readily distinguished from *Rorippa* by a combination of characters, including a perennial, almost always aquatic habit, hollow stems rooting at the submersed and lower nodes, pinnate emergent leaves with 1–9(–15) pairs of lateral leaflets that are never decurrent on the rachis, white flowers, curved, cylindric fruits, and reticulate seed coats, and by the absence of median nectar glands. Species of *Rorippa* are annuals or perennials of wet or mesic areas and almost always have taproots and only very rarely (e.g., *R. amphibia* and *R. fluvitalis* (E. Meyer ex Sonder) Thellung, both of which are yellow-flowered aquatics with simple leaves) root at the lowermost nodes. They have solid or rarely hollow stems, yellow or occasionally white flowers, nearly always median nectar glands, and colliculate, minutely rugose, papillose, verrucose, or reticulate seeds. The leaves of *Rorippa* range from entire to toothed, sinuate, or pinnatisect, and rarely form leafletlike lobes. When leafletlike lobes are present, they are always decurrent on the rachis, and the uppermost leaves are often simple. *Nasturtium* plants growing in water almost always produce simple leaves on submersed stems, but emergent

NOVON 8: 124–126. 1998.

shoots always produce compound leaves (Michaelis, 1976; Rollins, 1978). The fruits of *Rorippa* range from globose to ovoid, oblong, clavate, or cylindric.

Cardamine is readily distinguished from *Nasturtium* by its unique fruits that dehisce explosively, by its spirally coiled valves that lack a distinct midvein, and by its flattened replum. In *Nasturtium* the fruits do not dehisce explosively, the valves have a distinct midvein and do not coil after dehiscence, and the replum is rounded.

Nasturtium as circumscribed here includes five species: *N. officinale* (the type species, which is the watercress of commerce) and *N. microphyllum* Boenninghausen ex Reichenbach (both of which are native to Eurasia and northern Africa and widely naturalized elsewhere), the Moroccan *N. africanum* Braun-Blanquet, and the North American *N. gambellii* (California, Mexico), and *N. floridanum* (Al-Shehbaz & Rollins) Al-Shehbaz & Price (Florida). A new combination for the last is herein proposed.

Nasturtium valdes-bermejoi Castroviejo, which was described from Spain (Castroviejo, 1986), appears to be a minor variant of *N. microphyllum*. It was not recognized in the revised account of the genus for *Flora Europaea* (Valentine, 1993), and its alleged differences from *N. microphyllum* clearly fall within the variation range of that species.

Further study is needed of several morphologically anomalous species that appear to fit better into *Rorippa* than into *Nasturtium*. The recently discovered New Caledonian *Rorippa neocaledonica* Jonsell (Jonsell, 1995, 1997) is an annual with decurrent leafletlike lobes, yellow flowers, and verruculose seeds, and it does not produce adventitious roots at the lower nodes. We have not seen the type or other material of the species, and we believe that the species is a good member of *Rorippa*. It appears to be related to *R. sarmentosa* (DC.) J. F. Macbride of the Pacific Islands (Jonsell, 1997; Smith, 1981; Wagner et al., 1990). Though *R. sarmentosa* has compound leaves and is sometimes perennial with adventitious roots at the lower nodes, it clearly differs from *Nasturtium* in being a mesic rather than aquatic plant, and in having solid stems, median nectaries, yellow flowers, and papillose seeds (Jonsell, 1997). The Madagascar endemic *R. laurentii* Jonsell (Jonsell, 1979) has white flowers and pinnatisect leaves, but its erect stems that do not root at the lower nodes, broadly flattened fruits, median nectar glands, and ridged seeds clearly exclude it from *Nasturtium*.

Key to the Species of *Nasturtium*

1a. Seeds biseriate in each locule, coarsely reticulate, with 25–50(–60) areolae on each side; mature fruit (1.8–)2–3 mm wide . . . . . *N. officinale*

1b. Seeds uniseriate in each locule, moderately to minutely reticulate, with more than 100 areolae on each side; mature fruit 0.8–1.2(–1.8) mm wide.

2a. Emergent leaves not auriculate at base, 3- or very rarely 5-foliolate; seeds yellowish brown; style obsolete; plants endemic to Florida . . . . . *N. floridanum*

2b. Emergent leaves often minutely auriculate at the petiole base, (3 or)5–15-foliolate; seeds reddish brown; style distinct; plants of other parts of the world.

3a. Fruit abruptly ending in a style to 1 mm; leaflets entire to repand; seeds with 100–150(–175) areolae on each side . . . . . *N. microphyllum*

3b. Fruit attenuate into a slender style 1.5–2.5 mm; leaflets often coarsely dentate, rarely subsinuate-repand; seeds with 300–450 areolae on each side.

4a. Fruits 0.8–1(–1.5) mm wide; seeds not mucilaginous when wetted; plants of California and Mexico . . . . . *N. gambellii*

4b. Fruits 1.5–1.8 mm wide; seeds mucilaginous when wetted; plants of Morocco . . . . . *N. africanum*

Nasturtium floridanum (Al-Shehbaz & Rollins) Al-Shehbaz & Price, comb. nov. Basionym: *Rorippa floridana* Al-Shehbaz & Rollins, J. Arnold Arbor. 69: 68. 1988. *Cardamine curvisiliqua* Shuttleworth ex Chapman, Fl. South. U.S. 605. 1887; not *Rorippa curvisiliqua* (W. J. Hooker) Bessey ex Britton, Mem. Torrey Bot. Club 5: 169. 1894; not *Nasturtium curvisiliquum* (W. J. Hooker) Nuttall ex Torrey & A. Gray, Fl. N. Amer. 1: 73. 1838. *Nasturtium stylosum* Shuttleworth ex O. E. Schulz, in Engler & Prantl, Nat. Pflanzenfam., ed. 2, 17B: 553. 1936; not *N. stylosum* (DC.) O. E. Schulz ex Cheesman, Trans. & Proc. New Zealand Inst. 43: 179. 1911; not *Rorippa stylosa* (DC.) Allan, Fl. New Zealand 1: 188. 1961; not *R. stylosa* (Persoon) Mansfield & Rothmaler, Repert. Spec. Nov. Regni Veg. 49: 276. 1940. TYPE: U.S.A. Florida: "in uliginosis subsalsis ad fluv. St. Marks, prope St. Marks, April–May 1843," *Rugel s.n.* (lectotype, designated by Al-Shehbaz & Rollins (1988); isolectotype, GH).

Rorippa floridana was proposed by Al-Shehbaz and Rollins (1988) as a new name for the Florida endemic *Cardamine curvisiliqua* Shuttleworth ex Chapman, because the transfer of the latter name to *Rorippa* would have created a later homonym of *R. curvisiliqua* (W. J. Hooker) Bessey ex Britton, a

species restricted to the western United States (Alaska south into Wyoming and Montana to California). The transfer of the epithet *curvisiliqua* Shuttleworth ex Chapman to *Nasturtium* would also create a later homonym of *N. curvisiliqua* (W. J. Hooker) Nuttall ex Torrey & A. Gray. This species was also known as *N. stylosum* Shuttleworth ex O. E. Schulz, but this name is also a later homonym of the New Zealand endemic *N. stylosum* (DC.) O. E. Schulz ex Cheesman.

Literature Cited

Al-Shehbaz, I. A. 1988. The genera of Arabideae (Cruciferae; Brassicaceae) in the southeastern United States. J. Arnold Arbor. 69: 85–166.

——— & R. C. Rollins. 1988. A reconsideration of *Cardamine curvisiliqua* and *C. gambellii* as species of *Rorippa* (Cruciferae). J. Arnold Arbor. 69: 65–71.

——— & S. I. Warwick. 1997. The generic disposition of *Quidproquo confusum* and *Sinapis aucheri* (Brassicaceae). Novon 7: 219–220.

Castroviejo, S. 1986. *Nasturtium valdes-bermejoi*, sp. nov., de la Provincia de Huelva. Anales Jard. Bot. Madrid 43: 15–19.

Czerepanov, S. K. 1995. Vascular Plants of Russia and Adjacent States (the Former USSR). Cambridge Univ. Press, Cambridge.

Green, P. S. 1962. Watercress in the New World. Rhodora 64: 32–43.

Hedge, I. 1968. Arabideae. *In:* K. H. Rechinger (editor), Fl. Iranica 57: 193–218. Akademische Druck-u. Verlagsanstalt, Graz.

Jonsell, B. 1979. New taxa of Cruciferae from East Tropical Africa and Madagascar. Bot. Not. 132: 521–535.

———. 1988. Cruciferae. *In:* C. G. G. J. van Steenis (editor), Fl. Malesiana, ser. 1, 10: 541–560. Kluwer Academic Publishers, Dordrecht.

———. 1993. *Rorippa*. *In:* T. G. Tutin et al. (editors), Flora Europaea, ed. 2, 1: 344–345. Cambridge Univ. Press, Cambridge.

———. 1995. Une espèce nouvelle de *Rorippa* (Cruciferae) de Nouvelle-Calédonie. Bull. Mus. Natl. Hist. Nat., Paris, ser. 4, 17: 175–177.

———. 1997. Cruciferae (Brassicaceae). *In:* P. Morat (editor), Fl. Nouvelle-Calédonie 21: 81–117. Muséum national d'Histoire Naturelle, Paris.

Les, D. H. 1994. Molecular systematics and taxonomy of lake cress (*Neobeckia aquatica;* Brassicaceae), an imperiled aquatic mustard. Aquatic Bot. 49: 149–165.

Mabberley, D. J. 1997. The Plant-book, ed. 2. Cambridge Univ. Press, Cambridge.

Michaelis, F. B. 1976. Watercress (*Nasturtium microphyllum* (Boenn.) Rchb. and *N. officinale*) in New Zealand. Aquatic Bot. 2: 317–325.

O'Kane, S. L., Jr. & I. A. Al-Shehbaz. 1997. A synopsis of *Arabidopsis* (Brassicaceae). Novon 7: 323–327.

Rich, T. C. G. 1991. Crucifers of Great Britain and Ireland. Bot. Soc. British Isles Handbook 6: 1–336.

Rollins, R. C. 1978. Watercress in Florida. Rhodora 80: 147–153.

———. 1993. The Cruciferae of Continental North America. Stanford Univ. Press, Stanford.

Schulz, O. E. 1936. Cruciferae. *In:* A. Engler & K. Prantl (editors), Nat. Pflanzenfam., ed. 2, 17B: 227–658. Verlag von Wilhelm Engelmann, Leipzig.

Smith, A. C. 1981. Brassicaceae. Flora Vitiensis Nova 2: 703–711. Lawai, Hawaii.

Stuckey, R. L. 1972. Taxonomy and distribution of the genus *Rorippa* (Cruciferae) in North America. Sida 4: 279–430.

Valentine, D. H. 1993. *Nasturtium*. Pp. 345–346 *in* T. G. Tutin et al. (editors), Flora Europaea, ed. 2. Cambridge Univ. Press, Cambridge.

Wagner, W. L., D. R. Herbst & S. H. Sohmer. 1990. Brassicaceae. Pp. 398–414 *in* Manual of the Flowering Plants of Hawai'i, vol. 1. Univ. Hawaii Press & Bishop Museum Press, Honolulu.

Wannenmacher, R. 1986. Cruciferae. *In:* G. Hegi (editor), Illustrierte Fl. Mittel-europa 4(1): 73–514. Verlag Paul Parey, Berlin.

October 2, 1998

Dr. Ihsan Al-Shehbaz
Missouri Botanical Garden
P.O. Box 299
St. Louis, MO 63166-0299

Dear Dr. Al-Shehbaz:

Thank you very much for your reply; I look forward to receiving the Novon manuscript. The problem of identifying our specimens now is becoming very interesting. You are welcome to keep everything I sent you in the previous mail; I apologize for the poor material, but I did not have the seed packets I am sending you now. With this letter, I am sending you the following plant material for ID/confirmation/chromosome counts. Please return all this material (vouchers and seed packets), after extracting from it as much as you may need. You requested more plant material from the envelope 3 Vandenberg AFB (VAFB) population, and I am not sure whether you needed dried material or fresh. If you need fresh material, I cannot obtain it until next year; it is too late in the season. I had one more question. You mentioned biseriate refers to "the arrangement of seeds in one locule **or** on one side of the septum." The material in envelopes 1 and 4 from Oso Flaco Lake (OFL) shows the seeds were somewhat offset (not exactly in one row) in the locule, and were attached on 2 sides of the septum. Does that make them biseriate?

Enclosures

Voucher specimens, labelled 1-5 in pencil at the top right corner of the newspaper.

1. VAFB envelope 3 population, 9/6/98, collected by Parikh and Gale. Population relatively healthy.
2. Little Oso Flaco Lake population, 8/6/94, collected by Parikh and Gale. We saw these plants again in September, but this population now is completely gone, likely due to dredging in the drainage ditch where it was found.
3, 4. Black Lake Canyon population, 5/3/88, collected by Ferren *et al.*, ID by Bob Price. This population was relatively large through 1995, but has completely disappeared since then.
5. A photocopy of a 5/2/66 voucher (I cannot borrow it) of *Nasturtium officinale* which was annotated as *Cardamine gambellii* (the pencil mark above the label does not show up clearly), and then re-identified as *R. nasturtium-aquaticum*. This copy will not really help you with the current ID problem, but I had a copy, so I am sending it to you for your records.

Eight envelopes of seeds and fruit walls (plant #s are in circles in the top right corner), which will provide you much better material than I sent you earlier. Susan Mazer had these envelopes and she will use them for propagation, so she would like this material back (she would like to ask you about your propagation methods for *Nasturtium gambellii*, so I will give her your email address). The OFL envelopes are better samples of envelope 1 and the two vouchers previously sent you from the OFL population. The VAFB envelopes are from the envelope 3 population and current voucher 1. If you confirm that this is *N. gambellii*, it may be the only currently known extant population of this species.

I thought *N. microphyllum* was an eastern species? It is not reported in the California flora.

Once again, thank you very much for your time, and I look forward to hearing from you.

Regards,

Anuja Parikh

Anuja Parikh
Tel/Fax: 805-564-1352, email: ConsultFLx@aol.com

From: "Dr. Ihsan Al-Shehbaz" <ial-shehbaz@lehmann.mobot.org>
Organization: Missouri Botanical Garden
To: consultFLx@aol.com, mazer@lifesci.lscf.ucsb.edu
Date: Tue, 6 Oct 1998 17:14:21 CST6CDT
Subject: Nasturtium gambellii
Priority: normal

Dear Drs. Mazer and Parikh:

I have received the second evelope from you with the eight envelopes of seed and fruit samples. I will return them tomorrow morning.

Here are the results.

Seed and fruit envelopes:
Samples 17 and 22 , both from main gate VAFB, are DEFINITELY Nasturtium gambellii in all characters.

Samples 7 and 13, both from OSO Falco Lake, are NOT N. gambellii. I suspect that they are hybrids between N. officinale and N. gambellii. The seeds and fruit characters are intermediate between the two species.

Herbarium specimens:

1, from VAFP, is DEFINITELY N. gambellii
#2, from Oso Falco Lake, is intermediate between N. gambellii and N. officinale. In general features of the leaflets and styles it resembles N. gambellii, but the presence of auricles, larger seeds with large areolae, and wider fruits, it resembles N. officinale. You need to concentrate more on this population, do chromosome counts, critically study the morphology, etc. I will be glad to provide further when you study the populations further, and if you want to collaborate with me it is fine. If you want to udertake the work on your own, I will be absolutely supportive all the time. So please, do not hesitate to count on me at any stage.

#3 and #4, difficult to identify because of the lack of fruits and seeds.

#5 most likely N. officinale, but if you have the chance, do examine the seeds and follow the key I wrote in the Novon paper.

One last note on biseriate vs. uniseriate: if you remove one fruit valve, you will see the seeds arranged either in one row (N. gambellii), perfectly two rows (N. officinale), or in imperfect rows (plants in envelpes 7 and 13.

Nasturtium microphyllum is an introduced weed, and it is appearing in many new states as new records. I do not think that any of your plants belong to that species.

If I can be of any further help, please let me know.

All the best.
Ihsan

Ihsan Al-Shehbaz
Project co-director
Flora of China
Missouri Botanical Garden
P.O. Box 299
St. Louis, Missouri 63166-0299
U.S.A.
e-mail address: ial-shehbaz@lehmann.mobot.org
Tel.: (314)577-9556
Fax: (314)577-9438

From: "Dr. Ihsan Al-Shehbaz" <ial-shehbaz@lehmann.mobot.org>
Organization: Missouri Botanical Garden
To: "Susan J. Mazer" <mazer@lifesci.lscf.ucsb.edu>
Date: Wed, 7 Oct 1998 09:43:16 CST6CDT
Subject: Re: Nasturtium!
Priority: normal

Dear Susan:

I am really thrilled to know that you have done such a great job on Nasturtium. I think that this is one of the most fascinating biological problems becasue it deals with a native and introduced species, with their putative hybrids well established and on their own. There are so many facets that need to be looked at, and I am sure that you will cover all of them. It would be great to have somebody do the chromosome counts of N. gambellii and the hybrids, as well as check the seed viability, pollen stainability, and introgression. As for the seeds, please make sure to take SEM photos of random samples of each populations and do actual measurments for the size range of areolae (reticulations) on the same seed side throughout. This, along with seed weight, seeds length and width, and seed shape can provide a wealth of information on the identity of the taxa involved. Your observation on the leaflet margin is accurate, and that needs to be studied in some detail for the two species and the hybrids. Finally, measurments of style length, fruit width, presence of bracts, and development of petiolar auricles can all help.

I thank you for keeping me posted, and if there is anything that I can do to help, please let me know. I have already mailed your samples to Dr. Parikh.

All the very best.
Ihsan
Ihsan Al-Shehbaz
Project co-director
Flora of China
Missouri Botanical Garden
P.O. Box 299
St. Louis, Missouri 63166-0299
U.S.A.
e-mail address: ial-shehbaz@lehmann.mobot.org
Tel.: (314)577-9556
Fax: (314)577-9438

XI. Appendix I: Protocols and Methods used for DNA fingerprinting

IA. Introduction to Techniques

Introduction to the Polymerase Chain Reaction: documents obtained from the PCR Jumpstation website (http://www.horizonpress.com/gateway/pcr.html#pcrt)

Agarose Gel Electrophoresis: Extract from Molecular Cloning: a laboratory manual, 1989, Second Edition, by J. Sambrook, E. F. Fritsch and T. Maniatis (eds.), Cold Spring Harbor Laboratory Press.

Polyacrylimide Gel Electrophoresis: Extract from Molecular Cloning: a laboratory manual, 1989, Second Edition, by J. Sambrook, E. F. Fritsch and T. Maniatis (eds.), Cold Spring Harbor Laboratory Press.

IB. Several protocols specific to this report

Optimizing PCR with AmpliTaq Gold DNA Polymerase

GelStar Nucleic Acid Gel Stain Instructions

Use of Long Ranger gel solution for producing polyacrylimide gels

~~~ What The Heck is PCR? ~~~

Polymerase chain reaction (PCR) is a technique which is used to amplify the number of copies of a specific region of DNA, in order to produce enough DNA to be adequately tested. This technique can be used to identify with a very high-probability, disease-causing viruses and/or bacteria, a deceased person, or a criminal suspect.
In order to use PCR, one must already know the exact sequences which flank (lie on either side of) both ends of a given region of interest in DNA (may be a gene or any sequence). One need *not* know the DNA sequence in-between. The building-block sequences (nucleotide sequences) of many of the genes and flanking regions of genes of many different organisms are known. We also know that *the DNA of different organisms is different* (while some genes may be the same, or very similar among organisms, there will *always* be genes whose DNA sequences differ among different organisms - otherwise, would be the *same* organism (e.g., same virus, same bacterium, an identical twin; therefore, **by identifying the genes which are different, and therefore unique**, one can use this information to identify an organism).

A gene's building-block sequence is the *precise* order of appearance, one after the other, of 4 different components (deoxyribonucleotides) within a stretch of DNA (deoxyribonucleic acid). The 4 components are: Adenine, Thymidine, Cytosine and Guanine, abbreviated as: A, T, C and G, respectively (a 4-letter alphabet). The arrangement of the letters (one after the other) of this 4-letter alphabet generates a "sentence" (a gene sequence). The number of letters in the sentence may be relatively few, or relatively many, depending on the gene. If the sentence is 1000 letters-long, the sequence would be said to be 1 kilobase (1000 bases).
As an example:
ATATCGGGTTAACCCCGGTATGTACGCTA would represent part of one gene. DNA is double-stranded (except in some viruses), and the two strands pair with one another in a very precise way. **EACH** letter in a strand will pair with only one kind of letter across from it in the opposing strand: **A ALWAYS pairs with T; and, C ALWAYS pairs with G across the two strands.**
So:

TTAACGGGGCCCTTTAAA.....TTTAAACCCGGGTTT

Would pair with:

AATTGCCCCGGGAAATTT.....AAATTTGGGCCCAAA

Now, let's say that the above sequences "flank" (are on either end of..) the gene, which includes a long stretch of letters designated as:
.....
These are known, absolutely identified to be, the sequence of letters which ONLY flank a particular region of a particular organism's DNA, and NO OTHER ORGANISM'S DNA. This region would be a target sequence for PCR.

The first step for PCR would be to synthesize "primers" of about 20 letters-long, using each of the 4 letters, and a machine which can link the letters together in the order desired - this step is easily done, by adding one letter-at-a-time to the machine (DNA synthesizer). In this example, the primers we wish to make will be exactly the same as the flanking sequences shown above. We make ONE primer exactly like the lower left-hand sequence, and ONE primer exactly like the upper right-hand sequence, to generate:

TTAACGGGGCCCTTTAAA.....TTTAAACCCGGGTTT
AATTGCCCCGGGAAATTT.....>
and:
<.....*TTTAAACCCGGGTTT*
AATTGCCCCGGGAAATTT.....AAATTTGGGCCCAAA

Now. the ..... may be a very long set of letters in-between; doesn't matter. If you look at this arrangement, you can see that if the lower left-hand primer sequence (*italics*) paired to the upper strand could be extended to the right in the direction of the arrow, and the upper right-hand sequence paired to the lower strand could be extended to the left in the direction of the arrow (remembering that the ..... also represent letters, and opposite pairing will ALWAYS be A to T and C to G), one could successfully exactly duplicate the original gene's **entire sequence**. Now there would be four strands, where originally there were only two. If one leaves everything in there, and repeats the procedure, now there will be eight strands, do again - now 16, etc.. therefore, about 20 cycles will theoretically produce approximately one-million copies of the original sequences (2 raised to the 20th power).

Thus, with this amplification potential, **there is enough DNA in one-tenth of one-millionth of a liter (0.1 microliter) of human saliva (contains a small number of shed epithelial cells), to use the PCR system to identify a genetic sequence as having come from a human being!** Consequently, only a **very** tiny amount of an organism's DNA need be available originally. Enough DNA is present in an insect trapped within 80 million year-old amber (fossilized pine resin) to amplify by this technique! Scientists have used primers which represent present-day insect's DNA, to do these amplifications.

Here is how PCR is performed:
First step: unknown DNA is heated, which causes the paired strands to separate (single strands now accessible to primers).
Second step: add large excess of primers relative to the amount of DNA being amplified, and cool the reaction mixture to allow double-strands to form again (because of the large excess of primers, the two strands will always bind to the primers, instead of with each other).
Third step: to a mixture of all 4 individual letters (deoxyribonucleotides), add an enzyme which can "read" the opposing strand's "sentence" and extend the primer's "sentence" by "hooking" letters together in the order in which they pair across from one another - A:T and C:G. This particular enzyme is called a DNA polymerase (because makes DNA polymers). One such enzyme used in PCR is called *Taq*

polymerase (originally isolated from a bacterium that can live in hot springs - therefore, can withstand the high temperature necessary for DNA-strand separation, and can be left in the reaction). Now, we have the enzyme synthesizing new DNA in opposite directions - BUT ONLY THIS PARTICULAR REGION OF DNA.

After one cycle, add more primers, add 4-letter mixture, and repeat the cycle. The primers will bind to the "old" sequences as well as to the newly-synthesized sequences. The enzyme will again extend primer sentences ... Finally, there will be PLENTY of DNA - and ALL OF IT will be copies of just this particular region. Therefore, by using different primers which represent flanking regions of different genes of various organisms in SEPARATE experiments, one can determine if in fact, any DNA has been amplified. If it has not, then the primers did not bind to the DNA of the sample, and it is therefore highly unlikely that the DNA of an organism which a given set of primers represents, is present. On the other hand, appearance of DNA by PCR will allow precise identification of the source of the amplified material.

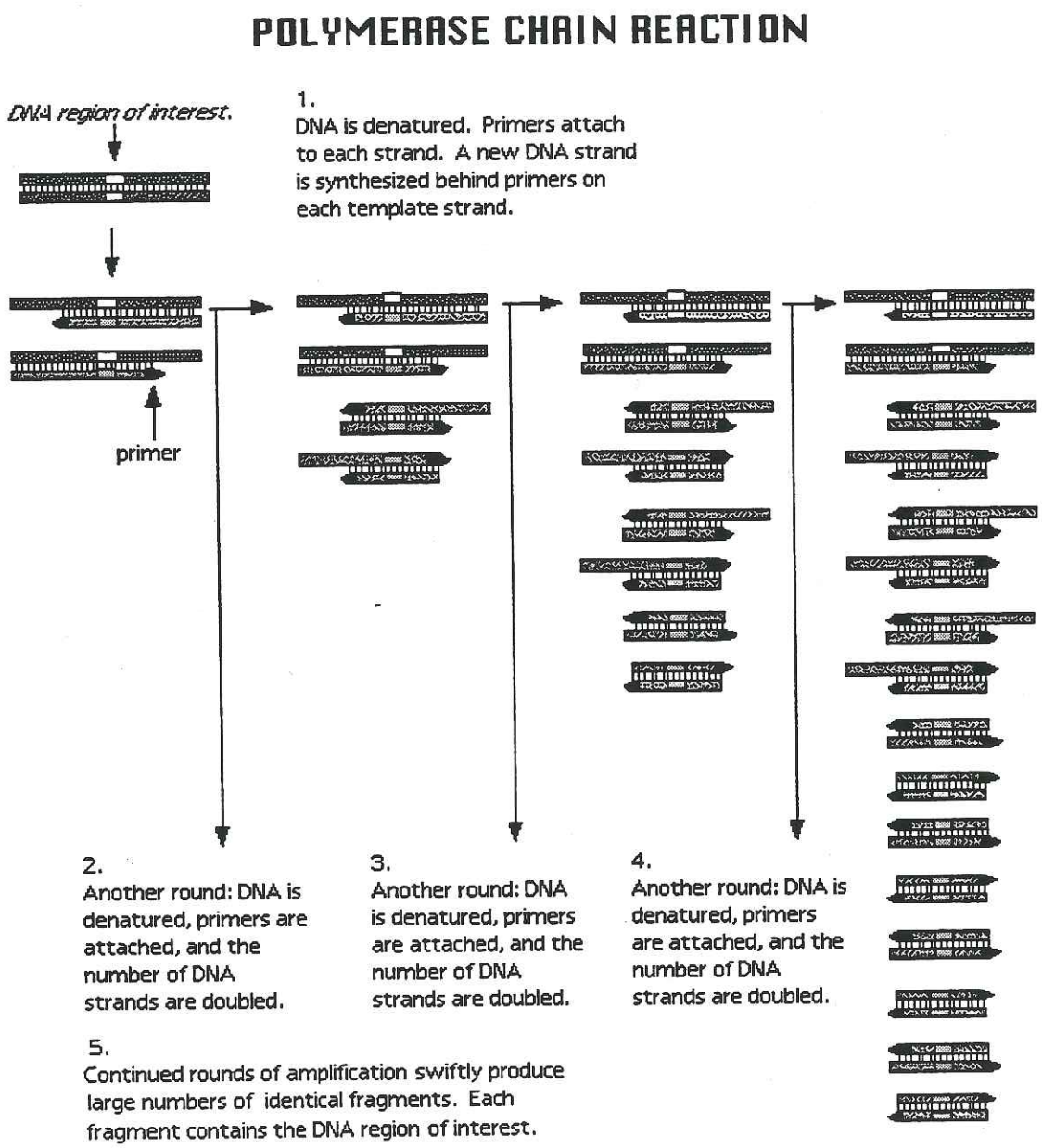
Copyright John C. Brown, 1995

[Top of Page | What the Heck?? | "Bugs" | My HomePage | KU Microbiology]

ACCESS EXCELLENCE
About Biotech

Graphics Gallery

Polymerase Chain Reaction (PCR)



Copyright 1999 Access Excellence @ the National Health Museum. All rights reserved.

Polymerase Chain Reaction (PCR)

The elegant technique of PCR, by which fragments of DNA can be made to replicate very rapidly, is illustrated.

Figure Legend:

Polymerase chain reaction (PCR), is a common method of creating copies of specific fragments of DNA. PCR rapidly amplifies a single DNA molecule into many billions of molecules.

Polymerase Chain Reaction - Xeroxing DNA

National Center for Human Genome Research, National Institutes of Health. "New Tools for Tomorrow's Health Research." Bethesda, MD: Department of Health and Human Services, 1992.

Copyright 1999 Access Excellence @ the National Health Museum. All rights reserved.

Who would have thought a bacterium hanging out in a hot spring in Yellowstone National Park would spark a revolutionary new laboratory technique? The polymerase chain reaction, now widely used in research laboratories and doctor's offices, relies on the ability of DNA-copying enzymes to remain stable at high temperatures. No problem for Thermus aquaticus, the sultry bacterium from Yellowstone that now helps scientists produce millions of copies of a single DNA segment in a matter of hours.

In nature, most organisms copy their DNA in the same way. The PCR mimics this process, only it does it in a test tube. When any cell divides, enzymes called polymerases make a copy of all the DNA in each chromosome. The first step in this process is to "unzip" the two DNA chains of the double helix. As the two strands separate, DNA polymerase makes a copy using each strand as a template.

The four nucleotide bases, the building blocks of every piece of DNA, are represented by the letters A, C, G, and T, which stand for their chemical names: adenine, cytosine, guanine, and thymine. The A on one strand always pairs with the T on the other, whereas C always pairs with G. The two strands are said to be complementary to each other.

To copy DNA, polymerase requires two other components: a supply of the four nucleotide bases and something called a primer. DNA polymerases, whether from humans, bacteria, or viruses, cannot copy a chain of DNA without a short sequence of nucleotides to "prime" the process, or get it started. So the cell has another enzyme called a primase that actually makes the first few nucleotides of the copy. This stretch of DNA is called a primer. Once the primer is made, the polymerase can take over making the rest of the new chain.

A PCR vial contains all the necessary components for DNA duplication: a piece of DNA, large quantities of the four nucleotides, large quantities of the primer sequence, and DNA polymerase. The polymerase is the Taq polymerase, named for Thermus aquaticus, from which it was isolated.

The three parts of the polymerase chain reaction are carried out in the same vial, but at different temperatures. The first part of the process separates the two DNA chains in the double helix. This is done simply by heating the vial to 90-95 degrees centigrade (about 165 degrees Fahrenheit) for 30 seconds.

But the primers cannot bind to the DNA strands at such a high temperature, so the vial is cooled to 55 decrees C (about 100 degrees F). At this temperature, the primers bind or "anneal" to the ends of the DNA strands. This takes about 20 seconds.

The final step of the reaction is to make a complete copy of the templates. Since the Taq polymerase works best at around 75 degrees C (the temperature of the hot springs where the bacterium was discovered), the temperature of the vial is raised.

The Taq polymerase begins adding nucleotides to the primer and eventually makes a complementary copy of the template. If the template contains an A nucleotide, the enzyme adds on a T nucleotide to the primer. If the template contains a G, it adds a C to the new chain, and so on to the end of the DNA strand. This completes one PCR cycle.

The three steps in the polymerase chain reaction - the separation of the strands, annealing the primer to the template, and the synthesis of new strands - take less than two minutes. Each is carried out in the same vial. At the end of a cycle, each piece of DNA in the vial has been duplicated.

But the cycle can be repeated 30 or more times. Each newly synthesized DNA piece can act as a new template, so after 30 cycles, 1 billion copies of a single piece of DNA can be produced! Taking into account the time it takes to change the temperature of the reaction vial, 1 million copies can be ready in about three hours.

PCR is valuable to researchers because it allows them to multiply unique regions of DNA so they can be detected in large genomes. Researchers in the Human Genome Project are using PCR to look for markers in cloned DNA segments and to order DNA fragments in libraries.

Go to next story: Recombination Up Close

See Graphics Gallery: Polymerase Chain Reaction

See Pioneer Profiles: Kary B. Mullis

PCR Technology

Connie Veilleux

Introduction

Polymerase chain reaction (PCR) has rapidly become one of the most widely used techniques in molecular biology and for good reason: it is a rapid, inexpensive and simple means of producing relatively large numbers of copies of DNA molecules from minute quantities of source DNA material--even when the source DNA is of relatively poor quality.

PCR involves preparation of the sample, the master mix and the primers, followed by detection and analysis of the reaction products. These steps are discussed below.

Sample Preparation

PCR is very versatile. Many types of samples can be analyzed for nucleic acids. Most PCR uses DNA as a target, rather than RNA, because of the stability of the DNA molecule and the ease with which DNA can be isolated. By following a few basic rules, problems can be avoided in the preparation of DNA for the PCR. The essential criteria for any DNA sample are that it contain at least one intact DNA strand encompassing the region to be amplified and that any impurities are sufficiently diluted so as not to inhibit the polymerization step of the PCR reaction.

Although any protocol is acceptable for PCR purposes, it is often best to use the fewest steps possible in DNA preparation in order to prevent accidental contamination with unwanted DNA. Usually a 1:5 dilution of the sample with water is sufficient to dilute out any impurities which may result from the purifying protocol.

The simplest method of isolating DNA from cells is as follows:

1. Cells can be obtained by using a toothpick to scrape under the fingernails, swabbing the inside of the mouth or from the roots of plucked hairs. Regardless of source, cells are resuspended in 20 ul of water. Skip to step four.

2. If you are using cells suspended in media, centrifuge at 1200- 1500Xg for 5 minutes. Resuspend the cell pellet in 1 ml of phosphate buffered saline (PBS) and repellet by spinning at 1200- 1500Xg for 5 minutes. Repeat. These PBS washes remove medium, and its inhibitory factors, from the surface of the cells. After the last wash resuspend the cell pellet in 20 ul of distilled water. Be aware that too much cell debris can inhibit the PCR reaction. If this happens, it may be necessary to further dilute the DNA sample. Go to step four.

3. For bacterial samples take a toothpick and scrape the teeth, or swab the throat, ears or between the toes. Resuspend material in 500ul of water. Freeze and thaw sample three times with vigorous shaking or vortexing between repetitions to break the bacterial cell wall. Although not all DNA will be released from the cells, there will be a sufficient quantity for PCR. Go to step four.

4. Place the sample in a 95oC heating block, or in boiling water, for 5 minutes. This step inactivates the DNase molecules that are found in the sample preparation. If left intact, DNase could clip the desired DNA template molecule into fragments which would be unsuitable for PCR. If there is very little DNA in the sample preparation, the DNA can be concentrated by ethanol precipitation. The sample is now ready for PCR.

DNA samples for PCR--regardless of preparation method--are generally run in duplicate in order to provide a control for the relative quality and purity of the original sample. Adding a small amount of DNA to the control just after the master mix step allows the detection of anything in the completed sample prep which would inhibit the PCR reaction.

Preparation of Master Mix

The Master Mix contains all of the components necessary to make new strands of DNA in the PCR process. The Master Mix reagents include:

Final Conc.	Component	Purpose
	Water	
1X	Buffer	keeps the master mix at the proper pH so the PCR reaction will take place.
200uM	Deoxynu-cleotides	provide both the energy and nucleosides for the synthesis of DNA. It is important to add equal amounts of each nucleotide (dATP, dTTP, dCTP, dGTP) to the master mix to prevent mismatches of bases.
0.2-1.0uM	Primers	Short pieces of DNA (20-30 bases) that bind to the

		DNA template allowing Taq DNA polymerase enzyme to initiate incorporation of the deoxynucleotides. Both specific and universal primers can be used.
2.5U/100ul	AmpliTaq polymerase	A heat stable enzyme that adds the deoxynucleotides to the DNA template.
0.05-1.0ug	Template DNA	The DNA which will be amplified by the PCR reaction.

Notes on the Master Mix

The Master mix buffer is often stored as a 10X stock solution (100 mM Tris-HCL, pH 8.3, 500 mM KCL, 1.5 mM MgCl2) which is diluted to 1X for use. Both the Master mix buffer and the purified water can be stored at room temperature. Store deoxynucleotides, primers and Taq DNA polymerase enzyme at -20oC.

Although 100ul of master mix per reaction is generally used, it is possible to use as little as 25 or 50ul to save on cost of reagents. Regardless of the total volume, be certain to keep the final concentrations of reagents constant.

Master mix reagents can be optained from a variety of companies. Often the initial concentration of the reagent will differ depending on which company produced it. It is easy to figure out how much stock reagent to use by following a simple formula:

```
(initial concentration) X ( volume needed ) =
                                (final concentration) X (volume of sample)
```

For example: I have 10X buffer, 10 mM of each nucleotide, 0.5 mM primers and Taq DNA polymerase at 5 Units/ul. I want to make one 50 ul reaction. Calculations are as follows:

10 X buffer: (10X) X (5 ul) = (1X) X (50 ul) Nucleotides: (10,000 uM) X (1 ul) = (200 uM) X (50 ul) (10mM=10,000uM) primers (500uM) X (O.1ul)= (1.0uM) X (50 ul) Since it is impossible to pipet 0.1ul accurately, a dilution needs to be made first. Add 10 ul of stock primer solution to 990 ul of water to get 5uM concentration of primers. This new primer dilution can be stored at 4oC. Calculation for 5uM stock: (5uM) X (10 ul) = (1.0 uM) X (50 ul) Taq DNA polymerase (5Units/ul) X (0.25 ul) = (.025 Units/ul) X (50 ul) 2.5 Units/100ul= Since it is impossible to pipet 0.25ul accurately, a .025 Units/ul dilution needs to be made first. Add 1.25 ul stock to 3.75 ul water to get a 1.25 Units/ul concentration. Discard and make fresh with each use. Calculation for 1.25 Units/ul stock: (1.25 Units/ul) X (1 ul) = (.025 Units/ul) X (50 ul)

To make the master mix for one reaction add:

- 5 ul 10X buffer
- 4ul Each nucleotide (1ul each of dATP, dCTP, dGTP, dTTP))
- 20 ul Each primer (10ul of each)
- 1 ul Taq DNA polymerase (Total volume = 30ul)
- add 15 ul of water
- 5 ul of template (Total volume = 50 ul)

If want to make 3 reactions, 3 X 50ul = 150ul. Use this number in the formula for "volume of sample."

Primers

A primer is a short segment of nucleotides which is complementary to a section of the DNA which is to be amplified in the PCR reaction. Primers are annealed to the denatured DNA template to provide an initiation site for the elongation of the new DNA molecule. Primers can either be specific to a particular DNA nucleotide sequence or they can be "universal." Universal primers are complementary to nucleotide sequences which are very common in a particular set of DNA molecules. Thus, they are able to bind to a wide variety of DNA templates.

Bacterial ribosomal DNA genes contain nucleotide sequences that are common to all bacteria. Thus, bacterial universal primers can be made by creating primers which are complementary to these sequences.
Examples of bacteria universal primer sequences are:
Forward 5' GAT CCT GGC TCA GGA TGA AC 3' (20 mer)
Reverse 5' GGA CTA CCA GGG TAT CTA ATC 3' (21 mer)

Animal cell lines contain a particular sequence known as the "alu gene". There are approximately 900,000 copies of the alu gene distributed throughout the human genome, and multiple copies distributed through the genome of other animal cells, as well. Thus, the alu gene provides the sequence for a universal primer for animal cell lines. The alu primer is especially useful in that it binds in both forward and reverse directions.
The alu universal primer seqeunce is as follows:
5' GTG GAT CAC CTG AGG TCA GGA GTT TC 3' (26mer)

When using universal primers the annealing temperature on the thermal cycler is lowered to 40-55 degrees C.

Sometimes primer units are listed in optical density reading (OD). If this is a problem you will need to convert to molarity using the

following equations: Change optical density reading of primer to molarity (uM units)-

1. N = # of primer bases
2. SIGMA 260 =~ 10,000 X N/ m X cm
3. Molecular weight =~ 330 X N
4. OD_{260} / SIGMA 260 X 10^6 = Concentration (uM)

For example- primer is 20 bases long/ OD_{260} = 10.

1. N = 20
2. SIGMA 260 =~ 10,000 X 20/m X cm = 20,000/m X cm
3. molecular weight =~ 330 X 20 = 6,600
4. 10 OD_{260}/20,000 $m^{-1}cm^{-1}$ X 10^6 = 50uM

Detection and analysis of the reaction product

The PCR product should be a fragment or fragments of DNA of defined length. The simplest way to check for the presence of these fragments is to load a sample taken from the reaction product, along with appropriate molecular-weight markers, onto an agarose gel which contains 0.8-4.0% ethidium bromide. DNA bands on the gel can then be visualized under ultraviolet trans-illumination. By comparing product bands with bands from the known molecular-weight markers, you should be able to identify any product fragments which are of the appropriate molecular weight.

1

Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

J. Sambrook
UNIVERSITY OF TEXAS SOUTHWESTERN MEDICAL CENTER

E.F. Fritsch
GENETICS INSTITUTE

T. Maniatis
HARVARD UNIVERSITY

1890–1990 CSH CENTENNIAL

Cold Spring Harbor Laboratory Press
1989

Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

All rights reserved
© 1989 by Cold Spring Harbor Laboratory Press
Printed in the United States of America

9

Book and cover design by Emily Harste

Cover: The electron micrograph of bacteriophage λ particles stained with uranyl acetate was digitized and assigned false color by computer. (Thomas R. Broker, Louise T. Chow, and James I. Garrels)

Cataloging in Publications data

Sambrook, Joseph
Molecular cloning : a laboratory manual / E.F. Fritsch, T. Maniatis—2nd ed.
p. cm.
Bibliography: p.
Includes index.
ISBN 0-87969-309-6
1. Molecular cloning—Laboratory manuals. 2. Eukaryotic cells--Laboratory manuals. I. Fritsch, Edward F. II. Maniatis, Thomas III. Title.
QH442.2.M26 1987
574.87'3224—dc19 87-35464

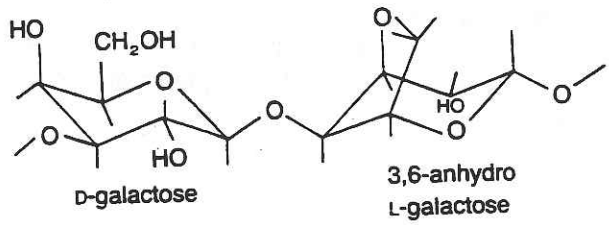
Researchers using the procedures of this manual do so at their own risk. Cold Spring Harbor Laboratory makes no representations or warranties with respect to the material set forth in this manual and has no liability in connection with the use of these materials.

Authorization to photocopy items for internal or personal use, or the internal or personal use of specific clients, is granted by Cold Spring Harbor Laboratory Press for libraries and other users registered with the Copyright Clearance Center (CCC) Transactional Reporting Service, provided that the base fee of $0.10 per page is paid directly to CCC, 21 Congress St., Salem MA 01970. [0-87969-309-6/89 $00 + $0.10] This consent does not extend to other kinds of copying, such as copying for general distribution, for advertising or promotional purposes, for creating new collective works, or for resale.

All Cold Spring Harbor Laboratory Press publications may be ordered directly from Cold Spring Harbor Laboratory Press, 10 Skyline Drive, Plainview, New York 11803. Phone: 1-800-843-4388. In New York (516) 367-8423. FAX: (516) 367-8432.

Agarose Gel Electrophoresis

Agarose, which is extracted from seaweed, is a linear polymer whose basic structure is



Commercially available agarose is not completely pure; it is contaminated with other polysaccharides, salts, and proteins. The amount of contamination varies from batch to batch of agarose and from manufacturer to manufacturer. These differences can affect both the migration of the DNA and the ability of the DNA recovered from the gel to serve as a substrate in enzymatic reactions. Because of the great increase in demand during the past 10 years, most manufacturers now prepare special grades of agarose that are screened for the presence of inhibitors and nucleases and for minimal background fluorescence after staining with ethidium bromide.

Some manufacturers also sell chemically modified forms of agarose that gel and melt at low temperature without significant deterioration in the strength of the hardened gel. Such chemically modified agaroses are used chiefly for preparative electrophoresis of DNA and for digestion of DNA with restriction enzymes in situ. Special grades of low-gelling-temperature agarose that can be used to analyze very small fragments of DNA (10–500 bp) are also available from some manufacturers. Gels made with agarose of this type have a greater resolving power than gels made with normal agarose, but the resolution obtained from these gels still cannot compare with the resolution obtained from polyacrylamide gels. Furthermore, since these gels contain a high concentration of agarose (4–10%), DNA fragments eluted from the gel are frequently contaminated with inhibitors that prevent further enzymatic manipulation.

Agarose gels are cast by melting the agarose in the presence of the desired buffer until a clear, transparent solution is achieved. The melted solution is then poured into a mold and allowed to harden. Upon hardening, the agarose forms a matrix, the density of which is determined by the concentration of the agarose. When an electric field is applied across the gel, DNA, which is negatively charged at neutral pH, migrates toward the anode. The rate of migration is determined by a number of parameters, which are discussed on the following pages.

Factors Affecting the Rate of DNA Migration in Agarose Gels

MOLECULAR SIZE OF THE DNA

Molecules of linear double-stranded DNA, which tend to become oriented in an electric field in an end-on position (Fisher and Dingman 1971; Aaij and Borst 1972), migrate through gel matrices at rates that are inversely proportional to the $\log_{10}$ of the number of base pairs (Helling et al. 1974) (Figure 6.1). Larger molecules migrate more slowly because of greater frictional drag and because they worm their way through the pores of the gel less efficiently than smaller molecules.

AGAROSE CONCENTRATION

A linear DNA fragment of a given size migrates at different rates through gels containing different concentrations of agarose. There is a linear relation-

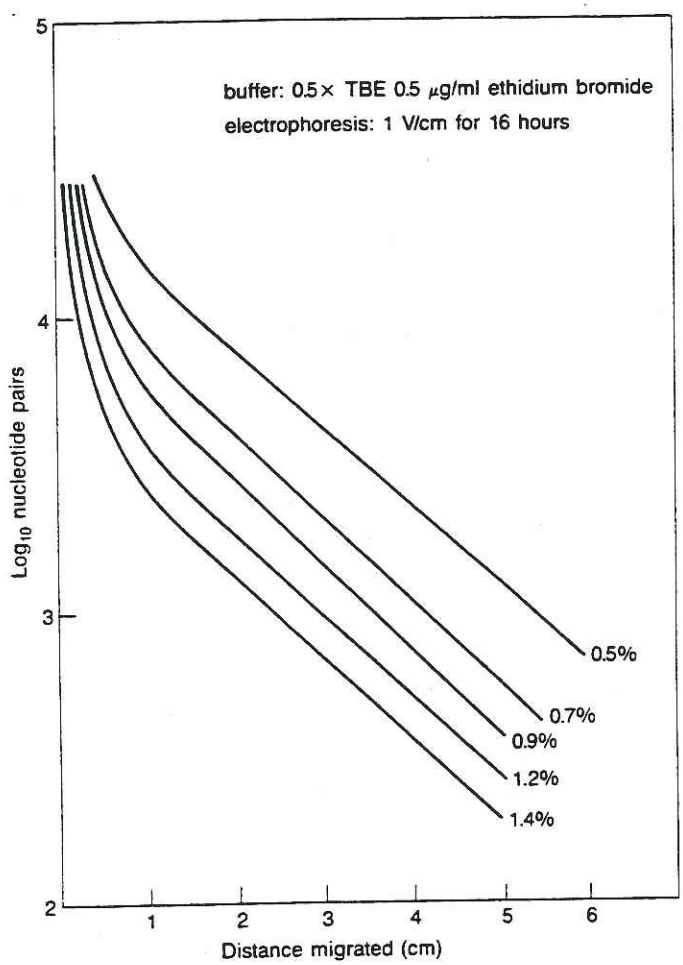


FIGURE 6.1
The relationship between the size of DNA and its electrophoretic mobility.

TABLE 6.1 Range of Separation in Gels Containing Different Amounts of Agarose

Amount of agarose in gel (% [w/v])	Efficient range of separation of linear DNA molecules (kb)
0.3	5–60
0.6	1–20
0.7	0.8–10
0.9	0.5–7
1.2	0.4–6
1.5	0.2–3
2.0	0.1–2

ship between the logarithm of the electrophoretic mobility of DNA (μ) and the gel concentration (τ), which is described by the equation:

$$\log \mu = \log \mu_o - K_r \tau$$

where μ_o is the free electrophoretic mobility of DNA and K_r is the retardation coefficient, a constant that is related to the properties of the gel and the size and shape of the migrating molecules. Thus, by using gels of different concentrations, it is possible to resolve a wide size range of DNA molecules (see Table 6.1).

CONFORMATION OF THE DNA

Superhelical circular (form I), nicked circular (form II), and linear (form III) DNAs of the same molecular weight migrate through agarose gels at different rates (Thorne 1966, 1967). The relative mobilities of the three forms depend primarily on the agarose concentration in the gel, but they are also influenced by the strength of the applied current, the ionic strength of the buffer, and the density of superhelical twists in the form I DNA (Johnson and Grossman 1977). Under some conditions, form I DNA migrates faster than form III DNA; under other conditions, the order is reversed.

An unambiguous method for identifying the different conformational forms of DNA is to carry out electrophoresis in the presence of increasing quantities of ethidium bromide. As the concentration of ethidium bromide increases, more of the dye becomes bound to the DNA. The negative superhelical turns in form I molecules are progressively removed, the radii of the molecules increase, and their rate of migration decreases. At the critical free-dye concentration, where no superhelical turns remain, the rate of migration of form I DNA reaches its minimum value. As still more ethidium bromide is added, positive superhelical turns are generated, the DNA molecules become more compact, and their mobility increases rapidly. Simultaneously, the mobilities of form II and form III DNA decrease differentially due to charge neutralization and the greater stiffness imparted to the DNA by the ethidium bromide. For most preparations of form I DNA, the critical concentration of free ethidium bromide is in the range of 0.1 μg/ml to 0.5 μg/ml.

APPLIED VOLTAGE

At low voltages, the rate of migration of linear DNA fragments is proportional to the voltage applied. However, as the electric field strength is raised, the mobility of high-molecular-weight fragments of DNA increases differentially. Thus, the effective range of separation in agarose gels decreases as the voltage is increased. To obtain maximum resolution of DNA fragments greater than 2 kb in size, agarose gels should be run at no more than 5 V/cm.

Distance is measured as the shortest path between the electrodes and is not merely the length of the gel itself.

DIRECTION OF THE ELECTRIC FIELD

DNA molecules larger than 50–100 kb in length migrate through agarose gels at the same rate if the direction of the electric field remains constant. However, if the direction of the electric field is altered periodically, the DNA molecules are forced to change course. Because larger molecules of DNA take longer to realign themselves to the new direction of the field, pulsed-field gel electrophoresis can be used to fractionate populations of extremely large molecules of DNA (up to 10,000 kb). Pulsed-field gel electrophoresis is discussed in more detail beginning on page 6.50.

BASE COMPOSITION AND TEMPERATURE

The electrophoretic behavior of DNA in agarose gels (in contrast to polyacrylamide gels [Allet et al. 1973]) is not significantly affected by either the base composition of the DNA (Thomas and Davis 1975) or the temperature at which the gel is run. Thus, in agarose gels, the relative electrophoretic mobilities of DNA fragments of different sizes do not change between 4°C and 30°C. In general, agarose gels are run at room temperature. However, gels containing less than 0.5% agarose and low-melting-temperature agarose gels are rather flimsy, and it is best to run them at 4°C, where they are stronger.

PRESENCE OF INTERCALATING DYES

Ethidium bromide, a fluorescent dye that is used to detect DNA in agarose and polyacrylamide gels (Sharp et al. 1973), reduces the electrophoretic mobility of linear DNA by about 15%. The dye intercalates between stacked base pairs, extending the length of linear and nicked circular DNA molecules and making them more rigid.

Ethidium bromide is a carcinogen and should be handled with care. All solutions containing ethidium bromide should be decontaminated before disposal (see pages 6.16–6.17).

COMPOSITION OF THE ELECTROPHORESIS BUFFER

The electrophoretic mobility of DNA is affected by the composition and ionic strength of the electrophoresis buffer. In the absence of ions (e.g., if electrophoresis buffer is omitted from the gel by mistake), electrical conductance is minimal and DNA migrates very slowly, if at all. In buffers of high ionic strength (e.g., if 10× electrophoresis buffer is used by mistake),

electrical conductance is very efficient and significant amounts of heat are generated. In the worst case, the gel melts and the DNA denatures.

Several different buffers are available for electrophoresis of native double-stranded DNA. These contain EDTA (pH 8.0) and Tris-acetate (TAE), Tris-borate (TBE), or Tris-phosphate (TPE) at a concentration of approximately 50 mM (pH 7.5–7.8) (Table 6.2). Electrophoresis buffers are usually made up as concentrated solutions and stored at room temperature.

For historical reasons, TAE is the most commonly used buffer. However, its buffering capacity is rather low, and it tends to become exhausted during extended electrophoresis (the anode becomes alkaline, the cathode acidic). Replacement of the buffer or recirculation between the two reservoirs is therefore advisable when carrying out electrophoresis for long periods of time at high current. Both TPE and TBE are slightly more expensive than TAE, but they have significantly higher buffering capacity. Double-stranded linear DNA fragments migrate approximately 10% faster through TAE than through TBE or TPE, but the resolving powers of these systems are almost identical, with the exception that the resolution of supercoiled DNAs is better in TAE than in TBE.

The most commonly used buffer for electrophoresis of denatured single-stranded DNA is 50 mN NaOH, 1 mM EDTA (alkaline electrophoresis buffer; see Table 6.2). As discussed below, agarose cannot be melted in the presence of NaOH. Therefore, the agarose must be melted in water before the concentrated NaOH/EDTA solution is added.

TABLE 6.2 Commonly Used Electrophoresis Buffers

Buffer	Working solution	Concentrated stock solution (per liter)
Tris-acetate (TAE)	1×: 0.04 M Tris-acetate 0.001 M EDTA	50×: 242 g Tris base 57.1 ml glacial acetic acid 100 ml 0.5 M EDTA (pH 8.0)
Tris-phosphate (TPE)	1×: 0.09 M Tris-phosphate 0.002 M EDTA	10×: 108 g Tris base 15.5 ml 85% phosphoric acid (1.679 g/ml) 40 ml 0.5 M EDTA (pH 8.0)
Tris-borate[a] (TBE)	0.5×: 0.045 M Tris-borate 0.001 M EDTA	5×: 54 g Tris base 27.5 g boric acid 20 ml 0.5 M EDTA (pH 8.0)
Alkaline[b]	1×: 50 mN NaOH 1 mM EDTA	1×: 5 ml 10 N NaOH 2 ml 0.5 M EDTA (pH 8.0)

[a]A precipitate forms when concentrated solutions of TBE are stored for long periods of time. To avoid problems, store the 5× solution in glass bottles at room temperature and discard any batches that develop a precipitate.

TBE was originally used at a working strength of 1× (i.e., a 1:5 dilution of the concentrated stock) for agarose gel electrophoresis. However, a working solution of 0.5× provides more than enough buffering power, and almost all agarose gel electrophoresis is now carried out with a 1:10 dilution of the concentrated stock.

TBE is used at a working strength of 1× for polyacrylamide gel electrophoresis (see page 6.39), twice the strength usually used for agarose gel electrophoresis. The buffer reservoirs of the vertical tanks used for polyacrylamide gel electrophoresis are fairly small, and the amount of electric current passed through them is often considerable. 1× TBE is required to provide adequate buffering power.

[b]Alkaline electrophoresis buffer should be freshly made.

Apparatuses Used for Agarose Gel Electrophoresis

Because agarose gel electrophoresis is both forgiving and adaptable, many successful configurations and sizes of electrophoresis tanks have been designed during the last 15 years. The choice among these different designs is largely a matter of personal preference. The most commonly used configuration is the horizontal slab gel, which was invented by Walter Schaffner and is superior to the old-fashioned vertical gels in several respects:

- Low agarose concentrations can be used because the entire gel is supported from beneath
- Gels can be cast in a wide variety of sizes
- The gels are very simple to load, pour, and handle
- The apparatus is durable and inexpensive to construct or buy

Horizontal slab gels are usually poured on a glass plate or plastic tray that can be installed on a platform in the electrophoresis tank. In a few designs, the gel is poured directly on the platform. Electrophoresis is carried out with the gel submerged just beneath the surface of the buffer. The resistance of the gel to the passage of electric current is almost the same as that of the buffer, and so a considerable fraction of the applied current passes along the length of the gel.

Among the factors to be considered when buying an electrophoresis tank are:

- *It should be easy to examine the gel by ultraviolet light during the run.* When the gel is poured on a glass plate or plastic tray, it can easily be removed, examined, and reinstalled at any stage. Most of the plastic trays currently supplied by commercial manufacturers are translucent to ultraviolet light, eliminating the need to transfer the gel from its support for examination. When the gel is poured directly on a platform within the tank, however, it may be more difficult to remove the gel from the tank both during and at the end of the run.
- *The apparatus should be supplied with a variety of combs to generate different numbers of wells of different sizes.* It is an advantage if the gel can accommodate two rows of wells, with at least 16 wells in each row. This makes it possible to analyze many samples of DNA simultaneously.
- *The apparatus should be fitted with a lid containing shielded electrical connections.* It should be impossible to remove the lid while electric current is flowing through the system. The tank should be designed to allow easy replacement of corroded or broken connections and the platinum electrodes.
- *The apparatus should be fitted with outlets to allow electrophoresis buffer to be removed easily and completely.* In many older designs, the buffer was removed by inverting the tank over a sink. This is not acceptable when the buffer contains ethidium bromide.
- *The outlets should be designed to allow circulation of buffer between the anodic and cathodic chambers.* This is important when RNA is analyzed by electrophoresis.

PREPARATION AND EXAMINATION OF AGAROSE GELS

Preparation of an Agarose Gel

1. Seal the edges of a clean, dry, glass plate (or the open ends of the plastic tray supplied with the electrophoresis apparatus) with autoclave tape so as to form a mold (Figure 6.2). Set the mold on a horizontal section of the bench (check with a level).

2. Prepare sufficient electrophoresis buffer (usually 1 × TAE or 0.5 × TBE; see Table 6.2, page 6.7) to fill the electrophoresis tank and to prepare the gel. Add the correct amount of powdered agarose (see Table 6.1, page 6.5) to a measured quantity of electrophoresis buffer in an Erlenmeyer flask or a glass bottle with a loose-fitting cap. The buffer should not occupy more than 50% of the volume of the flask or bottle.

   It is important to use the same batch of electrophoresis buffer in both the electrophoresis tank and the gel. Small differences in ionic strength or pH create fronts in the gel that can greatly affect the mobility of DNA fragments.

3. Loosely plug the neck of the Erlenmeyer flask with Kimwipes. When using a glass bottle, make sure the cap is loose. Heat the slurry in a boiling-water bath or a microwave oven until the agarose dissolves.

   Heat the slurry for the minimum time required to allow all of the grains of agarose to dissolve. Wearing an oven mitt, gingerly swirl the bottle or flask from time to time to make sure that any grains sticking to the walls enter the solution. *Take care—the agarose solution can become superheated and may boil violently if it has been heated for too long in the microwave oven.* Undissolved agarose appears as small "lenses" floating in the solution. Check that the volume of the solution has not been decreased by evaporation during boiling; replenish with water if necessary.

4. Cool the solution to 60°C, and, if desired, add ethidium bromide (from a stock solution of 10 mg/ml in water) to a final concentration of 0.5 μg/ml and mix thoroughly.

   Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described on pages 6.16–6.17.

   Stock solutions of ethidium bromide should be stored in light-tight containers (e.g., in a bottle completely wrapped in aluminum foil) at room temperature.

   When preparing gels that contain high concentrations of agarose (2% or above), cool the solution quickly to 70°C and pour the gel immediately. The higher the concentration of agarose, the quicker the gel hardens. Problems caused by premature hardening of the gel can be avoided by using sieving grades of low-gelling-temperature agarose. However, these grades of agarose are suitable only for analytical electrophoresis, since DNA fragments eluted from the gel are frequently contaminated with inhibitors that prevent further enzymatic manipulation.

5. Using a pasteur pipette, seal the edges of the mold with a small quantity of the agarose solution. Allow the seal to set. Position the comb 0.5–1.0 mm above the plate so that a complete well is formed when the agarose is added. If the comb is closer to the glass plate, there is a risk that the

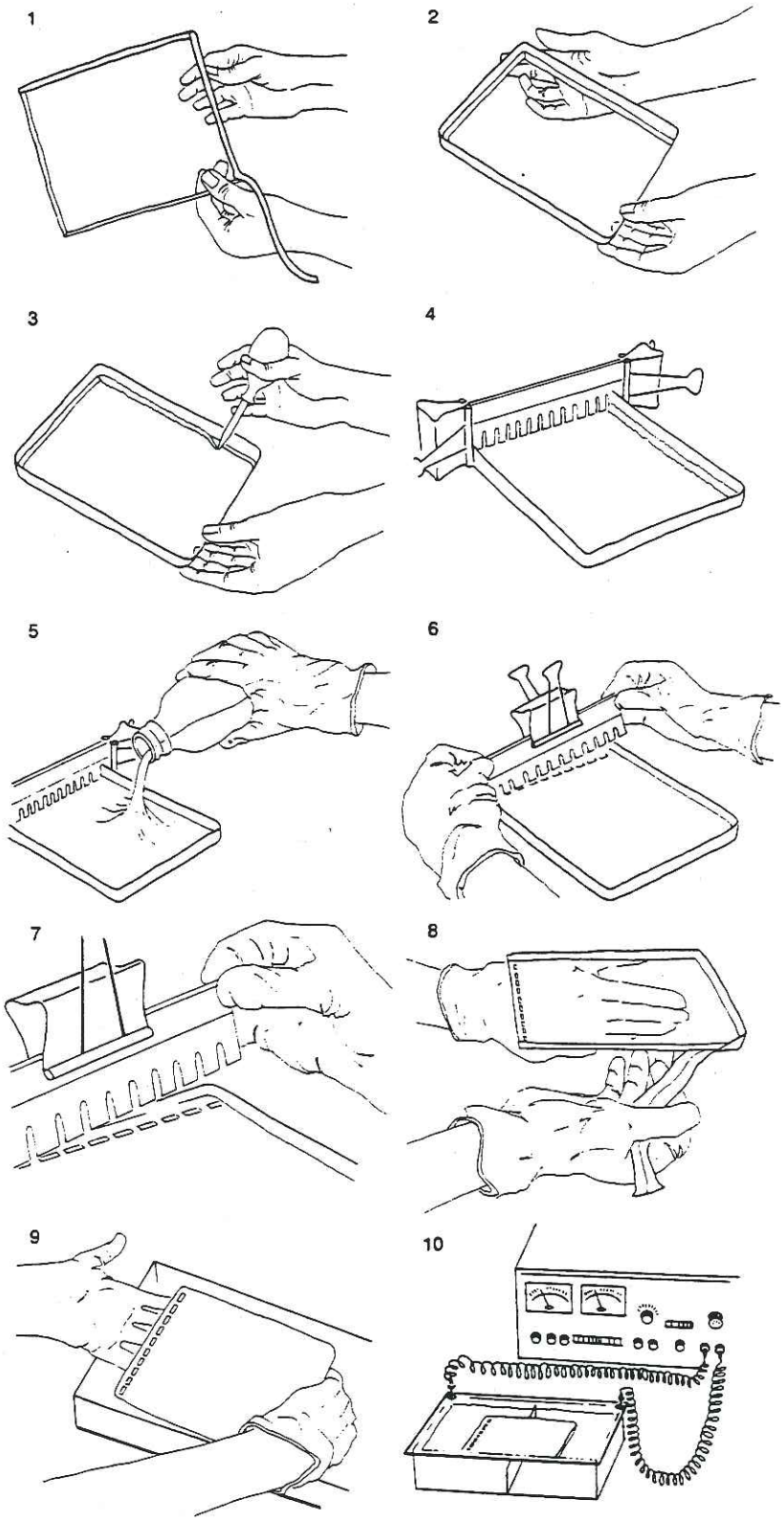


FIGURE 6.2
Pouring a horizontal agarose gel.

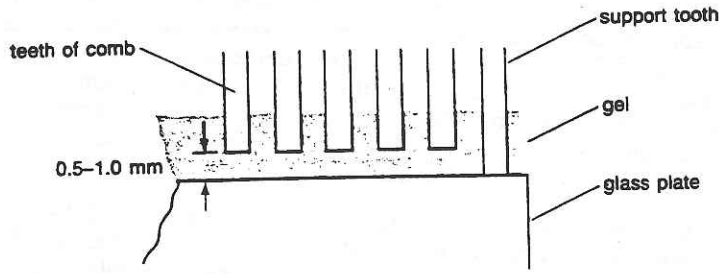


FIGURE 6.3
Diagram of a comb used to form slots in horizontal agarose gels.

base of the well may tear when the comb is withdrawn, allowing the sample to leak between the gel and the glass plate.

Some combs are designed with two outer teeth slightly longer than the internal teeth. When the comb is positioned above the plate, it is supported on the outer teeth; the inner teeth are automatically lifted clear of the glass plate (Figure 6.3). The disadvantage of this design is that the wells made by the outer teeth cannot be used during the subsequent electrophoresis.

6. Pour the remainder of the warm agarose solution into the mold. The gel should be between 3 mm and 5 mm thick. Check to see that there are no air bubbles under or between the teeth of the comb.

   When preparing gels that contain low concentrations of agarose (<0.5%), first pour a supporting gel (1% agarose) without wells. Allow this gel to harden at room temperature on the glass plate or plastic tray. Then pour the lower-percentage gel directly on top of the supporting gel. This reduces the chances that the lower-percentage gel will fracture during subsequent manipulations (photography, processing for Southern hybridization, etc.). Make sure that both gels are made from the same batch of buffer and that ethidium bromide is added to both gels or to neither.

7. After the gel is completely set (30–45 minutes at room temperature), carefully remove the comb and autoclave tape and mount the gel in the electrophoresis tank.

   Gels cast with low-melting-temperature agarose and gels that contain less than 0.5% agarose should be chilled to 4°C and run in the cold room.

8. Add just enough electrophoresis buffer to cover the gel to a depth of about 1 mm.

9. Mix the samples of DNA with the desired gel-loading buffer (Table 6.3). Slowly load the mixture into the slots of the submerged gel using a disposable micropipette, an automatic micropipettor, or, if you have a very steady hand, a pasteur pipette.

   Gel-loading buffers are usually made up as sixfold concentrated solutions. (See Table 6.3 for the preparation of standard 6× buffers.) Tenfold concentrated solutions can also be prepared if needed.

   The maximum amount of DNA that can be applied to a slot depends on the number of fragments in the sample and their sizes. The minimum amount of DNA that can be detected by photography of ethidium-bromide-stained gels is about 2 ng in a

0.5-cm-wide band (the usual width of a slot). If there is more than 500 ng of DNA in a band of this width, the slot will be overloaded, resulting in trailing and smearing—a problem that becomes more severe as the size of the DNA increases. When simple populations of DNA molecules (e.g., bacteriophage λ or plasmid DNAs) are to be analyzed, 100–500 ng of DNA should be loaded per 0.5-cm slot. When the sample consists of a very large number of DNA fragments of different sizes (e.g., restriction digests of mammalian DNA), however, it is possible to load 20–30 μg of DNA per slot without significant loss of resolution.

The maximum volume of solution that can be loaded is determined by the dimensions of the slot. (A typical slot [0.5 cm × 0.5 cm × 0.15 cm] will hold about 37.5 μl.) However, to reduce the possibility of contaminating neighboring samples, it is best to make the gel a little thicker or to concentrate the DNA by ethanol precipitation rather than to fill the slot completely.

For most purposes, it is not necessary to use a fresh pipette tip for every sample as long as the tip is thoroughly washed with buffer from the anodic chamber between samples. However, if the gel is to be analyzed by Southern hybridization or if bands of DNA are to be recovered from the gel, it is sensible to use a separate pipette tip for every sample.

Marker DNAs of known size (which can be purchased from commercial sources) should be loaded into slots on both the right and left sides of the gel. This makes it

TABLE 6.3 Gel-loading Buffers

Buffer type	6× Buffer	Storage temperature
I	0.25% bromophenol blue 0.25% xylene cyanol FF 40% (w/v) sucrose in water	4°C
II	0.25% bromophenol blue 0.25% xylene cyanol FF 15% Ficoll (Type 400; Pharmacia) in water	room temp.
III	0.25% bromophenol blue 0.25% xylene cyanol FF 30% glycerol in water	4°C
IV	0.25% bromophenol blue 40% (w/v) sucrose in water	4°C
V	*Alkaline loading buffer* 300 mN NaOH 6 mM EDTA 18% Ficoll (Type 400; Pharmacia) in water 0.15% bromocresol green 0.25% xylene cyanol FF	4°C

These gel-loading buffers serve three purposes: They increase the density of the sample, ensuring that the DNA drops evenly into the well; they add color to the sample, thereby simplifying the loading process; and they contain dyes that, in an electric field, move toward the anode at predictable rates. Bromophenol blue migrates through agarose gels approximately 2.2-fold faster than xylene cyanol FF, independent of the agarose concentration. Bromophenol blue migrates through agarose gels run in 0.5× TBE at approximately the same rate as linear double-stranded DNA 300 bp in length, whereas xylene cyanol FF migrates at approximately the same rate as linear double-stranded DNA 4 kb in length. These relationships are not significantly affected by the concentration of agarose in the gel over the range of 0.5% to 1.4%.

Which type of loading dye to use is a matter of personal preference. However, bromocresol green should be used as a tracking dye in alkaline gels because it displays a more vivid color than bromophenol blue at alkaline pH.

easier to determine the sizes of unknown DNAs if any systematic distortion of the gel should occur during electrophoresis.

When measuring the sizes of unknown DNAs, it is important that all samples be applied to the gel in the same buffer. The high concentrations of salt in certain restriction enzyme buffers (e.g., *Bam*HI or *Eco*RI) retard the migration of DNA and distort the electrophoresis of DNA in the adjacent wells.

10. Close the lid of the gel tank and attach the electrical leads so that the DNA will migrate toward the anode (red lead). Apply a voltage of 1–5 V/cm (measured as the distance between the electrodes). If the leads have been attached correctly, bubbles should be generated at the anode and cathode (due to electrolysis) and, within a few minutes, the bromophenol blue should migrate from the wells into the body of the gel. Run the gel until the bromophenol blue and xylene cyanol FF have migrated the appropriate distance through the gel.

During electrophoresis, the ethidium bromide migrates toward the cathode (in the direction opposite to that of the DNA). Extended electrophoresis can remove much of the ethidium bromide from the gel, making detection of small fragments difficult. If this occurs, restain the gel by soaking it for 30–45 minutes in a solution of ethidium bromide (0.5 μg/ml) as described on page 6.15.

The presence of ethidium bromide allows the gel to be examined by ultraviolet illumination at any stage during electrophoresis. However, some people feel that sharper bands of DNA are obtained when the gel is run in the absence of the dye. In this case, after electrophoresis is completed, stain the gel by soaking it for 30–45 minutes in a solution of ethidium bromide (0.5 μg/ml) as described on page 6.15.

11. Turn off the electric current and remove the leads and lid from the gel tank. If ethidium bromide was present in the gel and electrophoresis buffer, examine the gel by ultraviolet light and photograph the gel as described on page 6.19. Otherwise, stain the gel with ethidium bromide as described on page 6.15 and then photograph.

Solutions containing ethidium bromide should be decontaminated by one of the methods described on pages 6.16–6.17 before they are discarded.

Minigels

During the last 5 years, methods have been developed for analyzing small quantities of DNA very rapidly by using miniature agarose gels. Several types of miniature electrophoresis tanks are manufactured commercially. Because of the rather delicate milling involved in shaping the tiny combs and other parts, these tanks are quite expensive. However, inexpensive versions can be constructed easily in the laboratory.

A convenient electrophoresis chamber for submerged minigels is a small (2 1/2 inches × 6 1/2 inches) polystyrene snap-lock box. Four inches of platinum wire are required for each of the two electrodes, which are glued in place with silicone rubber cement. A platform composed of four 5-cm × 7.5-cm lantern slides glued together in a stack is cemented in the center of the box (Figure 6.4).

The gels themselves, consisting of 10–12 ml of melted agarose solution (0.5–2.0%) containing ethidium bromide (0.5 μg/ml), are poured on 5-cm × 7.5-cm lantern slides using a disposable 10-ml pipette. The solution flows better when the pipette tip is broken off. Each gel can accommodate eight slots, 2.5 mm long × 1 mm wide, which are formed by using a miniature Lucite comb of standard design. Several gels may be prepared simultaneously by mounting a long gel comb across slides placed side by side on a sheet of Parafilm.

Ethidium bromide is almost always included to reduce the time required between ending the run and seeing the result. By having a large number of lantern slides on hand, many gels can be poured at once. After hardening, the gels are wrapped in Saran Wrap, labeled (with the date and the concentration of the agarose), and stored in a drawer in the dark at room temperature. Either the whole minigel may be used at once or as many lanes as are

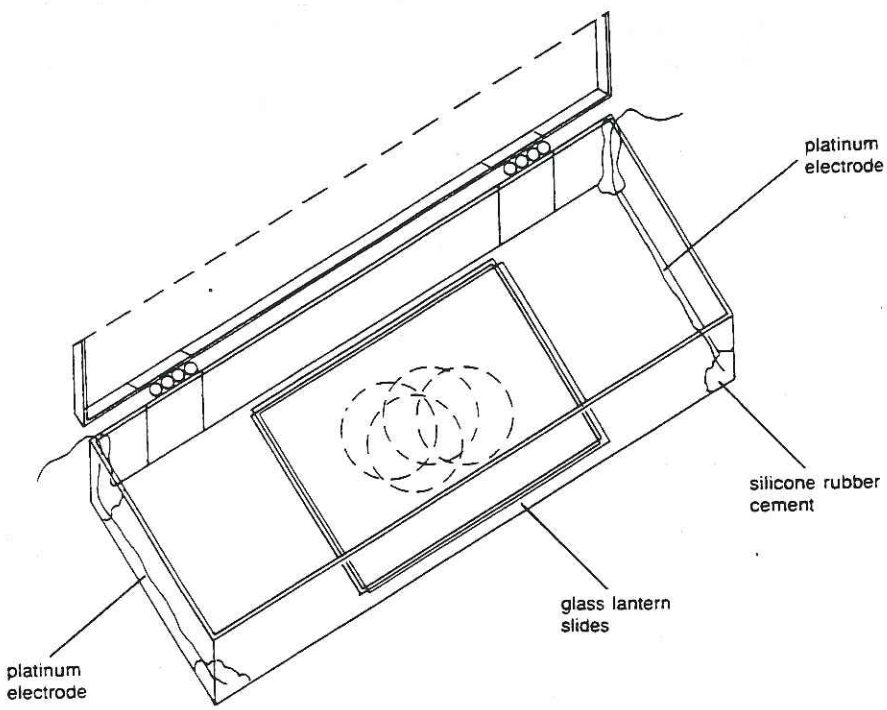


FIGURE 6.4
A minigel apparatus that can be constructed inexpensively in the laboratory.

required may simply be cut from the gel with a razor blade and carefully transferred to the electrophoresis apparatus.

Each gel slot holds 3–12 μl of fluid, depending on the thickness of the gel and the width of the teeth of the comb. Usually, 10–100 ng of DNA, in the gel-loading buffer of choice, are applied to a slot. The gel is run for 30–60 minutes at high voltage (5–20 V/cm) until the bromophenol blue and xylene cyanol FF have migrated the appropriate distance. The gel is then photographed as described on page 6.19.

Minigels are particularly useful when a rapid answer is required before the next step in a cloning protocol can be undertaken. Because the wells are smaller and the gels are thinner, less DNA than normal is required for visualization. Also, because the gels can be prepared in advance and run rapidly and because they require smaller amounts of reagents, there are considerable savings in both time and money. However, minigels are best suited for the analysis of small DNA fragments (<3 kb). Larger fragments are resolved poorly because of the high voltages that are generally used and the comparatively short length of the gel.

Staining DNA in Agarose Gels

The most convenient method to visualize DNA in agarose gels is staining with the fluorescent dye ethidium bromide (Sharp et al. 1973). This substance contains a planar group that intercalates between the stacked bases of DNA. The fixed position of this group and its close proximity to the bases cause dye bound to DNA to display an increased fluorescent yield compared to that of dye in free solution. Ultraviolet radiation at 254 nm is absorbed by the DNA and transmitted to the dye; radiation at 302 nm and 366 nm is absorbed by the bound dye itself. In both cases, the energy is re-emitted at 590 nm in the red-orange region of the visible spectrum. Because the fluorescent yield of ethidium bromide:DNA complexes is much greater than that of unbound dye, small amounts of DNA can be detected in the presence of free ethidium bromide in the gel.

Ethidium bromide can be used to detect both single- and double-stranded nucleic acids (both DNA and RNA). However, the affinity of the dye for single-stranded nucleic acid is relatively low and the fluorescent yield is comparatively poor.

Ethidium bromide is usually prepared as a stock solution of 10 mg/ml in water, which is stored at room temperature in dark bottles or bottles wrapped in aluminum foil. The dye is usually incorporated into the gel and the electrophoresis buffer at a concentration of 0.5 μg/ml. Although the electrophoretic mobility of linear double-stranded DNA is reduced by approximately 15% in the presence of the dye, the ability to examine the gel directly under ultraviolet illumination during or at the end of the run is often a great advantage. However, the gel may also be run in the absence of ethidium bromide and stained after electrophoresis is complete. In this case, the gel is immersed in electrophoresis buffer or water containing ethidium bromide (0.5 μg/ml) for 30–45 minutes at room temperature. Destaining is not usually required. However, detection of very small amounts (<10 ng) of DNA is made easier if the background fluorescence caused by unbound ethidium bromide is reduced by soaking the stained gel in water or 1 mM $MgSO_4$ for 20 minutes at room temperature.

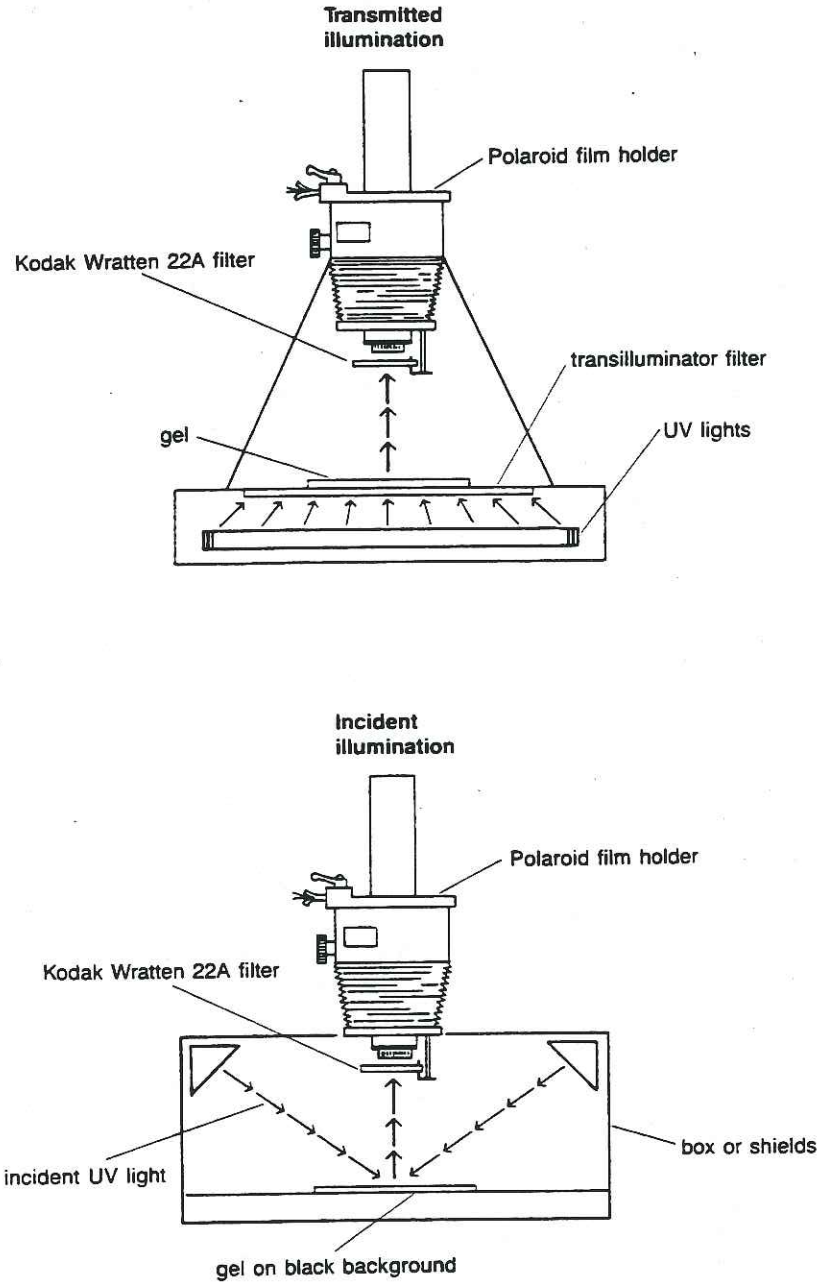


FIGURE 6.5
Photography of gels by ultraviolet illumination. The top diagram shows the arrangement of the ultraviolet light source, the gel, and the camera that is used for photography by transmitted light. The bottom diagram shows the arrangement that is used for photography by incident light.

Photography

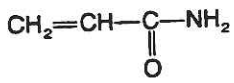
Photographs of gels may be made using transmitted or incident ultraviolet light (Figure 6.5). Most commercially available ultraviolet light sources emit ultraviolet light at 302 nm. The fluorescent yield of ethidium bromide:DNA complexes is considerably greater at this wavelength than at 366 nm and slightly less than at short-wavelength (254 nm) light. However, the amount of nicking of the DNA is much less at 302 nm than at 254 nm (Brunk and Simpson 1977).

The most sensitive film is Polaroid Type 57 or 667 (ASA 3000). With an efficient ultraviolet light source ($> 2500\ \mu W/cm^2$), a Wratten 22A filter, and a good lens ($f = 135$ mm), an exposure of a few seconds is sufficient to obtain images of bands containing as little as 10 ng of DNA. With a long exposure time and a strong ultraviolet light source, the fluorescence emitted by as little as 1 ng of DNA can be recorded on film. For detection of extremely faint bands, a lens with a shorter focal length ($f = 75$ mm) should be used in combination with conventional wet-process film (e.g., Eastman Kodak No. 4155). This allows the lens to be moved closer to the gel, concentrates the image on a smaller area of film, and allows for flexibility in developing and printing the image.

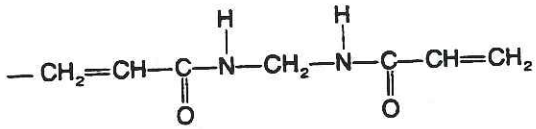
Caution: Ultraviolet radiation is dangerous, particularly to the eyes. To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light.

Polyacrylamide Gel Electrophoresis

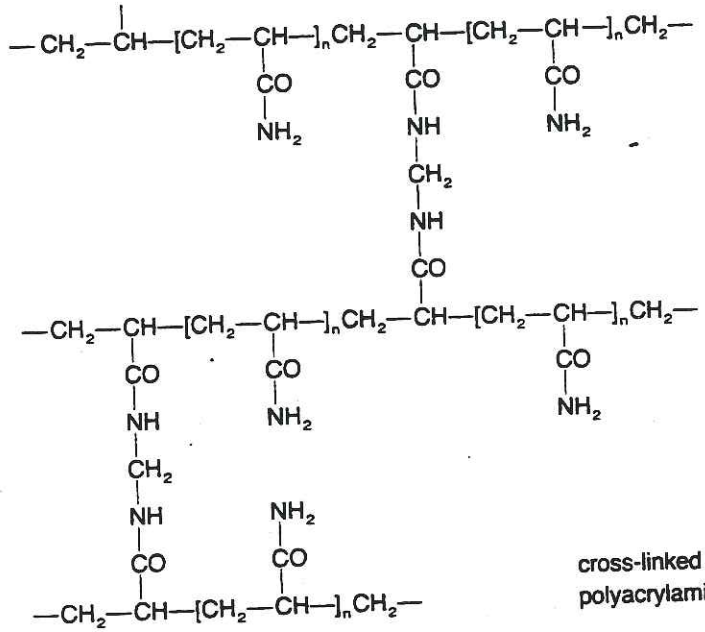
Acrylamide is a monomer whose structure is



In the presence of free radicals, which are usually supplied by ammonium persulfate and stabilized by TEMED (*N,N,N′,N′*-tetramethylethylenediamine), a chain reaction is initiated in which monomers of acrylamide are polymerized into long chains. When the bifunctional agent *N,N′*-methylenebisacrylamide is included in the polymerization reaction, the chains become cross-linked to form a gel, whose porosity is determined by the length of the chains and the degree of cross-linking.



N,N′-methylenebisacrylamide



cross-linked polyacrylamide

The length of the chains is determined by the concentration of acrylamide in the polymerization reaction (between 3.5% and 20%): 1 molecule of cross-linker is included for every 29 monomers of acrylamide. The effective range of separation in nondenaturing gels containing different concentrations of polyacrylamide is shown in Table 6.4.

Polyacrylamide gels are more of a nuisance to prepare and run than

agarose gels. They are almost always poured between two glass plates that are held apart by spacers and sealed by electrical tape. In this arrangement, most of the acrylamide solution is shielded from exposure to the air, so that inhibition of polymerization by oxygen is confined to a narrow layer at the top of the gel. Polyacrylamide gels can range in length from 10 cm to 100 cm, depending on the separation required; they are invariably run in a vertical position. However, they have three major advantages over agarose gels: (1) Their resolving power is so great that they can separate molecules of DNA whose lengths differ by as little as 0.2% (i.e., 1 bp in 500 bp). (2) They can accommodate much larger quantities of DNA than agarose gels: Up to 10 μg of DNA can be applied to a single slot (1 cm $\times$ 1 mm) of a typical polyacrylamide gel without significant loss of resolution. (3) DNA recovered from polyacrylamide gels is extremely pure and can be used for the most demanding purposes (e.g., microinjection of mouse embryos).

Two types of polyacrylamide gels are in common use:

- *Nondenaturing polyacrylamide gels for the separation and purification of fragments of double-stranded DNA.* These gels are poured and run in 1 $\times$ TBE at low voltage (1–8 V/cm) to prevent denaturation of small fragments of DNA by heat generated by the passage of electric current. Most species of double-stranded DNA migrate through nondenaturing polyacrylamide gels at a rate that is approximately inversely proportional to the $\log_{10}$ of their size. However, their electrophoretic mobility is also affected by their base composition and sequence, so that DNAs of exactly the same size can differ in mobility by up to 10%. This effect is believed to be caused by kinks that form at specific sequences in double-stranded DNA. Because it is impossible to know whether or not the migration of an unknown DNA is anomalous, electrophoresis through nondenaturing polyacrylamide gels cannot be used to determine the size of double-stranded DNAs.
- *Denaturing polyacrylamide gels for the separation and purification of single-stranded fragments of DNA.* These gels are polymerized in the presence of an agent (urea or, less frequently, formamide) that suppresses base pairing in nucleic acids. (Alkali cannot be used as a denaturing agent

TABLE 6.4 Effective Range of Separation of DNAs in Polyacrylamide Gels

Acrylamide (% [w/v])[a]	Effective range of separation (bp)	Xylene cyanol FF[b]	Bromophenol blue[b]
3.5	1000–2000	460	100
5.0	80–500	260	65
8.0	60–400	160	45
12.0	40–200	70	20
15.0	25–150	60	15
20.0	6–100	45	12

[a]*N,N'*-methylenebisacrylamide is included at 1/30th the concentration of acrylamide.
[b]The numbers given are the approximate sizes (in nucleotide pairs) of fragments of double-stranded DNA with which the dye comigrates.

because it deaminates acrylamide, and methylmercuric hydroxide cannot be used because it inhibits polymerization.) Denatured DNA migrates through these gels at a rate that is almost completely independent of its base composition and sequence. Among the uses of denaturing polyacrylamide gels are the isolation of radiolabeled DNA probes, the analysis of the products of nuclease-S1 digestions, and the analysis of the products of DNA sequencing reactions. Descriptions of denaturing polyacrylamide gels are given in Chapters 11 and 13.

PREPARATION OF NONDENATURING POLYACRYLAMIDE GELS

Most vertical electrophoresis tanks obtained from commercial sources are constructed to hold glass plates 20 cm × 40 cm. However, it is possible to run larger or smaller gels if suitable tanks are available. Spacers vary in thickness from 0.5 mm to 2.0 mm. The thicker the gel, the hotter it will become during electrophoresis; overheating results in "smiling" bands of DNA and other problems. Thinner gels are therefore preferred, since they produce the sharpest and flattest bands of DNA. However, it is necessary to use thicker gels when preparing large quantities of DNA (>1 μg/band). Below we describe the preparation and use of polyacrylamide gels.

1. Prepare the following solutions:

30% Acrylamide

acrylamide	29 g
N,N'-methylenebisacrylamide	1 g
H_2O to 100 ml	

Heat the solution to 37°C to dissolve the chemicals.

Caution: Acrylamide is a potent neurotoxin and is absorbed through the skin. The effects of acrylamide are cumulative. Wear gloves and a mask when weighing powdered acrylamide and methylenebisacrylamide. Wear gloves when handling solutions containing these chemicals. Although polyacrylamide is considered to be nontoxic, it should be handled with care because of the possibility that it might contain small quantities of unpolymerized acrylamide.

Cheaper grades of acrylamide and bisacrylamide are often contaminated with metal ions. Stock solutions of acrylamide can easily be purified by stirring overnight with about 0.2 volume of monobed resin (MB-1, Mallinckrodt), followed by filtration through Whatman No. 1 paper.

During storage, acrylamide and bisacrylamide are slowly converted to acrylic acid and bisacrylic acid. This deamination reaction is catalyzed by light and alkali. Check that the pH of the acrylamide solution is 7.0 or less, and store the solution in dark bottles at room temperature. Fresh solutions should be prepared every few months.

1 × TBE

89 mM Tris-borate
2 mM EDTA (pH 8.0)

TBE is usually made and stored as a 5 × stock solution (see Table 6.2, page 6.7). The pH of the buffer should be approximately 8.3.

TBE is used at a working strength of 1 × for polyacrylamide gel electrophoresis. This is twice the strength usually used for agarose gel electrophoresis. The buffer reservoirs of the vertical tanks used for polyacrylamide gel electrophoresis are fairly small and the amount of electric current passed through them is often considerable. 1 × TBE is required to provide adequate buffering power.

10% Ammonium persulfate

ammonium persulfate 1 g
H_2O to 10 ml

The solution may be stored at 4°C for several weeks.

2. Prepare the glass plates and spacers for pouring the gel. If necessary, clean them with KOH/methanol, which is prepared by adding ~5 g of KOH pellets to 100 ml of methanol.

   Caution: Handle the KOH and the KOH/methanol solutions with great care. Use gloves and a face protector.

   Then wash the glass plates and spacers in warm detergent solution and rinse them well, first in tap water and then in deionized water. Hold the plates by the edges so that oils from your hands do not become deposited on the working surfaces of the plates. Rinse the plates with ethanol and set them aside to dry. The glass plates must be free of grease spots to prevent air bubbles from forming in the gel.

   Treat one surface of each plate with a silicone solution. This prevents

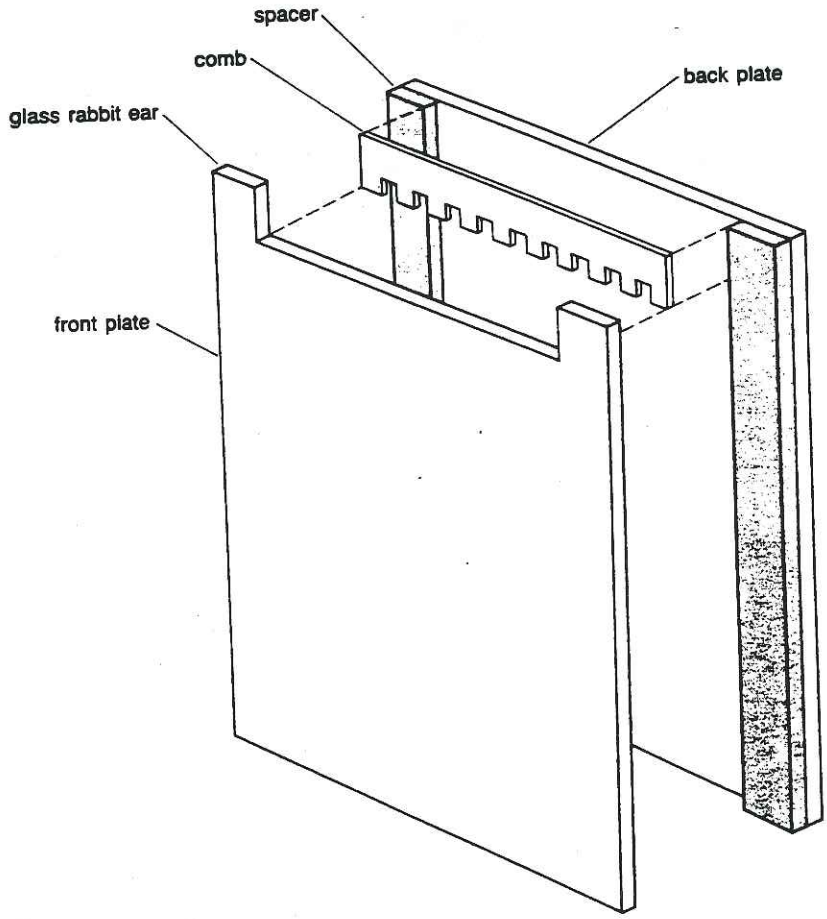


FIGURE 6.7

the gel from sticking tightly to both plates and reduces the possibility that the gel will tear when it is removed from the mold after electrophoresis is completed.

To siliconize a plate, lay the plate on a pad of paper in a fume hood and pour a small quantity of siliconizing fluid (e.g., Sigmacote) onto it. Wipe the fluid over the surface of the plate with a pad of Kimwipes, and then rinse the plate in deionized water. Dry the plate with a hairdryer. Wear gloves during siliconization.

3. There are many types of electrophoresis apparatus available commercially, and the arrangement of the glass plates and spacers differs slightly from manufacturer to manufacturer. In all cases, the aim is to form a watertight seal between the plates and the spacers so that the unpolymerized gel solution does not leak out. Typically, the two plates are of slightly different size and one of them is notched. Lay the larger (or unnotched) plate flat on the bench and arrange the spacers at each side parallel to the two edges (Figure 6.7). A couple of *minute* dabs of petroleum jelly help to keep the spacer bars in position during the next steps. Lay the inner (notched) plate in position, resting on the spacer bars. Bind the entire length of the two sides and the bottom of the plates with gel-sealing tape (3M yellow electrical tape; BRL catalog no. 1032ST) to make a watertight seal. Take particular care with the bottom corners of the plates, since these are the places where leaks often occur.

Several other methods have been used to prevent leakage of acrylamide solution from assembled gel molds, including:

- Sealing the edges with agarose
- Inserting a plastic spacer into the open space at the bottom of the mold during polymerization
- Sealing the bottom of the plate with a strip of filter paper impregnated with catalyzed acrylamide (Wahls and Kingzette 1988)

We recommend that you use whichever of these methods you find to be most reliable with the particular type of gel mold that is available.

4. Knowing the size of the glass plates and the thickness of the spacers, calculate the volume of acrylamide solution required. (See Table 6.5 for preparation of 100 ml of solution for different gel concentrations.)

5. (*Optional*) Place the required quantity of acrylamide solution in a clean sidearm flask. Deaerate the solution by applying vacuum, gently at first. Swirl the flask during deaeration until no more air bubbles are released.

Deaeration of the acrylamide solution is not essential, but it does reduce the chance that air bubbles will form when thick gels (>1 mm) are poured.

TABLE 6.5 Volumes of Reagents Used to Cast Polyacrylamide Gels

	Milliliters of reagents to cast gels of various concentrations (%)				
Reagents	**3.5%**	**5.0%**	**8.0%**	**12.0%**	**20.0%**
30% Acrylamide (see page 6.39)	11.6	16.6	26.6	40.0	66.6
Water	67.7	62.7	52.7	39.3	12.7
5× TBE (see Table 6.2, page 6.7)	20.0	20.0	20.0	20.0	20.0
10% Ammonium persulfate (see page 6.40)	0.7	0.7	0.7	0.7	0.7

6. Wearing gloves, perform the following manipulations over a tray so that any spilled acrylamide solution will not spread over the bench.

   a. Add 35 μl of TEMED (*N,N,N',N'*-tetramethylethylenediamine) to each 100 ml of acrylamide solution. Mix the solution by swirling.

   b. Draw the solution into the barrel of a 50-ml syringe. Invert the syringe and expel any air that has entered the barrel. Introduce the nozzle of the syringe into the space between the two glass plates. Expel the acrylamide solution from the syringe, filling the space almost to the top. Keep the remaining acrylamide solution at 4°C to reduce the rate of polymerization. If the plates were clean, there should be no trapped air bubbles, and if they were sealed well, no leaks. If air bubbles form, empty the gel mold and repour the gel, after thoroughly recleaning the glass plates.

   c. Lay the glass plates against a test-tube rack at an angle of approximately 10°. This decreases the chance of leakage and minimizes distortion of the gel.

7. Immediately insert the appropriate comb, being careful not to allow air bubbles to become trapped under the teeth. The tops of the teeth should be slightly higher than the top of the glass. Clamp the comb in place with a bulldog paper clip. If necessary, use the remaining acrylamide solution to fill the gel mold completely. Check that no acrylamide solution is leaking from the gel mold.

8. Allow the acrylamide to polymerize for 60 minutes at room temperature, adding additional acrylamide solution if the gel retracts significantly. When polymerization is complete, a schlieren pattern will be visible just beneath the teeth of the comb.

   Gels may be stored for 1–2 days in this state before they are used. After polymerization is complete, surround the comb and the top of the gel with paper towels that have been soaked in 1 × TBE. Then seal the entire gel in Saran Wrap and store at 4°C.

9. Carefully remove the comb. Immediately rinse out the wells with water. Using a razor blade or a scalpel, remove the electrical tape from the bottom of the gel.

   It is essential to wash out the wells thoroughly as soon as the comb is removed. Otherwise, small amounts of acrylamide solution trapped by the comb will polymerize in the wells, producing irregularly shaped surfaces that give rise to distorted bands of DNA.

10. Attach the gel to the electrophoresis tank, using large bulldog paper clips on the sides and three-prong clamps on the shoulders. The notched plate should face inward toward the buffer reservoir.

11. Fill the reservoirs of the electrophoresis tank with 1 × TBE. Use a bent

pasteur pipette or syringe needle to remove any air bubbles trapped beneath the bottom of the gel.

It is important to use the same batch of electrophoresis buffer in both of the reservoirs and in the gel. Small differences in ionic strength or pH produce buffer fronts that can greatly distort the migration of DNA.

12. Use a pasteur pipette to flush out the wells with 1 × TBE.

If remnants of unpolymerized acrylamide remain in the wells, diffuse, wavy bands of DNA will be observed.

13. Mix the DNA samples with the appropriate amount of 6 × gel-loading buffer (type I or type III; see Table 6.3, page 6.12). Load the mixture into the wells using a drawn-out glass micropipette or Hamilton syringe. Long, disposable, and expensive micropipette tips are also sold for this purpose by several manufacturers. Draw up all of the sample into the loading device, and then insert the tip of the device into the well. This should be done quickly, in a single movement, since the DNA sample tends to dribble out of the loading device after the tip is immersed in the electrophoresis buffer.

Usually, about 3–5 μl of DNA sample are loaded per well (0.5 cm × 0.3 cm × 1 mm). Raise the loading device as the sample is loaded into the well. The tip of the device should always be above the level of the sample in the well. Do not attempt to expel all of the sample from the loading device, since this almost always produces air bubbles that blow the sample out of the well.

The same device can be used to load many samples, provided it is thoroughly washed between each loading. However, it is important not to take too long to complete loading the gel; otherwise, the samples will diffuse from the wells.

14. Connect the electrodes to a power pack (positive electrode connected to the bottom reservoir). Nondenaturing polyacrylamide gels are usually run at voltage gradients between 1 V/cm and 8 V/cm.

If electrophoresis is carried out at a higher voltage, differential heating in the center of the gel may cause bowing of the DNA bands or even melting of the strands of small DNA fragments.

15. Run the gel until the marker dyes have migrated the desired distance (see Table 6.4, page 6.37). Turn off the electric power, disconnect the leads, and discard the electrophoresis buffer from the reservoirs. Detach the glass plates, and use a scalpel or razor blade to remove the electrical tape. Lay the glass plates on the bench (siliconized plate uppermost). Using a thin spatula, lift a corner of the upper glass plate. Check that the gel remains attached to the lower plate. Pull the upper plate smoothly away. Remove the spacers.

Occasionally, the gel remains attached to the siliconized plate. In this case, turn over the glass plates and remove the nonsiliconized plate.

16. Use one of the methods described on the following pages to detect the positions of bands of DNA in the polyacrylamide gel.

DETECTION OF DNA IN POLYACRYLAMIDE GELS

Staining with Ethidium Bromide

Because polyacrylamide quenches the fluorescence of ethidium bromide, it is not possible to detect bands that contain less than about 10 ng of DNA by this method.

1. Gently submerge the gel and its attached glass plate in staining solution (0.5 μg/ml ethidium bromide in 1 × TBE [see page 6.7]). Use just enough staining solution to cover the gel completely. After staining for 30–45 minutes at room temperature, remove the gel, using the glass plate as a support. Carefully blot excess liquid from the surface of the gel with a pad of Kimwipes. Do not use absorbent paper (to which the gel will stick). Cover the gel with a piece of Saran Wrap. Try to avoid creating air bubbles or folds in the Saran Wrap.

   Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described on pages 6.16–6.17.

   Try to minimize the movement of the staining solution across the surface of the gel during staining. The aim is to keep the gel attached to its supporting glass plate. If the gel becomes completely detached, it can usually be rescued from the staining solution on a large glass plate and transferred to a shallow water bath. In most cases, the gel can then be carefully unfolded and restored to its original shape. To avoid problems, some workers use a piece of plastic mesh (mesh size 1 cm, available from garden and hardware stores) to hold the gel in place during staining.

2. To photograph the gel, place a piece of Saran Wrap on the surface of an ultraviolet transilluminator. Invert the gel, and place it on the transilluminator. Remove the glass plate, leaving the gel attached to the Saran Wrap. Photograph the gel as described on page 6.19.

   Caution: Ultraviolet radiation is dangerous, particularly to the eyes. To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light.

Optimizing PCR with AmpliTaq Gold® DNA Polymerase

Introduction

Recently, PE Applied Biosystems introduced a new enzyme, AmpliTaq Gold® DNA Polymerase, to perform optimal PCR.

This guide is intended to help researchers understand many of the factors that influence the performance of PCR amplifications, allowing for efficient PCR optimization while taking advantage of the unique benefits of using AmpliTaq Gold enzyme.

In conventional PCR amplifications,[1-4] when active reaction components are subjected to lower than desired annealing temperatures, non-specific priming can occur, resulting in non-specific product formation and primer oligomerization.[2,3] Once non-specific products are formed, either during setup or in the initial cycle, they are amplified throughout the remaining PCR cycles. This, in turn, can reduce the sensitivity of the experiment either by decreasing the desired amplification signal or obscuring it with high background. When non-specific products are avoided, the polymerase is directed to the desired target only, increasing sensitivity and yield, and decreasing non-specific background amplification (Figure 1).

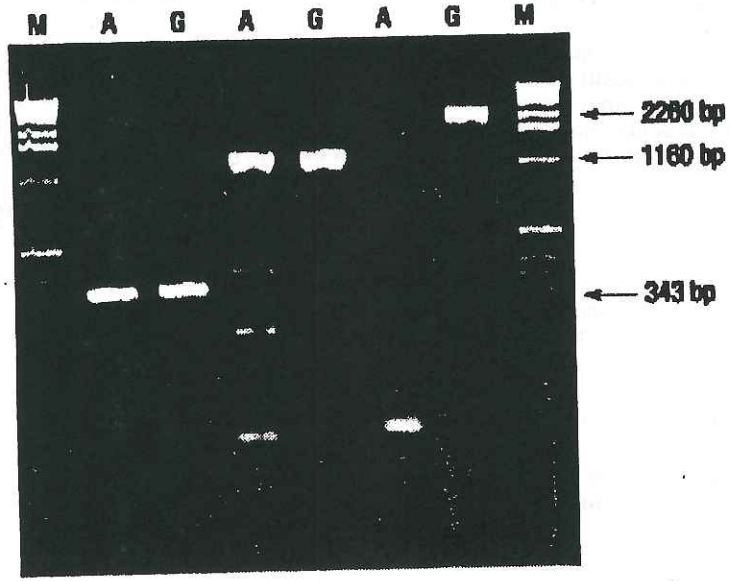


Figure 1. Increased Specificity and Yield of Desired Products with AmpliTaq Gold DNA Polymerase
AmpliTaq Gold DNA Polymerase (G lanes) eliminates amplification of non-specific products, resulting in a substantial increase in specificity and yield when compared to AmpliTaq DNA Polymerase (A lanes).
Staphylococcus aureus DNA was amplified for 35 cycles using three primer sets that target different regions of the fibronectin gene.
M: 0.5 µg of 1 kb ladder (LTI)
Cycling parameters: with AmpliTaq DNA Polymerase, 95 °C for 2 min. followed by 94 °C for 15 sec., 62 °C for 2 min. 30 sec. for 35 cycles. With AmpliTaq Gold enzyme, 95 °C for 10 min. followed by 94 °C for 15 sec., 62 °C for 2 min. 30 sec. for 35 cycles.

Hot Start PCR

To improve PCR specificity and sensitivity, the Hot Start technique was invented.[5-8] Hot Start PCR is a simple modification of the original PCR process in which the amplification reaction is started at an elevated temperature. This was initially performed manually, by adding an essential component to the reaction mixture only after that mixture had been heated above the desired annealing temperature (Figure 2). However, many researchers found this approach cumbersome and time-consuming, especially with large numbers of samples.

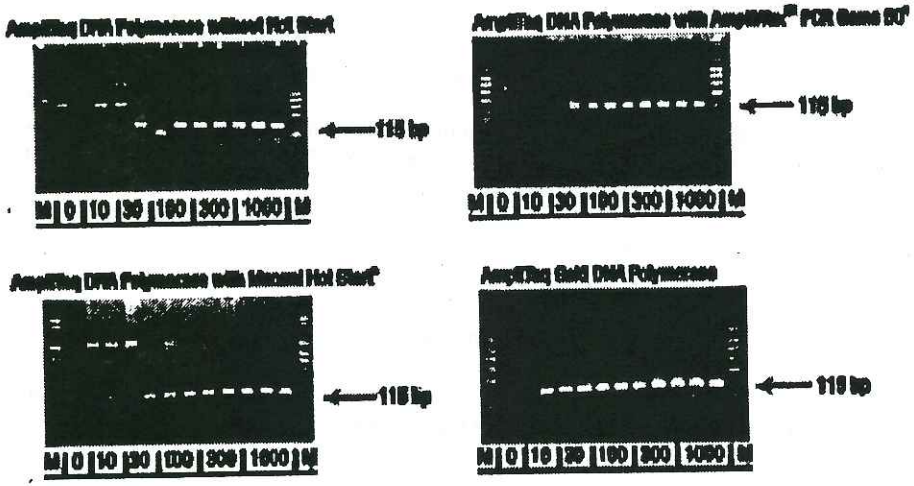


Figure 2. Increased Amplification Sensitivity of Low Copy Target DNA in the Presence of High Background
Compared to other Hot Start techniques, AmpliTaq Gold enzyme increases amplification sensitivity and specificity of low copy number HIV target DNA detection (10-1000 copies) in the presence of 1 µg of human placental DNA.
Duplicates of HIV-1 amplification reactions were analyzed after 40 cycles.
The copy number of the target DNA in each sample is indicated.

M: AmpliSize® DNA Standard (Bio-Rad)

Cycling parameters: with AmpliTaq DNA Polymerase 95 °C for 2 min. followed by 94 °C for 30 sec., 60 °C for 1 min. for 40 cycles. With AmpliTaq Gold enzyme 95 °C for 10 min. followed by 94 °C for 30 sec., 60 °C for 1 min. for 40 cycles.

AmpliTaq Gold® DNA Polymerase

AmpliTaq Gold DNA Polymerase is a chemically modified form of AmpliTaq® DNA Polymerase. This modification renders the enzyme inactive. Upon thermal activation the modifier is permanently released, resulting in active enzyme. A high-temperature incubation step is required to activate AmpliTaq Gold DNA Polymerase which ensures that active enzyme is generated only at temperatures in which primer is not annealed.

When AmpliTaq Gold enzyme is added to the reaction mixture at room temperature, primer extension does not occur because the enzyme is in an inactive state. Any low-stringency mispriming events that may have occurred will not be enzymatically extended, and will not be amplified (Figure 3). AmpliTaq Gold DNA Polymerase can be introduced into most existing amplification systems with minimal alterations to the amplification protocol. This results in higher specificity, sensitivity, and product yield.

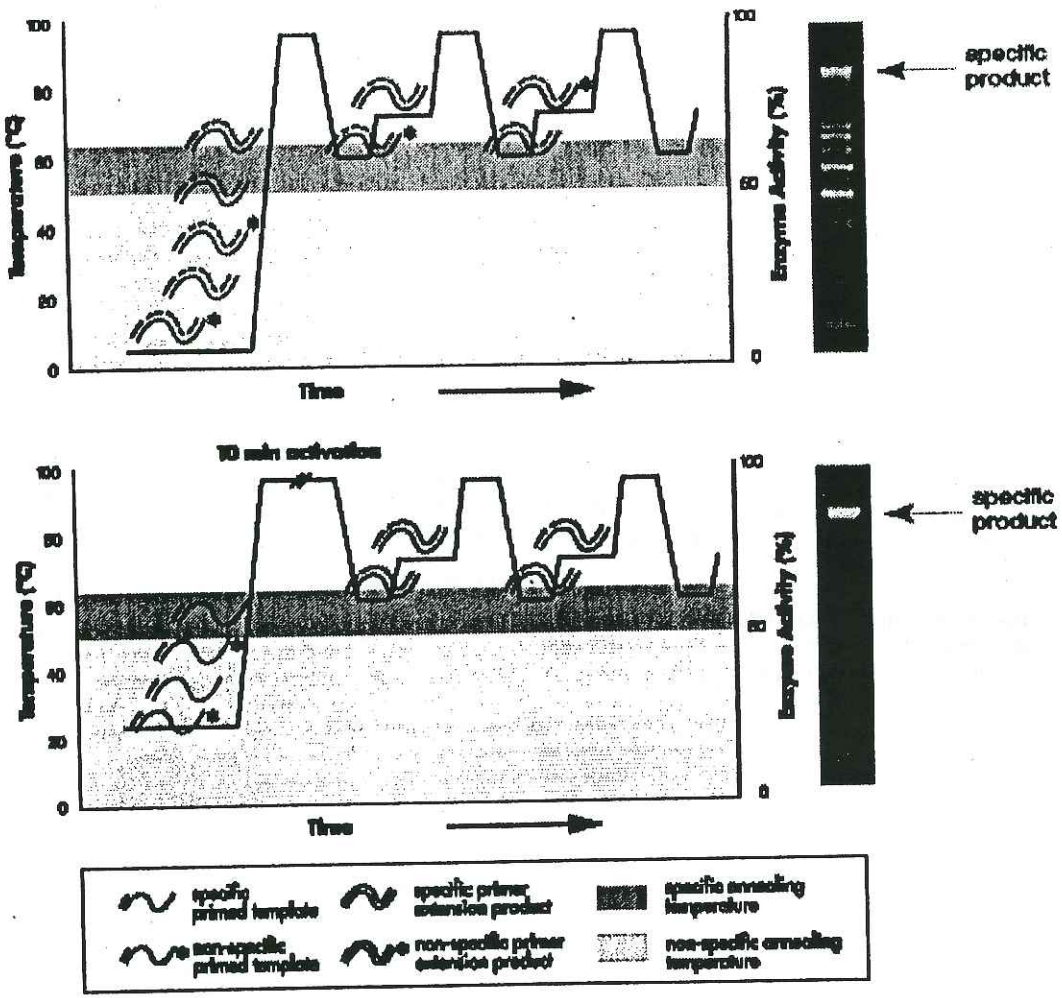


Figure 3. Schematic of PCR Set-up and Initial Cycle
The dynamics of PCR set-up and initial cycle vary, depending upon the sample temperature and the amount of active enzyme present. In conventional PCR (3a), mispriming events that occur on the initial upramp can lead to amplification of non-specific products because active enzyme is present throughout the entire process.

With AmpliTaq Gold DNA Polymerase(3b), active enzyme is present only at temperatures above the specific annealing temperature, which helps minimize extension of misprimed template. The 10-minute pre-incubation at 95 °C serves two purposes: to fully denature any mispriming that occurred during the initial upramp, and to initiate activation of the AmpliTaq Gold enzyme.

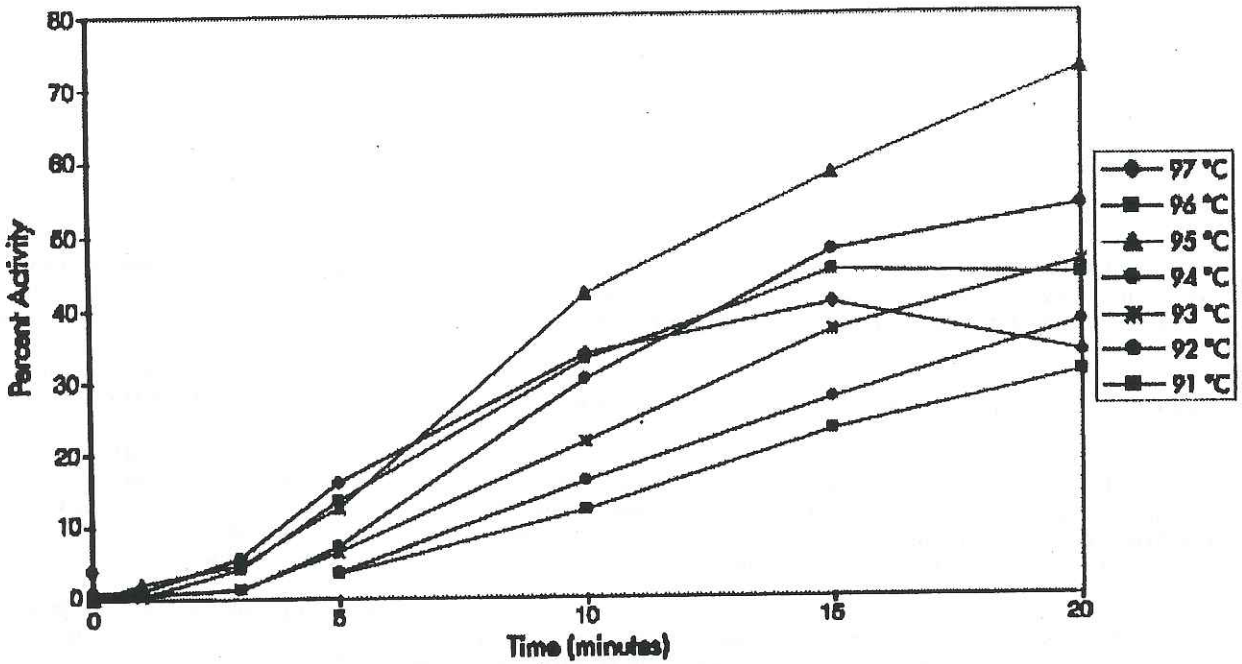


Figure 4. AmpliTaq Gold DNA Polymerase Activation at Various Temperatures and Times
Activity* of AmpliTaq Gold enzyme increases with incubation at temperatures up to 95 °C. At temperatures below 95 °C, longer incubation times are necessary to reach optimal activity.

*Samples are incubated at the temperatures and times specified above. They are then assayed for DNA polymerase activity. [9]

AmpliTaq Gold Activation

Efficient activation is critical for the success of AmpliTaq Gold DNA Polymerase, and depends on three important factors in the PCR amplification :

- incubation **temperature**
- incubation **time**
- reaction **pH**

The following recommendations are based on activation studies performed on PE Applied Biosystems GeneAmp® PCR Instrument Systems, using MicroAmp® plastic disposables and GeneAmp® PCR Buffer II.

Incubation Time and Temperature

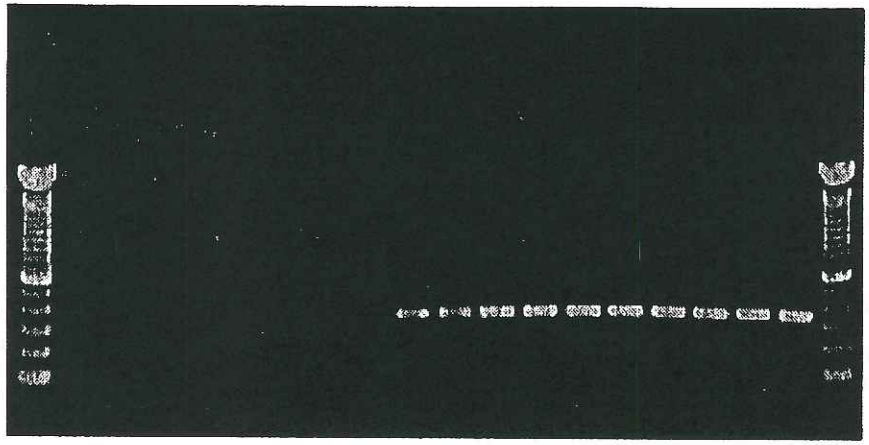
To activate AmpliTaq Gold DNA Polymerase, we recommend a minimum of 10 minutes pre-PCR incubation in GeneAmp® PCR Buffer II at 95 °C, followed by 25-35 cycles of amplification.

Pre-PCR incubation at 95 °C for 10 minutes results in the activation of ~40% of the enzyme, sufficient to perform efficient amplification during the early stages of the reaction when target is less abundant. Because more enzyme is activated at each cycle during the denaturation step, enzyme activity increases as the number of target molecules increases, providing optimal PCR performance (Figure 4).

A 10-minute incubation at 93 °C results in only ~20% activation and may provide insufficient enzyme activity if not compensated for by additional cycles or longer denaturation times. At temperatures greater than 95 °C, the enzyme is more susceptible to irreversible denaturation. We do not recommend enzyme exposure to temperatures > 95 °C. Optimal activation temperature and time may vary, depending upon the application and the thermal cycler model, and should be determined empirically.

When insufficient activation is suspected, we recommend increasing the incubation time rather than the incubation temperature.

Alternatively, activation can be achieved with **Time Release PCR**, a process in which the pre-PCR activation step is omitted and the number of cycles is increased. Since the pre-PCR incubation step is omitted, there is very little active enzyme present in the first few PCR cycles and at least 10 additional cycles are required to achieve desirable results (Figure 5).


5a
343 bp
M | 10 | 0 | 2.5 | 5 | 7.5 | 10 | 12.5 | 15 | 20 | M
No DNA
Pre-incubation at 95 °C (min)

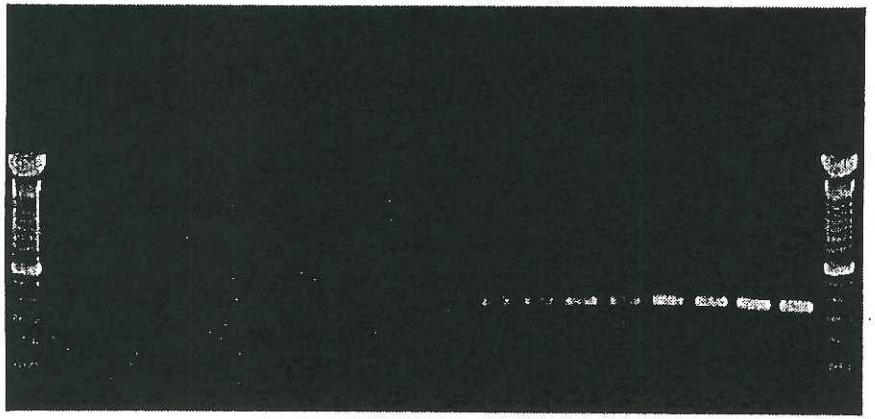

5b
343 bp
M | 35 | 35 | 38 | 41 | 44 | 47 | 50 | 53 | 56 | M
No DNA
Number of Cycles

Figure 5. Activation by Pre-PCR Incubation and Time Release PCR

a. Activation of AmpliTaq Gold DNA Polymerase by Pre-PCR Incubation
Duplicates of Staphylococcus aureus amplification reactions were analyzed after 35 cycles with varying pre-PCR incubation times at 95 °C.
b. Activation of AmpliTaq Gold DNA Polymerase through Time Release PCR.
The same target as above is amplified without performing a pre-PCR heating step. The number of PCR cycles is increased as shown.

Cycling parameters: 94 °C for 20 sec., 62 °C for 45 sec.

M: 0.5 µg of 100 bp DNA Ladder (LTI)

pH

We recommend the use of GeneAmp PCR Buffer II with AmpliTaq Gold DNA Polymerase. This is a Tris buffer with a delta pKa /°C of - 0.031. GeneAmp PCR Buffer II at the 1X concentration is 10 mM Tris-HCl and 50 mM KCl and has a pH of 8.3 at 25 °C. At temperatures >90 °C, the pH of the buffer drops below 7.0, which is required for efficient enzyme activation. We do not recommend non-Tris buffers or Tris buffers with pH >8.3.

Several factors can have a significant effect on the ionic conditions and/or the pH of the reaction:

- **Sample solution:** Contaminants of the nucleic acid template sample (including those carried over from the sample preparation process) may affect enzyme activation. If the template sample is basic enough to elevate the final pH of the reaction mixture above 8.3, activation efficiency will decrease. Diluting the sample in 1X GeneAmp PCR Buffer II minimizes potential ionic strength and pH variation. Dilutions should be empirically determined, and the lowest sample concentration at which desirable results are achieved, should be used. This reduces the effect of any potential alterations in pH and ionic strength, while taking advantage of the superior sensitivity of AmpliTaq Gold enzyme for low-copy amplification.
- **Sample volume:** We recommend that the sample not exceed 10% (v/v) of the total reaction volume. For example, use 1- 5 µL of sample in a 50- µL reaction. In most PCR amplifications, 10 starting copies of intact target DNA are sufficient for successful amplification.
- **Incubation time:** When contaminants that elevate the final reaction pH are suspected, lengthening activation time at
95 °C will increase activation efficiency.
- **Use of GeneAmp PCR Buffer II:** The stringent quality control associated with the manufacture of GeneAmp PCR Buffer II ensures accurate pH and purity.

Additional Factors that Affect the Efficiency of AmpliTaq Gold

Annealing Temperature and Primer Design

When designing primers for PCR amplification, it is important to:

- exclude sequences that are complementary between the primers, especially at the 3' ends
- keep their optimal annealing temperatures similar
- avoid internal secondary structures
- avoid long stretches of any one nucleotide, when possible

For best results, we recommend the use of primer design software.

The probability of mispriming events increases with:

- the complexity of the target sample (i.e., genomic DNA vs. plasmid DNA)
- the number of different primer sequences present in the same reaction.

Therefore, some of the most challenging PCR applications often involve the simultaneous amplification of multiple genomic targets in the same reaction (i.e., Multiplex PCR).[9] The amplification sensitivity and the specificity obtained as a result of the unique properties of AmpliTaq Gold enzyme simplify even these challenging applications (Figure 6).

The Hot Start feature of AmpliTaq Gold DNA Polymerase allows it to discriminate effectively between the amplification of very similar sequences, such as two different alleles of the same gene (Figure 7). Because the enzyme is only activated by temperatures at or above a stringent annealing temperature, assays can be designed such that only primers 100% homologous to the target sequence will hybridize and be extended.

In conventional PCR, primers can hybridize to similar (but not necessarily 100% homologous) sequences during PCR set-up and initial temperature ramp-up. At lower temperatures, these mismatched primers can be extended by the enzyme. These non-specific products will then contain homologous primer binding sites and be amplified efficiently even at stringent annealing temperatures.

The specificity afforded by AmpliTaq Gold enzyme may result in the apparent failure of a previously designed PCR assay that did not originally incorporate Hot Start. For example, in an assay in which primers are not 100% homologous to the initial template sequence, amplification of the target often depends on pre-PCR mispriming. When using AmpliTaq Gold DNA Polymerase or Hot Start, a slightly lower annealing temperature may be required. We therefore do not recommend the use of AmpliTaq Gold DNA Polymerase for applications in which low-stringency annealing is advantageous (i.e., RAPDs, degenerate PCR, etc.).

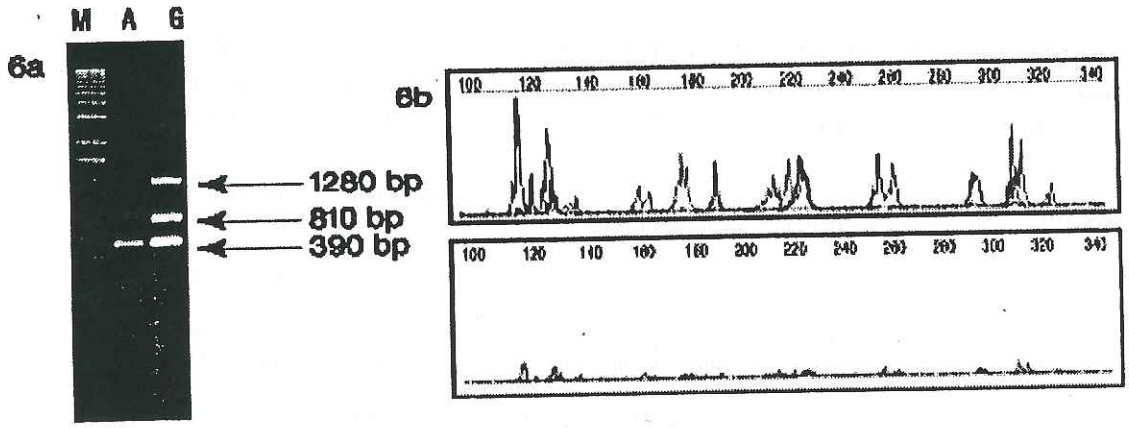


Figure 6. Reliable Co-amplification of Multiple Targets (Multiplex PCR)

a. AmpliTaq Gold DNA Polymerase (G) shows reliable co-amplification of three separate targets of a Staphylococcus aureus fibronectin gene, compared to AmpliTaq DNA Polymerase (A).

M: 0.5 µg of 1 Kb Ladder (LTI)

Cycling parameters: with AmpliTaq DNA Polymerase, 95 °C for 2 min. followed by 94 °C for 20 sec., 62 °C for 2 min. for 35 cycles. With AmpliTaq Gold DNA Polymerase 95 °C for 10 min. followed by 94 °C for 20 sec., 62 °C for 2 min. for 35 cycles.

b. AmpliTaq Gold DNA Polymerase shows reliable co-amplification of 13 markers from Panel 14 of the ABI PRISM™ Linkage Mapping Set. For more details, please refer to the "Optimized Genotyping with the ABI PRISM Linkage Mapping Set and AmpliTaq Gold™ DNA Polymerase application note. (Literature code 237413-001).

Figure 7. Use of AmpliTaq Gold DNA Polymerase in Allelic Discrimination
Amplifications of linearized plasmid DNA using two allele specific primers. In lanes 2 and 3, the forward primer has a single mismatch with the template at the 3' end. In lanes 4 and 5, the forward primer is a perfect match to the template. The same reverse primer is used in both amplifications. AmpliTaq Gold DNA Polymerase (lanes 3, 5) did not extend the mismatched primer, whereas AmpliTaq DNA Polymerase (lanes 2, 4) produced a variety of non-specific products. As expected with perfectly matched primer and target, the desired product was amplified with both enzymes, while background artifacts were minimized with AmpliTaq Gold enzyme.

Lane 1: 0.5 µg of 1 Kb Ladder (LTI)

Cycling parameters: with AmpliTaq DNA Polymerase, 95 °C for 2 min. followed by 94 °C for 15 sec., 60 °C for 30 sec. for 35 cycles. With AmpliTaq Gold DNA Polymerase 95 °C for 10 min. followed by 94 °C for 15 sec., 60 °C for 30 sec. for 35 cycles.

Magnesium Chloride Concentration

We recommend a $MgCl_2$ concentration range of 1.5-4.0 mM for AmpliTaq Gold enzyme. Some applications may require different $MgCl_2$ concentrations. We strongly suggest that the $MgCl_2$ concentration be determined empirically for each primer set in order to achieve optimal PCR performance. High concentrations of $MgCl_2$ may result in non-specific products, and too little $MgCl_2$ may reduce product yield. $MgCl_2$ concentration should also be adjusted when the concentrations of sample DNA and/or dNTPs change significantly.

Template

The purity of the DNA plays an important role in the success of the reaction. Sample preparations may include contaminants that inhibit enzyme activity. In addition, the concentration of the target DNA should be optimized for each amplification. When target DNA is present in excess, mis-priming and non-specific products are more likely to occur. Using AmpliTaq Gold DNA Polymerase routinely results in successful amplification, even when starting with as little as 10 copies of target DNA. The sensitivity obtained with AmpliTaq Gold enzyme allows for successful amplification even of degraded DNA samples, in which the effective starting copy number of intact template molecules can be very low.

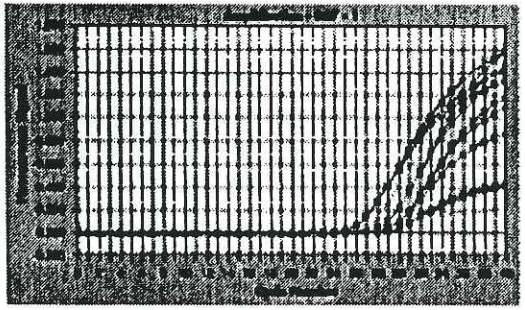
Deoxynucleotides

In standard PCR, 200 µM of each dNTP is sufficient for successful amplification. Some applications may require higher dNTP concentrations, especially when dNTP analogues are used. However, excess dNTPs can decrease enzyme fidelity. For high-fidelity applications, we suggest lower concentrations of dNTPs (greater than or equal to 20 µM each).

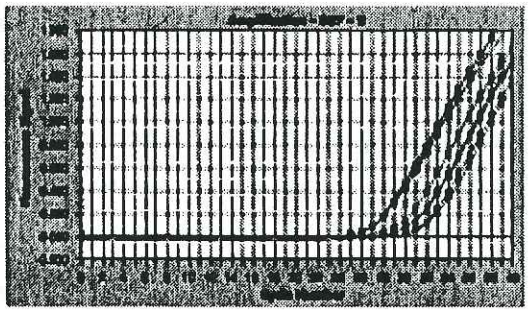
Thermal Parameters

In addition to the parameters discussed above, the success and the reproducibility of PCR amplification with AmpliTaq Gold DNA Polymerase depends on the thermal cycler. For optimal thermal performance and superior PCR amplification, we recommend regular diagnostic tests to verify temperature accuracy and to confirm cycle time reproducibility. For a typical amplification cycle, we recommend that the time of the denaturation step be a minimum of 15 seconds when using GeneAmp® PCR Instrument Systems 2400, 9600 or 9700 and 1 minute when using either the DNA Thermal Cycler or the DNA Thermal Cycler 480. Using AmpliTaq Gold DNA Polymerase with a high performance thermal cycler increases amplification reproducibility (Figure 8).

8a AmpliTaq DNA Polymerase



8b AmpliTaq Gold DNA Polymerase



Red-100 copies Blue-200 copies Green-400 copies Pink-800 copies Black-1600 copies

Figure 8. Low Copy Target Detection with High Reproducibility

Amplification plots from the ABI PRISM® 7700 Sequence Detection System* show cycle-to-cycle increases of ?-actin amplification product from human DNA, using AmpliTaq DNA Polymerase and AmpliTaq Gold DNA Polymerase. For each starting copy number (100, 200, 400, 800, and 1600 copies) was performed in triplicate. In amplifications using AmpliTaq Gold DNA Polymerase, higher reproducibility among triplicates, and higher yield at low copy number are consistently demonstrated.

Cycling parameters: with AmpliTaq DNA Polymerase, 95 °C for 2 min. followed by 94 °C for 20 sec., 60 °C for 1 min. for 40 cycles. With AmpliTaq Gold DNA Polymerase, 95 °C for 10 min. followed by 94 °C for 20 sec., 60 °C for 1 min. for 40 cycles.

* This system employs a probe technology that exploits the 5'-3' nuclease activity of AmpliTaq Gold DNA Polymerase to allow direct detection of the PCR product by the release of fluorescent reporter. Real-time detection on the ABI PRISM 7700 Sequence Detection System measures the increase of reporter fluorescence during PCR. For further details, please refer to the TaqMan® PCR Reagent Kit Protocol (PE Applied Biosystems Part Number 402823).

Conclusion

When used as recommended, AmpliTaq Gold enzyme increases amplification specificity and sensitivity, which improves product yield. This increased specificity makes AmpliTaq Gold DNA Polymerase ideal for multiplex PCR or allelic discrimination.

The enzyme efficiently amplifies low-copy targets even in the presence of high concentrations of complex DNA; thus, it is especially useful for pathogen detection and the amplification of degraded DNA samples.

Furthermore, because AmpliTaq Gold enzyme is inactive at room temperature, reactions can be set up in advance without the fear of amplifying non-specific sequences. This saves time without compromising performance.

Note

All PCR amplifications were performed on the GeneAmp® PCR System 9700 in 50-µL reaction volumes, with 1.25 units of AmpliTaq DNA Polymerase or AmpliTaq Gold DNA Polymerase, 0.2 µM of each primer, 200 µM each dNTP and 2.5 mM $MgCl_2$ in 1X GeneAmp PCR Buffer II. AmpliTaq Gold DNA Polymerase was subjected to a 10-minute pre-PCR activation step at 95 °C, unless otherwise specified. 5 µL of each amplification reaction was analyzed by ethidium bromide stained gel electrophoresis.

References

1. Saiki, R. K., *et al.* , (1985) *Science* **230:** 1350.
2. Saiki, R. K., *et al.* , (1988) *Science* **239:** 487?491.
3. Lawyer, F.C., *et al.* , (1989) *J. Biol. Chem.* **264:** 6427?6437.
4. Lawyer, F.C., *et al.* , (1993) *PCR Methods and Applications* **2:** 275?287.
5. Faloona, F.S., *et al.* , (1990) 6th Int'l Conf. AIDS, San Francisco, CA (Abstract No. 1019).
6. Chou, Q., *et al.* , (1992) *Nucleic Acids Res.* **20:** 1717?1723.
7. Kellog, D.E., *et al.* , (1994) *BioTechniques* **16:** 1134?1137.
8. Horton, R.M., *et al.* , (1994) *BioTechniques* **16:** 42?43.
9. Chamberlain, J. S., *et al.* , (1988) *Nucleic Acids Res.* **16:** 11141?11156.

Return to PCR

Information subject to change without notice.
Last updated September 1997

GelStar® Nucleic Acid Gel Stain Instructions

Before You Begin

Use a polypropylene container for diluting the stain and staining the gel
Use a 302 or 312 nm UV transilluminator for dye excitation
Use a GelStar filter for photography

- **For using GelStar stain in an agarose gel, see page 3**
- **For post-staining an agarose gel, see page 5**
- **For post-staining an MDE™ or polyacrylamide gel, see page 6**

Product Description

GelStar gel stain is a highly sensitive fluorescent stain for detecting nucleic acids in agarose and polyacrylamide gels. This single stain gives high sensitivity detection of double-stranded or single-stranded DNA, and RNA. Gels can be post-stained, or alternatively the stain can be added to agarose gels during gel casting. A comparison of GelStar gel stain with ethidium bromide is shown below. These results were obtained using a 300 nm UV light source and the appropriate filter for each stain.

Nucleic Acid	Sensitivity Increase Compared to Ethidium Bromide
dsDNA	4-16 fold
ssDNA or SSCP DNA	20-80 fold
Glyoxalated RNA	16 fold
Native RNA	3-10 fold
Formaldehyde-denatured RNA	2-3 fold

Catalog Number	Description	Quantity
50535	GelStar Nucleic Acid Stain	2 x 250 µL
50536	GelStar Nucleic Acid Stain photographic filter	1 each

Application Notes

- The powder used on some laboratory gloves may contribute to background fluorescence. We recommend the use of powder-free gloves and rinsing gloves prior to handling gels.
- Fibers shed from clothing or lab coats may be fluorescent; be cautious when handling gels.
- Detection of samples by addition of GelStar® gel stain to the sample loading buffer is not recommended.

Application Notes continued on next page

Application Notes (continued)

- Staining of nucleic acids with GelStar® stain has minimal impact on blotting efficiency. To ensure efficient hybridization, use of prehybridization and or hybridization solutions containing 0.1-0.3% SDS is important to remove stain retained during transfer.
- GelStar stain can be removed from DNA by ethanol precipitation.

Storage and Handling

- GelStar gel stain is supplied as a 10,000X concentrated stock solution in DMSO. This is stable for at least 6 months if stored at -20° C protected from light.
- Allow time for the stock solution to thaw totally. Removal of stain from partially thawed solutions will result in depletion of stain over time.
- GelStar gel stain may be diluted in most common electrophoresis buffers with a pH range from 7.0-8.5, or in TE buffer.
- Diluted stock solutions are stable for several days when stored at 4° C in a polypropylene container protected from light. Number of reuses depends upon the number and size of gels stained and the amount of nucleic acid in the gels.
- Prepare and store the stain in polypropylene containers such as Rubbermaid® containers or pipette-tip lids. The stain may adsorb to glass surfaces and some plastic surfaces, particularly if the surfaces carry residues of anionic detergents or reagents.

CAUTIONS

- The DMSO stock solution should be handled with caution as DMSO is known to facilitate entry of organic molecules into tissues. We strongly recommend using double gloves when handling the DMSO stock solutions. Please refer to the GelStar gel stain MSDS for more details. No data are available on the mutagenicity or toxicity of the GelStar gel stain.
- **This stain should be treated as a potential mutagen and used with appropriate care. Stain solutions should be disposed of by passing through activated charcoal followed by incineration of the charcoal. Solutions may also be treated using commercially available units designed to bind ethidium bromide.**

Excitation of GelStar-Stained Nucleic Acids

Either:
Illuminate the gel with a standard UV transilluminator (302 or 312 nm).
Or:
Excite the GelStar stain with an argon laser scanning system. Systems compatible with the detection of SYBR® Green stain should also be compatible with GelStar stain.

Continued on next page

Visualization by Photography

Photograph the gel with the filters and film in the table below. Polaroid® Type 55 positive/negative film can be used for photography of gels having relatively strong signals.

Exposure time varies with the strength of the illumination source and the filter used for photography.

Note: Use of a SYBR® Green filter or other filters results in at least a 2-fold lower detection sensitivity, particularly for ssDNA. However, this filter is also less sensitive to particulates in the gel.

Suggested Exposure Conditions For Different Film Types

Film	*f*-stop	Filter	Exposure
Type 57 or 667	4.5	GelStar	2-5 seconds
Type 55	4.5	GelStar	15-45 seconds

Visualization by Image Capture Systems

Due to the different spectral response of most video and CCD systems, the sensitivity seen with these systems may be lower than that obtained using Polaroid film. Nevertheless, GelStar® gel stain will still be more sensitive than ethidium bromide. We suggest you contact your system's manufacturer for details on recommended filters. The excitation and emission maxima of GelStar gel stain are 493 nm and 527 nm (532 nm for RNA) respectively.

Protocol for Adding GelStar Stain to Agarose Gels

Detection of DNA or RNA in Agarose Gels by Pre-staining

Step	Action
1	Remove GelStar stain from -20° C storage and thaw at room-temperature for 10 to 20 minutes.
2	Spin the stain vial briefly in a microcentrifuge to deposit the solution in the bottom of the vial.

Continued on next page

Detection of DNA or RNA in Agarose Gels by Pre-staining (continued)

3	Prepare the gel solution and cool the solution to 55-65° C.
4	Add a volume of stock GelStar® stain to the tempered gel solution as indicated below: **For DNA:** Use a final concentration of 1X, e.g., 5 µL of stain stock added to each 50 mL of gel solution. **For RNA:** Use a final concentration of 2X, e.g., 10 µL of stain stock added to each 50 mL of gel solution.
5	Mix the gel solution by swirling, stirring, or inversion to thoroughly distribute the stain into the gel solution.
6	Immediately cast the gel and allow to solidify. Avoid extended light exposure and extended exposure (>10 min) to a buffer overlay.
7	Run the gel using your standard protocol.
8	Visualize the results by photography, scanning, or image capture as described above. No destaining is normally required, although we recommend a brief rinse with buffer to minimize deposition of stain on work surfaces.

Notes:

- The effect of GelStar stain on DNA migration is similar to ethidium bromide's, i.e, DNA migration is slower compared to gels with no stain added.
- GelStar stain is sensitive to the presence of particulates in the gel buffer and dust/debris on gel trays. For optimal results, filter the buffer used to prepare the gel solution.
- Including GelStar stain in vertical gels is not recommended.

Continued on next page

Detection of DNA and RNA in Agarose Gels by Post-Staining

Protocol

Step	Action
1	Separate the samples by electrophoresis as normal.
2	Remove GelStar® stain from -20° C storage and thaw at room-temperature for 10 to 20 minutes.
3	Spin the stain vial briefly in a microcentrifuge to deposit the solution in the bottom of the vial.
4	Dilute stock GelStar stain in buffer in a polypropylene container. **For DNA:** Dilute in TE, TAE, or TBE to give a final concentration of 1X, e.g., 5 µL of stain stock added to each 50 mL of buffer. **For RNA:** Add stock GelStar stain in 1X MOPS buffer to give a final concentration of 2X, e.g., 10 µL of stain stock added to each 50 mL of buffer. Prepare enough stain solution to cover the gel during staining.
5	Mix the stain solution to distribute the stain thoroughly into the solution.
6	Place the gel and the stain solution in a polypropylene container and incubate with gentle agitation. Protect from direct exposure to strong light during staining. Staining is normally complete within 30 minutes. Exceptionally thick gels (> 4mm) or high concentration gels may require longer staining times for optimal results.
7	Visualize the results by photography, scanning, or image capture as described above. No destaining is normally required, although we recommend a brief rinse with buffer to minimize deposition of stain on work surfaces.

Notes:

- GelStar stain is compatible with post-staining of DNA in agarose gels >4 mm thick such as FMC BioProducts' Reliant® precast agarose gels.
- GelStar stain gives excellent detection of RNA that has not been denatured, as well as RNA denatured by a variety of methods, e.g., glyoxal, formamide, or formaldehyde. The increase in detection sensitivity in comparison to ethidium bromide staining varies depending upon the sample and gel preparation methods used (see note below and Table on page 1).
- Detection of glyoxalated RNA with GelStar stain is optimal in gels that are ≤4 mm thick. To see the full sensitivity enhancement of GelStar stain use gels of this thickness or include the stain in the gel for detection.

Continued on next page

Detection of Nucleic Acids on Vertical Gels by Post-Staining

Protocol

Step	Action
1	Separate the samples by electrophoresis as normal.
2	Remove GelStar® stain from -20° C storage and thaw at room-temperature for 10 to 20 minutes.
3	Spin the stain vial briefly in a microcentrifuge to deposit the solution in the bottom of the vial.
4	Add stock GelStar stain to buffer (TE, TAE, MOPS, or TBE) to give a final concentration of 2X, e.g. 10 µL of stain stock added to each 50 mL of buffer. Prepare enough stain solution to cover the surface of the gel during staining.
5	Mix the stain solution by swirling, stirring, or inversion to distribute the stain thoroughly into the solution.
6	Open the cassette, and leave the gel in place on one plate.
7	Place the plate, gel side up, in a staining container.
8	Gently pour the stain over the surface of the gel; a disposable pipette may be used to help distribute the stain evenly over the gel surface
9	Incubate for 30 minutes to stain. No destaining is required, although we recommend a brief rinse with buffer to minimize deposition of stain on work surfaces.
10	Visualize the results by photography, scanning, or image capture as described above. For highest sensitivity the gel should be carefully removed from the plate and placed directly on the transilluminator or scanning stage. Alternatively, if a relatively low fluorescence plate is used, the results may be visualized by placing the gel and plate gel side down on the transilluminator and photographing or by scanning the gel directly on the plate.

Notes:

- As an alternative to the protocol presented for staining gels on the cassette plate, smaller gels such as mini-gels may be removed from both plates then stained using the protocol for post-staining agarose gels.
- Treatment of one plate with a "release" agent, such as Gel Slick™ solution (FMC Cat #50640), increases the ease of separating the glass plates while keeping the gel in place on the other plate for staining.
- Handling or compression of gels (particularly polyacrylamide-type gels) can lead to regions of high background after staining. If possible gels should not be handled directly; use a spatula (or other tool) and a squirt bottle to slide the gel off the plates and into the stain or onto the light box.

(Notes continued on next page)

Notes (continued)

- Addition of 50% glycerol to the staining buffer is recommended when using GelStar® stain with MDE™ gels (FMC Cat #50620) for heteroduplex or SSCP analyses. This minimizes swelling of the gel during staining, and improves gel handling and staining intensity.

Related Products

Catalog Number	Description	Quantity
50620	MDE Gel Solution	250 mL
50560	Glyoxal Sample Buffer for RNA Electrophoresis	1.7 mL
54922	Reliant® RNA gel, 1.25% SeaKem® Gold Agarose in MOPS Buffer	20 gels/box

For Research Use Only.

FMC BioProducts
191 Thomaston Street
Rockland, Maine 04841
207-594-3400

Customer Service: 800 341-1574
Order Fax: 800 362-5552
Technical Service: 800 521-0390
Tech. Service Fax: 800 362-1133
E-mail: biotechserv@fmc.com
homepage: http://www.bioproducts.com

FMC BioProducts Europe
Risingevej 1
DK-2665 Vallensbaek Strand
Denmark
Telephone: 45 43 73 11 22
Fax: 45 43 73 56 92

FMC, GelSlick, SeaKem, Reliant, MDE and GelStar are trademarks of FMC Corporation. Rubbermaid is a trademark of Rubbermaid, Inc. Polaroid is a trademark of Polaroid Corp. SYBR is a trademark of Molecular Probes, Inc. SYBR Green stain is covered by US Patents 5,436,134 and 5,658,751. GelStar stain is covered bu US Patent 5,436,134. Other US and foreign patents pending.
Copyright © 1997 FMC Corporation. All rights reserved.

18111-0498-2

Technical Information Index

- Agarose Physical Chemistry
- Electrophoresis of DNA in Agarose Gels
- Dissolving and Casting Agarose Gels
- Visualizing DNA in Agarose Gels
- GelStar® Nucleic Acid Stain
- SYBR® Green I and II Gel Stains
- Vacuum Drying Agarose Gels
- In-Gel Applications
- DNA Recovery
- DyeDeoxy™ Cycle Sequencing of Agarose Gel Purified PCR Products after ß-agarase Digestion
- Fast Running Protocols for High Resolution in MetaPhor® Agarose Gels
- Southern Blotting from Agarose Gels
- Protocol for In-Gel Hybridization of DNA <=1 kb Using GelBond® Film
- PAGEr™ Precast Gels for Protein Electrophoresis NEW
- Protein Electrophoresis in Agarose Gels

Manual Sequencing with Long Ranger™ Gel Solution

Long Ranger™ Gel Solution

Protocol

Precautions

Wear gloves and use all safety precautions routinely used when handling acrylamide solutions. Please read the material safety data sheet (MSDS) for this product prior to use. This product is for research use only and not intended for diagnostic or human use.

Procedure

Gel Preparation and Pouring

Glass plates must be clean and free of dried gel and soap residues. To remove residues, apply ethanol to both plates and wipe dry.

Ensure that the gel will not stick to the glass plates by silanizing one plate.

Assemble glass plates according to manufacturer's instructions.

Long Ranger gel solution is supplied as a 50% stock solution. For maximum readable bases (multiple loads), we recommend a 5% or 6% gel in 1X TBE and a running buffer of 1X TBE. For fast runs of 200 to 300 bases, prepare a 5% or 6% gel in 0.6X TBE gel and running buffers. If the sequence you intend to read is closer to the primer, pour an 8% Long Ranger gel in 0.6X TBE and use running buffer of 0.6X TBE. See Table 13 for 10X TBE buffer recipe.

- SYPRO™ Red Protein Gel Stain
- Prosieve® 50 Gel Solution for Protein Analysis
- Reliant® RNA Gel Protocol
- Mutation Detection
- Heteroduplex Analysis with MDE™ Gel Solution
- SSCP Analysis with MDE™ Gel Solution
- Manual Sequencing with Long Ranger™ Gel Solution
- Protocols for Long Ranger Singel™ Pack Use
- Use of Long Ranger™ Gel Solution with Automated Sequencers
- Long Ranger™ Solution Reuse for STR Analysis NEW

TABLE 13

10X TBE Buffer for Long Ranger Gels

Maniatis

To 800 mL distilled water add:

		5X	
Tris Base	108 g	54	108
Boric Acid	55 g	27.5	55
$Na_2EDTA \cdot 2H_2O$	7.4 g	3.7	20ml of 0.5N

Dissolve solids, then add water to 1000 mL. We do not recommend using TBE buffers made with 0.5M EDTA stock solutions.

TABLE 14

Preparation of Long Ranger Gels (50 mL Total Volume)

COMPONENTS	GEL % IN 0.6X TBE 5%	6%	8%
Urea	21 g	21 g	21 g
10X TBE	3 mL	3 mL	3 mL
Long Ranger gel solution (50%)	5 mL	6 mL	8 mL
Deionized water (fill to)	50 mL	50 mL	50 mL
TEMED	25 µL	25 µL	25 µL
10% APS	250 µL	250 µL	250 µL

For extended runs or multiple loads, the gel should contain 1X TBE. Use 5 mL of 10X TBE to prepare 50 mL of diluted gel.

Note: Previous protocols have suggested using 0.6X TBE (running buffer) and 1.2X TBE (gel buffer) for maximum read. While effective, the sample wells may degenerate after long runs (5 or more hours) making multiple loading difficult.

Place the specified quantity of the first three components from Table 14 into a clean beaker or 50 mL centrifuge tube and mix gently by swirling or inversion. To facilitate dissolving the urea, the solution may be warmed, but cool the solution to room temperature before polymerization. After the urea has dissolved, bring the final volume to 50 mL with deionized water.

Note: This recipe should be proportionately adjusted based on the amount of gel needed to fill your plates.

If desired, and after the urea has dissolved, filter the solution through Whatman® #1 filter paper or a Nalgene® cellulose acetate filter (<=0.45 um).

Add the specified amounts of TEMED and 10% APS and mix the solution by inversion or gentle swirling.

Pour the gel solution into the plates using the standard procedure for acrylamide. Insert comb (in an inverted position if using a sharkstooth comb), and allow to polymerize for at least 30 minutes at room temperature.

Remove the comb, and rinse the wells or top surface of the gel well with buffer.

Mount the gel cassette onto the sequencing apparatus according to the manufacturer's instructions.

Prepare a sufficient quantity of running buffer to fill both anodal and cathodal chambers by diluting 10X TBE stock to 0.6X or 1X, as appropriate.

Electrophoresis

Prerun the gel for 10 to 15 minutes before loading the samples. Use 28 to 35 watts for 35 mL gels (40 cm x 20 cm x 0.4 mm) or 55 to 66 watts for 70 mL gels (40 cm x 40 cm x 0.4 mm).

Prepare DNA samples as you would for standard sequencing gels: denature samples for 2 to 5

minutes at 75° C or the temperature recommended for the enzyme you are using, and immediately chill on ice.

After the prerun, rinse the well(s) thoroughly with buffer. Reinsert the sharkstooth comb so it just touches the gel, and load samples.

Adjust the power so that the temperature of the glass plates is between 40° and 50° C. We recommend using 28 to 35 watts for 35 mL gels and 55 to 66 watts for 70 mL gels. Monitor the temperature of the plates with Gel Temperature Monitoring Strips, and adjust wattage to maintain the desired temperature.

Monitor the run time with the marker dyes bromophenol blue and xylene cyanol (often contained in sequencing kit stop solutions). When doing multiple loads, the second loading should be added when the bromophenol blue is approximately 3 cm from the bottom of the gel (see Tables 15, 16).

TABLE 15

Approximate Running Times of 5% Long Ranger™ Gels

Bases Read	250	400–500	>600
Prerun	10–15 minutes	10–15 minutes	10–15 minutes
Run Time	2 hours	4 hours	6–8 hours

TABLE 16

Dye Migration in Long Ranger Gels

GEL %	BROMOPHENOL BLUE	XYLENE CYANOL
5%	40 bp	175 bp
6%	20 bp	138 bp
7%	19 bp	123 bp
8%	11 bp	98 bp

Autoradiography

When the run is complete, turn off the power supply, disconnect electrodes and remove the plates from the apparatus.

Long Ranger does not require fixing or removal of urea for ^{35}S labeled reactions. If your application requires fixing, soak in 20% ethanol and 10% acetic acid for 10 to 20 minutes.

Allow the plates to cool briefly before separating. Transfer the gel onto Whatman® 3MM filter paper.

Place the Whatman 3MM paper on a flat surface with gel side up and cover with plastic wrap.

Dry the gel (not required for 32P-labeled gels) under vacuum at 70° to 80° C for 30 to 60 minutes. Remove the plastic wrap and expose gel to X-ray film using standard techniques.

Formamide in Long Ranger Gels

Unreadability or errors in interpretation on a sequencing film are often caused by the phenomenon of compressions. A compression is usually associated with intermolecular base pairing in a primer extension which is G-C rich. The folded structure or hairpin loop persists during the electrophoresis and runs faster through the gel matrix than the equivalent unfolded structure. The resulting autoradiogram of such a DNA sequence is diagnosed by bands running very close together, usually with a gap or increased band spacing in the region above. One method of eliminating gel compressions is to increase the denaturing conditions in the gel matrix by adding formamide (in addition to urea).

Prepare and assemble glass plates as described above.

As with a standard polyacrylamide gel, it is necessary to increase the Long Ranger gel concentration to 8% when formamide is added. For making 50 mL of 8% Long Ranger gel containing 40% formamide, follow Table 17. Adjust the quantity of each component proportionately for making up greater than 50 mL of gel solution.

TABLE 17

Components of 8% Long Ranger Gel with Formamide

COMPONENTS	AMOUNT
Long Ranger	8 mL
Formamide	20 mL
Urea	21 g
10X TBE	5 mL
Deionized water (fill to)	50 mL
TEMED*	60 µL
10% APS*	400 µL

**APS and TEMED are increased as compared to standard Long Ranger.*

Place the specified quantity of the first four components into a clean beaker or 50 mL centrifuge tube and mix gently by swirling or inversion. Once urea has dissolved, bring final volume to 50 mL with deionized water.

If desired and after urea has dissolved, filter the solution through Whatman® #1 filter paper or Nalgene® cellulose acetate filter (<=0.45 uM).

Add the specified amounts of TEMED and 10% APS and mix the solution by inversion or gently swirling.

Pour the gel solution into the plates and allow to polymerize for at least 30 minutes at room temperature.

Once polymerized, transfer plates to electrophoresis apparatus and prepare samples as described above.

Long Ranger with formamide should be run at the same power settings as a regular Long Ranger gel in 1X TBE.

The running time should be increased to 4 to 5 hours since the addition of formamide slows the DNA migration about 50%.

When the run is complete, turn off the power supply, disconnect electrodes and remove the plates from the apparatus.

Allow the plates to cool briefly before separating. A formamide gel must be fixed prior to drying. To minimize swelling, a 20% ethanol and 10% acetic acid fixing solution must be used. Place the glass plate with the gel still adhered in the fixing solution for 15 minutes. (Leaving the gel on the plate inhibits the gel from swelling during fixing.)

Remove the plate from the fixing solution and drain off excess fixing solution. Transfer the gel to Whatman® 3MM paper. Cover with plastic wrap prior to drying.

Dry the gel under vacuum at 70° to 80° C for 30 to 60 minutes. Remove plastic and expose to X-ray film using standard techniques.

Troubleshooting

Symptoms	Causes	Solutions
Fuzzy bands	Buffer problems	Borate can precipitate out of concentrated solutions of TBE. This will change the buffer capacity, ionic strength, and the pH of the solution. Solutions of TBE containing visible precipitate should be discarded.
	Gel overheating	Do not use electrophoretic conditions that cause the gel to become hotter than 50° C.
	Prerun not adequate	The prerun is essential to warm the gel to assist in keeping the samples denatured. Follow protocol for prerun times and conditions.
	Gel not warm enough	If the gel runs too cool, partial DNA renaturation can occur, leading to fuzzy bands. This can also occur during double loads if the gel cools during the second load.

Slow, fast or incomplete polymerization	Ammonium persulfate and TEMED problems	APS solutions should be made just prior incomplete TEMED problems to use. Solid APS should consist of free-polymerization flowing crystals, not clumps. TEMED should be used within the manufacturer's stability date.
		Use recommended volumes and concentrations of APS and TEMED. Too fast a polymerization can result in a poor gel structure leading to resolution difficulties.
	Long Ranger™ solution temperature	The gel solution must be at room temperature before adding APS and TEMED. Dissolution of urea is endothermic and will cool the solution. If not allowed to come to room temperature, slow polymerization may result. If the solution is heated to dissolve the urea, cool to room temperature before adding APS and TEMED.
		If using the premix solution, allow the 75 mL bottle for equilibrate toroom temperature (about 1 hour) before adding APS and TEMED.
Gel sticking or swelling	Silanization problems	If gel sticks to both plates, there could be no silane used, silane on both plates, or a combination of anti-wetting agents used to coat the plates. Strip both plates (soak in 2 N NaOH for 0.5 hour), and recoat one plate only.
		If gel swells out of top, there is either too much silane, or silane on both plates. Strip and reapply as described above.
	Buffer problems	If buffer has broken down, this can cause heating at interface of gel and buffer. This can cause gel to swell out of top or bottom.
	Polymerization problems	Poor polymerization can cause gel to be sticky. This can result in the gel adhering to both plates despite silanization.
Sample loading difficulties	Over-silanization	Over-silanization can cause gel to detach from plates and result in sample flow between gel and plates.
	Use of discontinuous buffers, or buffer breakdown	Discontinuous buffers or buffer breakdown will cause heat build-up at the interface between the gel and buffer. This can cause the gel to distort, and make multiple loading difficult.
	Polymerization problems	Poor polymerization can result in gel distortion in the area around the comb where the gel comes into contact with the air.
Swelling during fixation of foramide gels	Wrong fixative	Use the correct fixative as stated in the fixation of protocol.
	Gel detaching from plate	Silanize one plate only. Lower the gel carefully into the fixative to avoid detachment of the gel from the plate.
Bubbles during gel casting	Dirty plates	Plates must be completely clean and free of oils, detergents and dirt.
	Silanization problems	Over-silanization can result in difficulty with pouring bubble-free gels.

© Copyright 1999 FMC Corporation